Cytokine expression in dogs with natural *Leishmania infantum* infection

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SUMMARY

The aim of this study was to evaluate cytokine expression in 22 *Leishmania infantum* naturally infected dogs, in order to correlate this parameter with the clinical status of infected animals. After 4 and 8 months from the first diagnosis of *Leishmania* infection, clinical and laboratory examination of dogs was performed and peripheral blood mononuclear cells (PBMC) were isolated. The cytokine profile was analysed in terms of IFN-gamma, IL-4, IL-10 and TNF-alpha mRNA expression in cultured PBMC by a semi-quantitative reverse transcriptase-PCR. Thirteen out of 22 *Leishmania*-infected dogs remained asymptomatic in the follow-up, while 9 showed clinical signs of leishmaniasis. IL-4, IL-10, TNF-alpha and IFN-gamma mRNA levels were not significantly different in asymptomatic compared to symptomatic animals 4 months from the diagnosis of *Leishmania* infection, but were significantly higher in symptomatic *versus* asymptomatic dogs after 8 months from diagnosis. In addition, IL-4, IL-10 and TNF-alpha mRNA levels significantly increased only in symptomatic dogs at 8 months, in comparison to the levels found at 4 months. These results show a mixed Th1 and Th2 cytokine response in *Leishmania*-infected dogs, with higher cytokine expression in dogs with manifest clinical disease, during the second follow-up after 8 months from the first diagnosis of infection.

Key words: *Leishmania*, canine leishmaniasis, IFN-gamma, IL-10, IL-4, TNF-alpha.

INTRODUCTION

Protozoa of the *Leishmania* genus infect phagocytes of several mammalian species, including humans. Human leishmaniasis comprises 2 major diseases, visceral leishmaniasis (VL), which is fatal if untreated, and the cutaneous form (CL) that can heal spontaneously but often leaves disfiguring scars. VL causes an estimated 59 000 deaths annually (a rate surpassed among parasitic diseases only by malaria), and the loss of 2 357 000 disability-adjusted life years (DALYs), with an estimated increasing burden in poor social and economic settings (Alvar *et al*. 2006) and in HIV-infected patients (Alvar *et al*. 2008). The incidence of *Leishmania* infection in humans and dogs has increased in Italy since the 1990s, with new foci being detected within the traditional boundaries of endemic transmission but also in northern regions previously regarded as non-endemic (Dujardin *et al*. 2008; Maroli *et al*. 2008; Schönian *et al*. 2008).

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The dog is the main reservoir host for *Leishmania infantum*, which is responsible for human leishmaniasis in China, Mediterranean countries and the Americas (Dantas-Torres, 2007). Infected dogs can be asymptomatic or symptomatic. The latter form ranges from limited to multifocal tissue damage which gives rise to a chronic, non-self-healing visceral disease (Baneth *et al*. 2008). Transmission of *Leishmania* spp. occurs when phlebotomine sandfly vectors feed on symptomatic or asymptomatic infected dogs, even if the infectiousness appears to be higher in dogs with clinical disease (Michalsky *et al*. 2007). This highlights the importance of dogs in spreading human disease, also in the light of a possibly prolonged subpatent period (Oliva *et al*. 2006). In Southern Italy a yearly incidence rate of canine leishmaniasis (CanL) of 9·52% has been estimated in both farm and kennel dogs (Paradies *et al*. 2006), with a high percentage (53·1%) of serologically positive asymptomatic animals (Brandonisio *et al*. 1992).

The current control strategies for VL include the detection and treatment of infected and diseased animals and humans (Chappuis *et al*. 2007), the development of an affordable and effective vaccine for humans and dogs (Palatnik-de-Sousa, 2008), and the use of insecticides and repellents to avoid

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sandfly bites (Otranto *et al*. 2007). In this respect, knowledge of the immune mechanisms involved in animal protection plays a pivotal role in understanding the pathogenesis and clinical course of the disease, as well as in fostering the development of vaccines. In particular, no conclusive data are available on the immunological mechanisms responsible for resistance or disease progression in canine leishmaniasis, and studies on the expression of different cytokines (reviewed by Barbiéri, 2006; Baneth *et al*. 2008; Carrillo and Moreno, 2008) have often yielded conflicting results. Cellular immune responses toward a Th1 subset mediated by IFN-γ and TNF-α seem to predominate in asymptomatic dogs, exhibiting apparent resistance to visceral leishmaniasis, while the role of Th2 cytokines, such as IL-4 and IL-10, in symptomatic animals is still controversial.

The aim of this study was to monitor cytokine expression in cultured peripheral blood mononuclear cells (PBMC) from asymptomatic and symptomatic dogs in the course of natural *Leishmania* infection, in order to correlate the cytokine pattern with the clinical status in these animals. In particular, interferon (IFN)-γ, interleukin (IL)-10, IL-4 and Tumour Necrosis Factor (TNF)-α gene expression was investigated in dog PBMC by a semi-quantitative reverse transcriptase-PCR (RT-PCR), after 4 and 8 months from the first diagnosis of *Leishmania* infection.

MATERIALS AND METHODS

Parasites

Parasites were isolated from the bone marrow of a *Leishmania*-infected dog and cultured on Tobie-Evans medium at 24 °C. The isolated strain was typed by the Istituto Superiore di Sanità (Rome, Italy) and shown to belong to the *L. infantum* species, zymodeme MON1. Promastigotes at day 4 of culture were used, since the maximum percentage of metacyclic (virulent) promastigotes is detected on the 4th day of growth for *L. infantum* (Louassini *et al*. 1998). The liquid phase of the tubes was collected and centrifuged at 350 ***g*** for 10 min. Supernatants were then discarded and the pellets were suspended in phosphate-buffered saline (PBS) pH 7·2, and washed 3 times by centrifugation in PBS at 350 ***g*** for 10 min as previously described (Brandonisio *et al*. 2002). Finally, promastigotes were counted using a haemocytometer after immobilization with 2–3 drops of 70% ethanol.

Leishmania *soluble antigen* (*LSA*) *preparation*

LSA was used as stimulator antigen for lymphocyte activation. LSA was prepared according to the method of Pinelli *et al*. (1994). Briefly, washed cultured *L. infantum* promastigotes at a concentration of 2×10^8/ml in PBS were frozen and thawed 3 times, sonicated, centrifuged (8000 ***g***, 30 min, 4 °C) and the resulting supernatants were collected. The protein content in the supernatants was evaluated by the Bradford method (Bradford, 1976) and aliquots (1 mg/ml) of LSA were stored at −20 °C until use.

Animal sampling and clinical examination

The animals came from 2 kennels in the Apulian region, southern Italy (latitude 42° and 39° North, longitude 15° and 18° East), where endemic CanL has been reported over the previous years (Paradies *et al*. 2006). A total of 22 dogs that were negative for *L. infantum* (in parasitological and serological tests, see below) in March 2005 but newly infected by *L. infantum* by March 2006 (after one sandfly season) were enrolled in the study. All selected animals were positive at parasitological (i.e. PCR on skin biopsy and/or microscopic examination of lymph node smears) and/or serological tests for *Leishmania* but did not exhibit clinical signs of leishmaniasis or laboratory abnormalities in March 2006 (Otranto *et al*. 2007). Serological tests for the presence of anti-*Leishmania* antibodies (IFAT) and PCR were performed using the procedures already described (Paradies *et al*. 2006; Otranto *et al*. 2007).

Clinical examination of dogs was performed monthly, in order to evaluate a possible disease progression. Clinical signs indicative of CanL (e.g. weight loss, alopecia, dermatitis, conjunctivitis, onychogryphosis, lymphadenopathy, etc.) were recorded on each dog's file. In July and November 2006, at 4 and 8 months from the diagnosis of *Leishmania* infection (first and second follow-up), 20 ml heparinized peripheral blood samples and serum samples were collected from the brachial or jugular vein of each dog. Animals were handled and sampled with the owners' consent, and approval was obtained from the Ethics Committee of the University of Bari. Serological tests for the presence of anti-*Leishmania* antibodies (IFAT) were performed at each follow-up time using the procedures already described (Paradies *et al*. 2006).

Isolation and stimulation of peripheral blood mononuclear cells (*PBMC*)

PBMC were isolated from 20 ml of heparinized peripheral blood, diluted with sterile Hanks' balanced salt solution (HBSS; Gibco-Invitrogen, Carlsbad, CA, USA) at a 1:1 (v/v) ratio and centrifuged on the cell separation medium Lympholyte-H (CEDARLANE, Burlington, Ontario, Canada) at 700 ***g*** for 15 min at room temperature. Then, isolated PBMC were extensively washed and suspended in RPMI-1640 complete medium, supplemented with

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2 mM glutamine, 10% heat-inactivated (56 °C for 30 min) foetal calf serum (FCS), 100 μl/ml streptomycin and 100 IU/ml penicillin. Cell viability was evaluated by trypan blue dye exclusion and resulted higher than 98%. Isolated cells were cultured at a density of 10×10^6/ml in 25 cm^2 ventilated tissue culture flasks (Falcon) at 37 °C in 5% CO_2 supplemented with 200 mM glutamine, 10% FCS, 100 μg/ml streptomycin and 100 IU/ml penicillin.

After 24 h of incubation in order to stabilize cell culture (Schmelzer *et al*. 2009), PBMC were submitted to the following *in vitro* treatments (1) stimulation with LSA (10 μg/ml) (2) stimulation with the mitogen phytohaemagglutinin (PHA, Sigma-Aldrich) (5 μg/ml). Unstimulated cells were incubated as negative controls.

Detection of dog cytokine gene expression by reverse transcription (*RT*)*-PCR*

After 48 h of incubation in the same culture conditions, total cellular RNA was extracted from PBMC cultured in tissue flasks as indicated above, by the RNAzolTMbee isolation reagent (Tel-Test, Inc., Friendswood, TX, USA), according to the manufacturer's instructions. The RNA extracted was stored at −80 °C until use. The expression of dog cytokine genes was analysed by RT-PCR. Briefly, total cellular RNA extracted from dog PBMC cultures was submitted to reverse transcription to obtain the cDNA. Reverse transcription was performed in a final volume of 20 μl containing 3 μg of total RNA, 40 U of RNase Out (Recombinant Ribonuclease Inhibitor, Life Technologies, Milan, Italy), 40 mU of oligo dT with 0·5 mM dNTP (PCR Nucleotide Mix, Roche Diagnostics, Mannheim, Germany), 40 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Roche Diagnostics). The reaction tubes were incubated at 37 °C for 60 min, then at 95 °C for 5 min and at 4 °C to stop the reaction. The obtained cDNAs were then amplified by a Perkin Elmer 2400 thermal cycler under the following conditions: 95 °C for 4 min, 95 °C for 1 min, annealing temperature according to the primers used for 1 min and 72 °C for 1 min (40 cycles of amplification). At the end of the reaction, tubes were incubated at 72 °C for 7 min and then at 4 °C to stop the reaction. Each tube contained, in a final volume of 50 μl, 2 μl of cDNA, 200 μM dNTP (PCR Nucleotide Mix), 4 U TaqDNA Polymerase (both from Roche Diagnostics), 5 μl of $MgCl_2$ buffer stock solution, and 50 pmol of primers (Invitrogen, Paisley, UK) specific for dog cytokine genes, whose GenBank Accession numbers were AF 327899 for TNF-α; AF 327901 for IFN-γ; AF 328930 for IL-10; AF 239917 for IL-4, and AF 327898 for GADPH, used as housekeeping gene control. The resulting products were separated with agarose gel (1·2%) and stained with ethidium bromide.

Densitometric analysis

PCR product bands were analysed and the average intensity of the bands was determined using software 1D Image Analysis Software (Kodak Digital Science) in a grey-scale mode. The image was digitally inverted, so that the integration of bands was reported as positive values. The pixel density was determined after background subtraction and used to calculate the integrated density of a selected band. Values of integrated density were reported as Arbitrary Units (AU), corresponding to volume units of pixel intensity per mm^2. The integrated density of each band was reported as the mean of 3 different measurements of the same band for each sample run in triplicate. The amount of cytokine expression was established as a ratio referred to GADPH, a housekeeping gene, which is expressed at a constant rate independent of the immunological activation state (Gröne *et al*. 1996). Data were collected in terms of average intensity of bands of the different cytokines per average intensity of bands of GADPH, and imported to a spreadsheet (Excel; Microsoft, Redmond, Washington, USA). Data plotted in the final graphs represent the mean and standard deviation (S.D.) of the 3 mean values obtained for each experimental set.

Statistical analysis

Our data do follow a normal distribution, therefore, the results were analysed by one-way ANOVA and a P-value $<0{\cdot}05$ was considered statistically significant. The statistical analysis was performed using the software package MINITAB® Release 14.1 (Minitab Ltd, Coventry, UK).

RESULTS

Thirteen out of 22 *Leishmania*-infected dogs enrolled in the study remained asymptomatic during the follow-up, while 9 developed clinical signs of leishmaniasis (i.e. dermatitis, lymphadenopathy, conjunctivitis, and skin ulcers) and/or hypergammaglobulinaemia within the first 4 months after the diagnosis of infection; these symptoms were still present 8 months after diagnosis. Six (66·6%) and 4 (30·7%) symptomatic and asymptomatic dogs, respectively, were positive in IFAT at the first and/or second follow-up, whereas the rest remained negative (data not shown).

The expression of the cytokines IL-4, IFN-γ, IL-10 and TNF-α in PBMC was investigated by RT-PCR in both symptomatic and asymptomatic dogs.

Analysis of IL-4 expression. Results of densitometric analysis of bands obtained by electrophoresis of RT-PCR products demonstrated that IL-4 mRNA levels obtained from LSA-stimulated PBMC

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were not significantly different in asymptomatic ($0{\cdot}02 \pm 0{\cdot}01$, mean ± S.D.) compared to symptomatic animals ($0{\cdot}01 \pm 0{\cdot}00$) 4 months from the diagnosis of *Leishmania* infection, but were significantly ($P = 0{\cdot}02$) higher in LSA-stimulated PBMC from symptomatic ($0{\cdot}10 \pm 0{\cdot}01$) *versus* asymptomatic ($0{\cdot}04 \pm 0{\cdot}02$) dogs after 8 months from diagnosis (Fig. 1A).

In addition, IL-4 mRNA levels were significantly ($P = 0{\cdot}001$) increased in LSA-stimulated PBMC from symptomatic dogs from 4 months in comparison to 8 months after diagnosis of infection. No significant differences were observed in LSA-stimulated PBMC from asymptomatic dogs between 4 and 8 months after diagnosis of infection.

However, expression of IL-4 by LSA-stimulated PBMC was very low and not significantly different from unstimulated PBMC (used as controls) both in symptomatic dogs at 4 months and in asymptomatic dogs after 8 months from diagnosis of infection.

Analysis of IL-10 expression. Results of densitometric analysis of RT-PCR products demonstrated that IL-10 mRNA levels obtained from LSA-stimulated PBMC were not significantly different in asymptomatic ($0{\cdot}55 \pm 0{\cdot}06$) compared to symptomatic animals ($0{\cdot}30 \pm 0{\cdot}09$) 4 months from the diagnosis of *Leishmania* infection, but were significantly ($P = 0{\cdot}001$) higher in LSA-stimulated PBMC from symptomatic ($0{\cdot}97 \pm 0{\cdot}27$) *versus* asymptomatic dogs ($0{\cdot}45 \pm 0{\cdot}06$) after 8 months from diagnosis (Fig. 1B).

In addition, IL-10 mRNA levels were significantly ($P = 0{\cdot}002$) increased in LSA-stimulated PBMC from symptomatic dogs, 8 months in comparison to 4 months after diagnosis of infection. No significant differences were observed in LSA-stimulated PBMC from asymptomatic dogs between 8 and 4 months after diagnosis of infection.

Comparison between unstimulated PBMC and LSA-stimulated PBMC showed that stimulation induced a significant increase of IL-10 expression in all examined dog samples.

Analysis of TNF-α expression. Results of RT-PCR products demonstrated that TNF-α mRNA levels obtained from LSA-stimulated PBMC were not significantly different in asymptomatic ($0{\cdot}24 \pm 0{\cdot}04$) compared to symptomatic animals ($0{\cdot}13 \pm 0{\cdot}05$) at the first follow-up from the diagnosis of *Leishmania* infection, but were significantly ($P = 0{\cdot}03$) higher in LSA-stimulated PBMC from symptomatic ($0{\cdot}57 \pm 0{\cdot}15$) *versus* asymptomatic dogs ($0{\cdot}28 \pm 0{\cdot}05$) at the second follow-up from diagnosis (Fig. 1C).

In addition, TNF-α mRNA levels were significantly ($P < 0{\cdot}01$) increased in LSA-stimulated PBMC of symptomatic dogs at the second follow-up, in comparison to the levels found at the first follow-up, but were not significantly different during follow-up in asymptomatic dogs.

Comparison between unstimulated PBMC and LSA-stimulated PBMC showed that stimulation induced a significant increase of TNF-α mRNA levels in all examined samples.

Analysis of IFN-γ expression. IFN-γ mRNA levels in LSA-stimulated PBMC were not significantly different in asymptomatic ($0{\cdot}26 \pm 0{\cdot}11$) compared to symptomatic animals ($0{\cdot}43 \pm 0{\cdot}14$) 4 months from diagnosis of the infection, but were significantly ($P = 0{\cdot}001$) higher in PBMC of symptomatic dogs ($0{\cdot}71 \pm 0{\cdot}22$) in comparison to asymptomatic animals ($0{\cdot}19 \pm 0{\cdot}08$) after 8 months from diagnosis. However, no significant differences in the IFN-γ mRNA levels were observed between the first and second follow-up in either asymptomatic or sick dogs (Fig.1D).

Comparison between unstimulated PBMC and LSA-stimulated PBMC showed that stimulation induced a significant increase of IFN-γ mRNA expression in all examined samples.

Fig. 2 shows results of a representative experiment of agarose gel electrophoresis of RT-PCR products and staining with ethidium bromide.

Overall, PBMC from both asymptomatic and symptomatic dogs expressed IL-10, TNF-α and IFN-γ cytokines mRNA levels after LSA stimulation at 4 and 8 months after diagnosis. However, mRNA levels for all cytokines tested were significantly higher in LSA-stimulated PBMC of symptomatic dogs in comparison to asymptomatic ones at 8 months post-diagnosis. Moreover, an increased expression of IL-4, IL-10 and TNF-α in LSA-stimulated PBMC was recorded in symptomatic dogs at the second follow-up after the diagnosis of infection. Conversely, no increase in cytokine expression was observed in asymptomatic dogs during the follow-up. However, it should be noted that the very low IL-4 production observed in symptomatic dogs after LSA stimulation when compared to controls does not allow drawing any definitive conclusion about this cytokine production.

DISCUSSION

It is well known that in the murine model of infection with *Leishmania major*, a clear dichotomy is observed between cytokine production by draining lymph node cells of susceptible *versus* resistant mouse strains (Wilson *et al*. 2005). In resistant mouse strains, infection with *L. major* results in the development of a protective T helper-1 (Th1) immune response with high levels of IFN-γ and resistance to re-infection. By contrast, infection of susceptible mouse strains leads to the development of a Th2 immune response characterized by the production of IL-4, IL-6, IL-10 and IL-13, whose role can differ according to the *Leishmania* species (Tacchini-Cottier and Launois, 2008).

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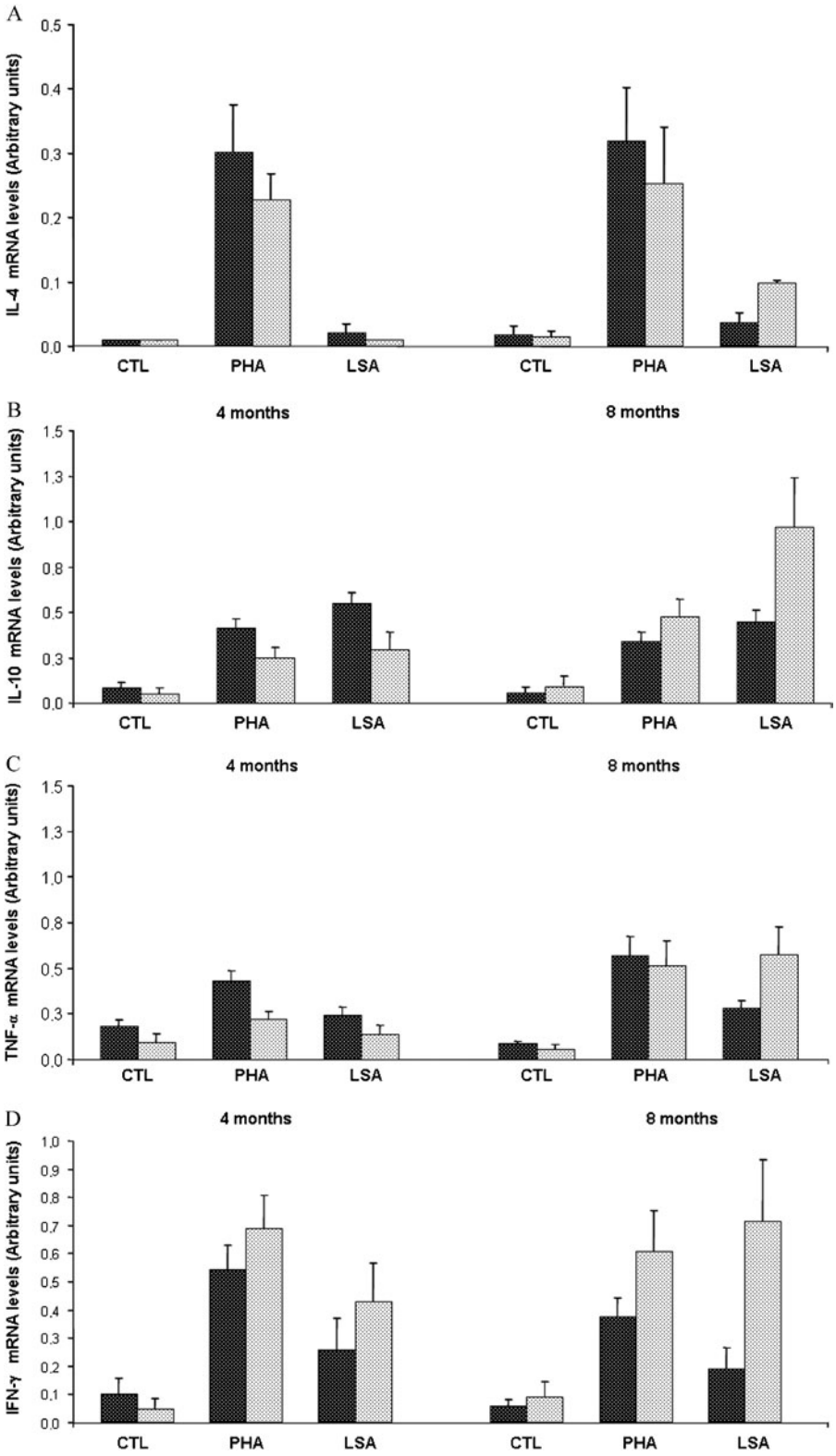

Fig. 1. IL-4 (Panel A), IL-10 (Panel B), TNF-α (Panel C), IFN-γ (Panel D) mRNA expression in PBMC from 13 asymptomatic (black columns) and 9 symptomatic (white columns) dogs in samples taken at first and second follow-up (i.e. 4 and 8 months after the diagnosis of canine leishmaniasis). Results of densitometric analysis of RT-PCR bands are expressed as arbitrary units (means $\pm$ S.D.). CTL, controls; PHA, phytohaemagglutinin; LSA, *Leishmania* soluble antigen. See text for statistically significant differences.

However, the paradigm Th1-protection/Th2-susceptibility is largely based on the cure/no cure responses to *L. major* infection in mice, whereas visceral disease in humans, dogs and other rodents does not show a clear Th1/Th2 dichotomy pattern (Strauss-Ayali *et al*. 2007). Finally, the paradigm

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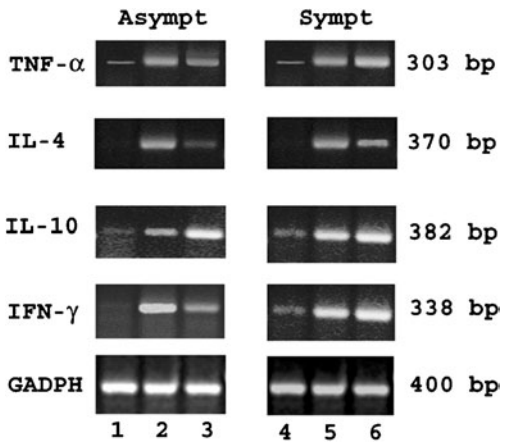

Fig. 2. Gel electrophoretic analysis of RT-PCR products derived from PBMC total mRNA of asymptomatic (left) and symptomatic (right) dogs. Lanes 1 and 4: unstimulated PBMC; lanes 2 and 5: PHA stimulated PBMC; lanes 3 and 6: LSA stimulated PBMC. The figure is representative of 22 experiments carried out on 13 asymptomatic and 9 symptomatic dogs.

Th1/protection has recently been debated, especially in view of results obtained in human visceral leishmaniasis (Mansueto *et al*. 2007) or in vaccination experiments, since many leishmanial antigens which stimulated a Th1 immune response did not show a protective action (Campos-Neto, 2005; Khalil *et al*. 2005).

The results presented herein suggest that *L. infantum* infection induces a mixed Th1/Th2 response in both asymptomatic and symptomatic dogs, with higher cytokine expression in dogs exhibiting manifest disease at 8 months follow-up than at 4 months follow-up.

This is in accordance with results of other investigations, showing a mixed cytokine response in canine leishmaniasis. In fact, in the spleen of dogs with natural *L. infantum chagasi* infection, both IL-10 and IFN-γ were associated with disease progression (Lage *et al*. 2007), and in the spleen of experimentally infected dogs, both IL-4 and IFN-γ may have a role in allowing persistence of the parasite (Strauss-Ayali *et al*. 2007). Finally, a recent longitudinal study in experimentally infected dogs suggested an early expression of both Th1 (IFN-γ) and Th2 (IL-4, IL-13) cytokines in dogs that develop clinical leishmaniasis (Sanchez-Robert *et al*. 2008).

As regards IFN-γ, our results showing a higher expression of its levels at the second follow-up in dogs with overt disease are not in accordance with previous investigations in which IFN-γ production by LSA-stimulated PBMC was correlated with protection or asymptomatic infection (Strauss-Ayali *et al*. 2005; Chamizo *et al*. 2005; Carrillo *et al*. 2007). In addition, in experimental *L. infantum* infection, a protective role for a Th1-mediated immune response was suggested by the finding of enhanced levels of IFN-γ in cultured PBMC (Pinelli *et al*. 1995). In natural infection, in animals that remained asymptomatic a Th1 dominance mediated by IL2, IFN-γ and IL-18 was recorded (Manna *et al*. 2006). Accordingly, dogs vaccinated with killed promastigotes produced IFN-γ (Panaro *et al*. 2001), and stimulation with the recombinant proteins (HSP-70, PFR-2 and KMP-11) induced an up-regulation of IFN-γ mRNA expression in dog PBMC (Carrillo *et al*. 2008). Among the vaccines revealing a high efficacy in the field (reviewed by Miró *et al*. 2008), purified excreted-secreted antigens from *L. infantum* promastigotes with muramyl dipeptide correlate with a specific Th1-type cellular immune response and increased IFN-γ levels 8 months after vaccination (Lemesre *et al*. 2007).

However, in accordance with results presented here, there was a positive correlation between IFN-γ production and progress of infection in freshly stimulated PBMC (Poot *et al*. 2006; Sanchez-Robert *et al*. 2008), bone marrow (Quinnell *et al*. 2001) and spleen samples (Lage *et al*. 2007; Strauss-Ayali *et al*. 2007). The higher expression of IFN-γ levels found in the present work in dogs with overt disease at the second follow-up may suggest that *Leishmania*-infected macrophages become non-responsive to the activating stimulus of IFN-γ (Olivier and Gregory, 2008). Nonetheless, IFN-γ may exert a dual, i.e protective or disease-promoting, role in CanL, as shown in *Toxoplasma gondii* infection (Pfaff *et al*. 2007).

The correlation between disease progression and higher expressions of IL-4, IL-10 and TNF-α recorded herein suggests a counter-protective role for these cytokines in CanL. As regards IL-4 and IL-10, our results are in accordance with other publications showing correlation between IL-4 expression and parasite load in skin samples during CanL (Brachelente *et al*. 2005). A decreased expression of IL-4 mRNA was also observed in freshly isolated PBMC of asymptomatic dogs compared to control dogs (Chamizo *et al*. 2005). In addition, stimulation with the P-8 amastigote antigen or recombinant proteins (HSP-70, PFR-2 and KMP-11) induced low levels of IL-4 mRNA expression in dog PBMC, with no measurable induction of IL-10 (Carrillo *et al*. 2007, 2008). The inhibitory effect of IL-4 and IL-10 on signal transduction for the inducible enzyme iNOS2 (Bogdan *et al*. 2000), responsible for nitric oxide production by activated macrophages and involved in the long-term protection of dogs against natural *L. infantum* infection (Panaro *et al*. 2008; Zafra *et al*. 2008), should be borne in mind.

IL-10 plays an important counter-protective role in the course of *Leishmania* infection also in humans and mice (Nylén and Sacks, 2007; Anderson *et al*. 2008). However, a possible low implication of IL-10 during CanL is suggested by other studies, in which the disease severity was not related with the

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expression of IL-10 by PBMC of experimentally infected dogs (Santos-Gomes *et al*. 2002) and the expression levels of IL-10 did not change during the infection in spleens of *L. infantum*-infected dogs (Strauss-Ayali *et al*. 2007). Similarly, Corrêa and colleagues (2007) showed higher levels of IL-10 than of IFN-γ in the spleen and liver of both symptomatic and asymptomatic dogs naturally infected by *L. chagasi*, with no clear correlation with the clinical status.

As regards TNF-α, its increased expression in LSA-stimulated PBMC recorded only in animals presenting clinical signs of CanL at the second follow-up after the diagnosis of infection, is not in accordance with previous studies showing its up-regulation in PBMC (Carrillo *et al*. 2007) and lymph nodes (Alves *et al*. 2008) of asymptomatically infected dogs. In this context, a better investigation of the genetic background of dogs predisposing to overt disease would be of particular interest (Baneth *et al*. 2008), since allelic associations between polymorphisms at the TNF locus within the major histocompatibility complex and susceptibility to the severe mucocutaneous form of leishmaniasis caused by *L. braziliensis* was demonstrated in humans (Blackwell, 1999).

Overall, studies concerning the production of different cytokines in CanL are often difficult to compare because of their different experimental designs, i.e. in the course of natural or experimental infection, or in response to vaccination at different times during the infection and using different tissues (i.e. skin, spleen, liver, bone marrow, cultured or freshly isolated PBMC). The methods used for cytokine evaluation were also different, mainly involving dosage by ELISA or evaluation of mRNA expression by RT-PCR or real-time RT-PCR. Further investigation should be made of the PBMC fraction involved in the enhanced cytokine expression observed in the present study, possibly using dog CD markers and assessing canine regulatory T cell (TREG) populations.

Finally, results from serological tests have shown that 66·6% of animals showing clinical symptoms and 30·7% of asymptomatic dogs were positive at serology at the first and/or second follow-up, whereas the remaining dogs were negative. This confirms that the diagnostic and prognostic predictive value of serodiagnosis in dog leishmaniasis is scant (Otranto *et al*. 2009).

In conclusion, this study indicates that the cell-mediated immune response mechanisms underlying natural CanL in the Mediterranean area could be different from those in other animal models in which the classical paradigm of Th1/Th2 was associated with resistance or susceptibility. A better understanding of the complex profile of cytokine expression in CanL should contribute to the design of more efficient vaccines and therapies.

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