Vaccine (2008) **26**, 1902–1911

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/vaccine

Immunogenicity of HSP-70, KMP-11 and PFR-2 leishmanial antigens in the experimental model of canine visceral leishmaniasis

Eugenia Carrillo[a,1], Martín Crusat[a], Javier Nieto[a], Carmen Chicharro[a], Maria del Carmen Thomas[b], Enrique Martínez[c], Basilio Valladares[c], Carmen Cañavate[a], Jose María Requena[d], Manuel Carlos López[b], Jorge Alvar[a], Javier Moreno[e,*]

[a] *WHO Collaborating Centre for Leishmaniasis, Centro Nacional de Microbiología, Inst. de Salud Carlos III, Majadahonda, Spain*
[b] *Inst. de Parasitología y Biomedicina López Neyra, CSIC, Granada, Spain*
[c] *Facultad de Farmacia, Univ. de La Laguna, Tenerife, Spain*
[d] *Centro de Biología Molecular Severo Ochoa, Univ. Autónoma de Madrid, Cantoblanco (Madrid), Spain*
[e] *Centro de Investigaciones Biológicas, CSIC, Madrid, Spain*

Received 18 September 2007; received in revised form 9 January 2008; accepted 21 January 2008
Available online 13 February 2008

KEYWORDS
Leishmania;
Canine;
Cytokine;
Immunogenicity;
KMP-11;
HSP-70;
PFR-2

Summary Zoonotic visceral leishmaniasis (ZVL) is a parasitic disease caused by *Leishmania infantum*/*L. chagasi* that is emerging as an important medical and veterinary problem. Dogs are the domestic reservoir for this parasite and, therefore, the main target for controlling the transmission to humans. In the present work, we have evaluated the immunogenicity of the *Leishmania infantum* heat shock protein (HSP)-70, paraflagellar rod protein (PFR)-2 and kinetoplastida membrane protein (KMP)-11 recombinant proteins in dogs experimentally infected with the parasite. We have shown that peripheral blood mononuclear cells (PBMC) from experimentally infected dogs proliferated in response to these recombinant antigens and against the soluble leishmanial antigen (SLA). We have also quantified the mRNA expression level of the cytokines induced in PBMC upon stimulation with the HSP-70, PFR-2 and KMP-11 proteins. These recombinant proteins induced an up-regulation of IFN-γ. HSP-70 and PFR-2 also produced an increase of the TNF-α transcripts abundance. No measurable induction of IL-10 was observed and low levels of IL-4 mRNA were produced in response to the three mentioned recombinant antigens. Serum levels of specific antibodies against HSP-70, PFR-2 and KMP-11 recombinant proteins were also determined in these animals. Our study showed that HSP-70, KMP-11 and PFR-2 proteins are recognized by infected canines. Furthermore, these antigens produce a Th1-type immune response, suggesting that they may be involved in protection. The identification

* Corresponding author. Tel.: +34 918 373 112.
E-mail address: javier.moreno@cib.csic.es (J. Moreno).
[1] Present address: Centro de Investigaciones Biológicas, CSIC, C/Ramiro de Maeztu 9, 28040 Madrid, Spain. Tel.: +34 918 373 112.

0264-410X/$ – see front matter
doi:10.1016/j.vaccine.2008.01.042

as vaccine candidates of *Leishmania* antigens that elicit appropriate immune responses in the canine model is a key step in the rational approach to generate a vaccine for canine visceral leishmaniasis.

Introduction

Zoonotic visceral leishmaniasis (ZVL) caused by *Leishmania infantum*/*Leishmania chagasi* is an endemic disease in the Mediterranean basin, Asia, Central and South America, representing a serious public health problem [1]. The parasite is transmitted by the bite of phlebotomine sandflies. Dogs as the domestic reservoirs for the parasite play a key role in the transmission cycle to human. The prevalence of infected dogs in endemic areas is high [2], but the outcome of the disease after infection is variable and only part of the animals develop symptoms while the rest remain asymptomatic; nevertheless, in addition to symptomatic animals, the capability of subclinically infected dogs to infect sandflies has been demonstrated [3,4]. Current control strategy to interrupt transmission by culling seropositive animals is ineffective and although chemotherapy reduces or eliminates clinical symptoms [5—8] it does not eliminate the parasite, implying that treated dogs could still represent an epidemiological risk [9]. A vaccine against canine visceral leishmaniasis would constitute an important tool and a cost-effective strategy for controlling canine visceral leishmaniasis [10]. Furthermore, an effective immunization of dogs could significantly reduce the incidence of human visceral leishmaniasis caused by *L. infantum* [11].

Previous studies have used experimentally infected animals to study the immunogenic capability of defined leishmanial antigens [12,13]. The canine model of leishmaniasis has demonstrated its utility to evaluate both infection treatment and vaccine efficacy. However, there is a low number of immunization/efficacy assays performed in the canine model of visceral leishmaniasis in comparison to that reported in the murine model [14]. *Leishmania* antigens such as the fucose-mannose ligand [15], protein Q [16], purified excreted/secreted antigens from *L. infantum* [17], Histone 1 and HASPB1 proteins [18], TSA-LmsT11-LeIF trifusion protein [19], *Leishmania* homologue of receptors for activated C kinase (LACK) [20] and cysteine proteinases [21,22] have been used in vaccine trials with variable success in providing protection to dogs against a parasite challenge. Therefore, identification of new vaccine candidates with immunogenic and protective capacity in the canine reservoir is needed for the development of an effective vaccine to prevent canine visceral leishmaniasis and parasite transmission.

In this work, we evaluated the antigenicity of *Leishmania* recombinant proteins HSP-70, PFR-2 and KMP-11 in the experimental canine model of visceral leishmaniasis. HSP-70 is a major heat shock protein, a group of evolutionarily conserved proteins that have been described as antigens in infectious diseases caused by bacteria, protozoa, fungi and nematodes [23]. It has been shown that HSP-70 from *Leishmania braziliensis* induced the secretion of IFN-γ and IL-2 in absence of IL-10 and IL-4 in patients with mucocutaneous leishmaniasis [24]. Further, the immunization of mice with HSP-70 produced a Th1-type immune response [25,26]. PFR is a potent immunogen able to induce a protective Th1 response against the experimental infection with *Trypanosoma cruzi* [27]. In cutaneous leishmaniasis, PFR-2 from *Leishmania mexicana* induced protection against the experimental infection with *L. panamensis*, showing that PFR-2 is a conserved immunogen between *Leishmania* species [28]. KMP-11 is a surface membrane protein associated to lipophosphoglycan (LPG), which is expressed differentially in amastigote and promastigote stages of *Leishmania* [29—31]. Patients cured from visceral leishmaniasis caused by *L. donovani* showed cellular proliferation against KMP-11 from *L. donovani* and also production of IFN-γ and IL-4 [32,33]. It has been described that the DNA vaccine of KMP-11 protected hamsters experimentally infected with *L. donovani* [34].

We examined the immunogenicity of HSP-70, PFR-2 and KMP-11 in dogs experimentally infected with *Leishmania infantum* evaluating the lymphoproliferative responses of PBMCs and their cytokine mRNA gene expression elicited upon stimulation with these proteins. In addition, we determined the serum antibody levels against HSP-70, PFR-2 and KMP-11 in these animals.

Materials and methods

Animals

Beagle dogs purchased to a local breeder, ranging in age from 24 to 30 months were used in this study. The animals were kept at the facilities of the National Centre for Microbiology in Majadahonda (Madrid). All dogs received routine vaccinations and both the anti-leishmanial antibody and cellular immune responses to soluble leishmanial antigen (SLA) were negative in all animals. After quarantine, these dogs were experimentally infected by intravenous (iv) inoculation with 10^8 *L. infantum* promastigotes (MCAN/ES/98/LLM-724). Dogs were monitored for 1.5 years after infection, and a complete characterization of the animals was carried out, including haematological, parasitological and immunological aspects. These animals were kept and handled in the facilities under veterinary care, following ethical guidelines in accordance with national and European Union regulations. The various analyses employed a total of 13 dogs representative of each clinical group: 4 asymptomatic, 5 oligosymptomatic and 4 polysymptomatic dogs [35,36]. In addition, two non-infected dogs were used as controls; these animals were negative for anti-leishmanial antibody, lymphoproliferative response to SLA and parasitological studies performed.

Purification of leishmanial recombinant antigen

L. infantum HSP-70 was purified as described previously [37]. Briefly, *L. infantum* HSP-70 was produced in *Escherichia coli* transformed with pQE/HSP-70 clone. The protein was purified by affinity chromatography on a nitrilotriacetic acid-Ni^{2+} column (Qiagen). Finally, proteins were passed through a polymixin-agarose column (Sigma) to eliminate endotoxins. Residual endotoxin was measured with Quantitative Chromogenic Limulus amebocyte assay (QCL-1000, BioWhittaker), showing that preparations were essentially endotoxin-free (less than 30 ng/mg of recombinant protein).

The KMP-11 and PFR-2 genes were isolated by PCR amplification of genomic DNA of *L. infantum* using oligonucleotides that correspond with the 5′ and 3′ ends, respectively, of each gene. The amplified DNA fragments were cloned, respectively, into the pQE30 and pQE32 vector (Quiagen) using BamHI and SphI sites for KMP-11 and BamHI and XbaI sites for PFR-2. The restriction sites were generated *ad hoc* in the amplified fragments. The *E. coli* M15 strain was chosen as host bacteria and the expressed proteins were purified by Ni^{2+} affinity chromatography. KMP-11 recombinant protein was overexpressed in *E. coli* after induction for 3 h at 37 °C with 0.1 mM IPTG. Thus, the protein was solubilized in phosphate buffer (50 mM $NaHPO_4$, 300 mM NaCl) pH 8 and bound to the Ni^{2+}-NTA resin for 2 h. The resin was washed with the same buffer at pH 8 followed by buffer at pH 7.5, pH 6 and pH 5.5. The recombinant protein was finally eluted with phosphate buffer at pH 4.

The PFR-2 recombinant protein was expressed in *E. coli* cells (M15 strain) after addition of IPTG at 1 mM concentration and grown for 3 h. The cells were pelleted and resuspended in buffer (0.1 M Na_2HPO_4, 0.01 M Tris—HCl, 1 mM PMSF and 8 M urea) at pH 8, broken by sonication, and centrifuged at 10,000 × *g* for 10 min to pellet cellular debris. The soluble fraction was used for protein purification by affinity chromatography after incubation with Ni-NTA columns for 2 h at room temperature. The resin was subsequently washed twice with the previously described buffer at pH 8 and at pH 7.5. The recombinant PFR-2 protein was eluted with resuspension buffer at pH 6. The purified recombinant protein was extensively dialysed against PBS.

The purity degree of recombinant proteins was evaluated by SDS-PAGE and Coomassie blue staining. Moreover, all purified recombinant protein preparations were tested by the E-Toxate reaction kit (Limulus amebocyte lysate (LAL), Sigma, St. Louis, MO) showing that the endotoxin levels were below the detection limit of the kit (<0.1 endotoxin units per ml). Protein concentration was measured using the Bradford method.

ELISA tests

Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 200 ng of HSP-70, 750 ng of KMP-11; 400 ng of PFR-2 or 1000 ng of SLA per well in carbonate buffer (15 mM Na_2CO_3, 28 mM $NaHCO_3$, pH 9.6) and then blocked with 200 μl of blocking buffer (PBS containing 1% BSA and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. After washing the plates three times with PBS (0.15 M NaCl, 0.05 M Na_2PO_4, pH 7.4) containing 0.01% Tween 20, 100 μL of sera diluted 1:100 in dilution buffer (PBS containing 0.1% BSA and 0.1% Tween 20) were added to each well and incubated for 30 min. Plates were washed and then incubated for 30 min with horseradish peroxidase (HRP)-conjugated sheep anti-canine IgG (Bethyl Laboratories, Montgomery, TX; 1:2500). After intensive washing, 50 μl of a substrate solution containing 0.8 mg/ml 2,2′-azino-bis(3-ethylbenzthioline-6-sulfonic acid) diluted in phosphate-citrate buffer (ABTS; Sigma—Aldrich, St. Louis MO, USA) were added to each well. The absorbance at 405 nm was measured using a microELISA reader (Benchmark, Biorad, USA). Results were expressed as mean values of duplicate samples.

Cell isolation

PBMC were prepared by density gradient centrifugation of heparinized blood samples obtained from dogs (Lymphocyte Isolation Solution, RAFER, Spain). Erythrocytes were removed after treatment with ACK erythrocyte lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2 EDTA, pH 7.4). PBMC were washed twice in PBS (pH 7.4), counted and adjusted up to 2.5×10^6 cells/ml in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES and 10% heat-inactivated foetal calf serum).

Cell-proliferation assay

Cells were cultured in 96-well plates at a density of 2×10^5 PBMC per well for 6 days with either the recombinant antigens (5 μg/ml), SLA (10 μg/ml) or 10 μg/ml of concanavalin A (ConA) in a final volume of 200 μl per well. Plates were pulsed during the last 18 h with BrdU and cell proliferation was determined using a non-radioactive ELISA technique, according to manufacturer's instruction (BrdU Cell Proliferation Assay Kit, Biotrak, Amersham, UK). All tests were determined in triplicate and results were expressed as the mean increase of optical density regarding to the blank.

PBMC stimulation and RNA extraction

PBMC (5×10^6 cells/well) from infected animals (four asymptomatic, five oligosymptomatic and four polysymptomatic dogs) and controls (two non-infected) were cultured in a 6-well plate (Nunc, Roskilde, Denmark) with either complete media (unstimulated), 5 μg/ml recombinant antigens, 10 μg/ml SLA or 10 μg/ml ConA. Cells were incubated at 37 °C for 24 h in 5% CO_2 atmosphere. Total RNA was extracted using a RNA extraction kit according to the manufacturer's recommendations (SV-total RNA isolation system, Promega, Madison, USA). The concentration of total RNA was determined spectrophotometrically (ND-1000 UV-V Spectrophotometer, NanoDrop Technology, USA) and each sample was adjusted to a final working concentration of 5 ng/μL with nuclease-free H_2O and stored at −80 °C until use. RNA was free of genomic DNA as determined by PCR.

Cytokine mRNA quantification by real-time-PCR

Real-time-PCR (qRT-PCR) was performed using the ABI Prism 7000 DNA sequence detection system (PE Applied Biosystems, Foster City, CA, USA). Reverse transcription (RT) and PCR amplifications were carried out in a single well by using TaqMan PCR Core Reagent Kit (PE Applied Biosystems). The amplification conditions, the gene-specific primers and probes are as described previously [12]. Briefly, a final volume of 25 μl reaction mixture was used containing 50 ng of template RNA, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U of AmpliTaq Gold, 6.25 U of MultiScribe reverse transcriptase, 10 U of RNase inhibitor, each primer at 200 nM and 100 nM of TaqMan probe. Parallel reactions were performed for the detection of canine IL-10, TNF-α, IFN-γ, IL-4 and IL-18 transcripts from PBMC.

The response to SLA in different groups of infected dogs is shown as the amount of the target RNA relative to the control gene β-actin, expressed as $2^{-\Delta Ct}$ value [38]. Data presented are the averaged mRNA levels of PBMC from four asymptomatic, five oligosymptomatic, four polysymptomatic and two control non-infected dogs. The effect of specific antigen stimulation in canines is shown as the cytokine expression of individual animals: asymptomatic (A-1, A-2 and A-3) and oligosymptomatic (O-1, O-2 and O-3) infected dogs and a non-infected control animal (C-1). For these results, the β-actin gene was used as the control gene (for calculation of ΔCt) and unstimulated samples for each animal were used as the calibrator (for calculation of $\Delta\Delta$Ct) [38]. Differences in gene transcription between stimulated and unstimulated cells are expressed as *n*-fold difference relative to the calibrator. Basal expression of IL-4 was not detected and a Ct value of 35 was assigned to the undetected unstimulated PBMCs for calculation of $\Delta\Delta$Ct in IL-4 gene expression.

Statistical analysis

The effect of antigen stimulation on cytokine gene expression of PBMC from different groups of infected dogs was analyzed using the Mann–Whitney *U*-test. Logarithmic transformation was performed for all mRNA levels before the data were analyzed. Significance was set at $P \leq 0.05$.

Results

Antibody response to leishmanial antigens

The sera from five dogs experimentally infected with *Leishmania* were collected every 2 months post-infection. IgG antibody levels measured by ELISA are shown in Fig. 1. Two control non-infected dogs were also examined and these animals were negative for anti-leishmanial antibody, as well as in their lymphoproliferative responses to leishmanial antigens (data not shown).

The pattern of HSP-70-specific IgG antibody response observed post-infection was similar to that against SLA and showed to be consistently high throughout the observation period (Fig. 1). The levels of IgG antibodies against KMP-11 were lower than those against HSP-70 and were detected later. In general, IgG antibodies against PFR-2 were undetectable.

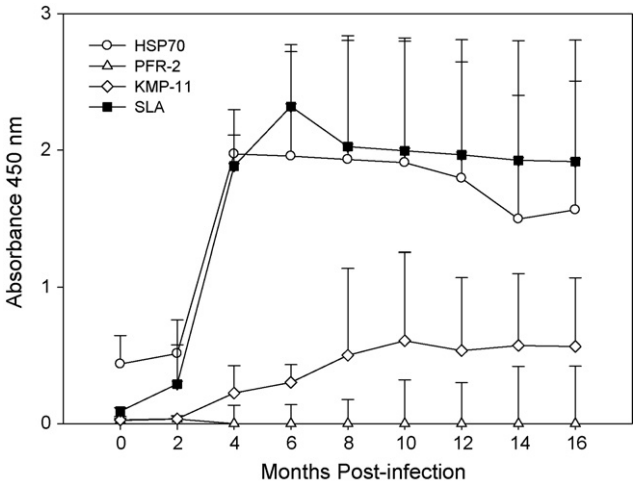

Figure 1 Antibody responses in five oligosymptomatic infected dogs to SLA (■) and the recombinant antigens HSP-70 (O), PFR-2 (△) and KMP-11 (◊). The cut-off was 0.427 (HSP-70), 0.104 (KMP-11), 0.000 (PFR-2) and 0.161 (SLA).

Lymphoproliferative response to leishmanial antigens

The proliferative responses to HSP-70, PFR-2 and KMP-11 as well as SLA were examined in asymptomatic and oligosymptomatic dogs at 20 month post-infection. As shown in Fig. 2, PBMC proliferated upon exposure to HSP-70, PFR-2 and KMP-11, even though it was lower than the proliferation induced by SLA. Stimulation with ConA induced high lymphoproliferative responses in PBMC from both asymptomatic and oligosymptomatic infected dogs.

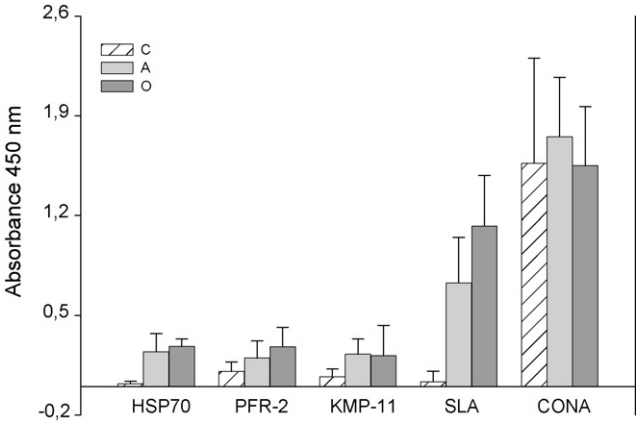

Figure 2 *In vitro* cell proliferative response to HSP-70, PFR-2, KMP-11 and SLA of PBMC from six asymptomatic (A) and five oligosymptomatic (O) *L. infantum* infected dogs at 20-month post-infection and three healthy control animals (C).

Expression of cytokine transcripts in SLA-stimulated PBMC from dogs experimentally infected with *L. infantum*

We have analyzed the cytokine pattern elicited in PBMC stimulated for 24 h with SLA from infected dogs with different clinical status. Both asymptomatic and oligosymptomatic dogs significantly ($P = 0.016$) up-regulated IFN-γ expression in comparison to unstimulated PBMC (Fig. 3); however polysymptomatic dogs showed no changes after SLA stimulation. Furthermore, SLA stimulation produced different IFN-γ expression in PBMC from asymptomatic and oligosymptomatic animals in comparison to polysymptomatic ($P = 0.032$ and $P = 0.029$, respectively).

Up-regulation of TNF-α mRNA expression in the SLA-stimulated PBMC also occurred in asymptomatic and oligosymptomatic dogs, but reached significant levels in the latter group ($P = 0.016$). We have also found significant differences in the TNF-α expression of PBMC from oligosymptomatic animals in comparison to polysymptomatic ($P = 0.029$).

Asymptomatic and oligosymptomatic animals showed a tendency to express the IL-10 transcripts below the basal levels of the control unstimulated cells; this decrease was statistically significant in the oligosymptomatic group ($P = 0.016$). While the expression of IL-4 by unstimulated PBMC was below the detection limit, SLA stimulation produced detectable IL-4 in infected animals. However, these levels of IL-4 did not differ significantly among clinical groups. We have found that polysymptomatic animals had a tendency (not significant) to produce higher expression levels of IL-18 (Fig. 3).

Expression of cytokine transcripts in HSP-70, KMP-11 and PFR-2-stimulated PBMC from dogs experimentally infected with *L. infantum* compared with controls

To determine the potential of these leishmanial recombinant antigens to stimulate PBMC from *L. infantum* infected dogs to produce specific cytokines, we have studied the expression of cytokines related to the pathogenesis or control of visceral leishmaniasis. The specific cytokine profile induced by HSP-70, PFR-2 and KMP-11 was studied in asymptomatic and oligosymptomatic dogs and compared to the cytokine profile induced by SLA as internal control (standard) for this response. For this purpose, PBMC from three asymptomatic and three oligosymptomatic dogs were stimulated with SLA or the recombinant antigens for 24 h and the levels of cytokine mRNA were quantified. We have found similar cytokine expression in asymptomatic and oligosymptomatic animals after specific leishmanial stimulation as previously described [12,39]. Therefore, in Discussion section we have analyzed the results as pertaining to a single group independently of their clinical status of the animals.

HSP-70, PFR-2 and KMP-11 stimulation produced an up-regulation of the IFN-γ transcript abundance in PBMC from six, four and three animals, respectively (Fig. 4). The incubation with the recombinant antigens induced 6.0—14.7-fold (HSP-70), 7.6—8.3-fold (PFR-2) and 3.9—4.9-fold (KMP-11) increase in IFN-γ mRNA expression compared to unstimulated cells. The IFN-γ expression of SLA-stimulated cells increased 20.3—54.8-fold (Fig. 3), while the unspecific amplification of this cytokine in the PBMC from the control dogs after antigenic stimulation was 3.9 (HSP-70), 3.6 (PFR-2), 1.3 (KMP-11) and 2.6 (SLA).

TNF-α expression in PBMC from the infected dogs was also up-regulated after stimulation with HSP-70 and PFR-2 antigens (2.6—6.8- and 3.6—8.8-fold increase, respectively) (Fig. 4) and it was slightly higher than that observed for SLA stimulation (1.9—4.5-fold increase). Healthy (non-infected) dogs presented an unspecific TNF-α mRNA production after antigenic stimulation with HSP-70 and PFR-2 (1.6- and 1.7-fold increase, respectively), whereas no TNF-α expression was observed in response to incubation with KMP-11 or SLA (0.9- and 1.1-fold increase, respectively).

IL-10 and IL-18 mRNA abundance in PBMC after stimulation with SLA or the recombinant antigens was similar to those found for unstimulated cells. The stimulation with HSP-70 produced IL-4 transcripts in two infected dogs, stimulation with PFR-2 produced IL-4 transcripts in one animal, while KMP-11 did not induce a detectable amount of IL-4 transcripts.

Discussion

It has been proposed that the development of a successful vaccine could be the most effective approach for the control of canine leishmaniasis [10]. For this purpose, it is necessary to evaluate the immunogenicity of vaccine candidates. The induction of a Th1-type cytokine response (IFN-γ, TNF-α) would indicate the vaccine potential to confer protection against *Leishmania* infection. For example, *in vitro* assays of candidate vaccine antigens like P-8 using PBMC from *Leishmania infantum* infected dogs have shown to induce mainly antigen specific Th1-like responses during an ongoing infection [12].

In the present work, we have studied the potential as vaccine candidates for canine leishmaniasis of the leishmanial antigens HSP-70, KMP-11 and PFR-2. We have determined the cell proliferation and cytokine pattern elicited by these proteins in PBMC from dogs infected with *Leishmania infantum* and compared them with that obtained after stimulation with SLA. Our results showed that the stimulation with SLA from *L. infantum* produced a strong proliferation and IFN-γ mRNA expression in PBMC from asymptomatic and oligosymptomatic animals, as previously described [12,40]. SLA stimulation also elicited a high expression of TNF-α and low IL-4 transcript abundance in PBMC from asymptomatic dogs without changes in the expression of IL-10 or IL-18. These data confirm that a preferential Th1-like response is associated with disease resistance and protection in canine visceral leishmaniasis [12,17,22,41—43].

Sera from dogs infected with *Leishmania infantum* showed a strong specific reaction against the recombinant *L. infantum* HSP-70 throughout the study period. The recognition of HSP-70 isolated from *L. infantum* and *L. donovani* by sera from dogs with visceral leishmaniasis has been previously described [44], as well as the recognition of HSP-70 isolated from *L. braziliensis* by sera from cutaneous, mucocutaneous and visceral leishmaniasis patients [45—47]. The mitogenic capability of HSP-70 from *L. infantum* for murine

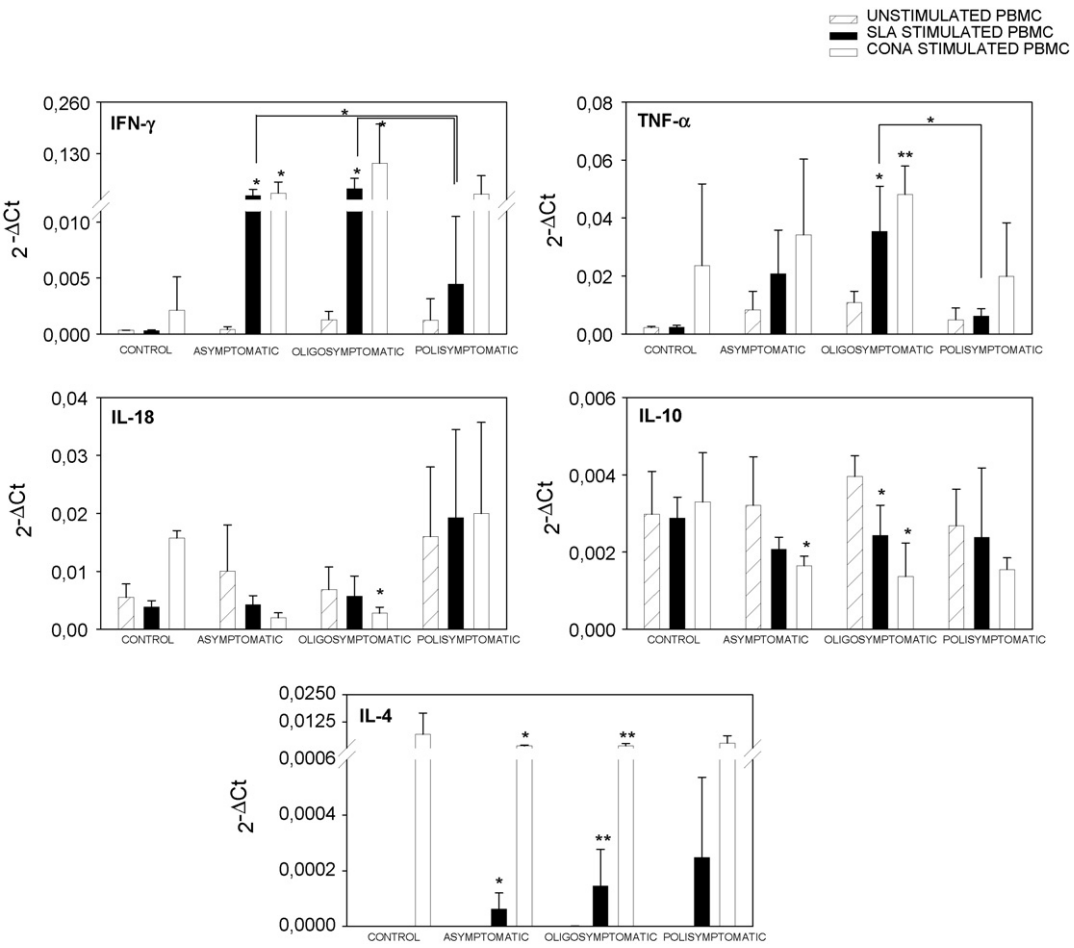

Figure 3 Cytokine gene expression in PBMC from *L. infantum* experimentally infected dogs and healthy control dogs. PBMC were stimulated with soluble leishmanial antigen (SLA) from *L. infantum* and ConA. Asymptomatic and oligosymptomatic dogs showed antigen specific proliferation to SLA. RNA isolation and qRT-PCR were performed after 24 h of stimulation as described in Material and methods. qRT-PCR data were analyzed by using the $2^{-\Delta Ct}$ method. The average of the mRNA levels of PBMC from four asymptomatic, five oligosymptomatic, four polysymptomatic and two control non-infected dogs are presented. Striped bars represent unstimulated PBMC, black bars represent SLA and open bars represent ConA. Results were considered statically significant when $p \leq 0.050$ (*) and $p \leq 0.010$ (**).

B cells has also been described [48]. Further, HSP-70 isolated from *L. braziliensis* induced cell proliferation and production of cytokines in PBMC from *L. chagasi*, *L. guyanensis* and *L. amazonensis* infected patients [24]. We have shown that HSP-70 from *L. infantum* produced a moderate lymphoproliferative response in the asymptomatic and oligosymptomatic dogs studied. Consequently, the results point to the presence of T-cell immunodominant epitope(s) in the HSP-70 antigen and the conservation of the T-cell epitope(s) between some species. *In vitro* exposure to HSP-70 of cultured PBMC from cured patients of mucocutaneous leishmaniasis, resulted in increased levels of mRNA for IFN-γ and IL-2 in the absence of IL-10 and IL-4 [24]. Our study in the canine model of visceral leishmaniasis, the stimulation of PBMC with HSP-70 from *L. infantum* produced an increase in the IFN-γ and TNF-α expression in all animals but not changes in IL-10 mRNA. Therefore, *L. infantum* HSP-70 induced a moderate Th1-like response. The B and T epitope(s) of HSP-70 and the Th1-like expression pattern elicited *in vitro* by this molecule in canine PBMC suggest that HSP-70 is immunogenic under the conditions used in this study. The conservation of epitope(s) among *Leishmania* species and the capacity of HSP-70 to generate a specific response against *Leishmania* in distinct hosts (human patients and dogs) and different clinical manifestations (cutaneous, mucocutaneous and visceral) show the potential of HSP-70 as a candidate for vaccine trials.

Paraflagellar rod proteins (PFR) are strong immunogens against infection with *Trypanosoma cruzi* [49,50] able to induce a Th1-like immune response [27]. Despite PFR proteins are highly conserved among kinetoplastid parasites, there are few studies about the immunogenicity of this antigen against *Leishmania*. In fact, this is the first study of the PFR-2 immunogenic profile in visceral leishmaniasis. In cutaneous leishmaniasis, it has been described that PFR-2 from *L. mexicana* induced protection against the experimental infection with *L. panamensis*. Hence, it seems that PFR-2 is conserved among *Leishmania* species [28]. In our study in the canine model of visceral leishmaniasis, PFR-2 isolated from *L. infantum* was slightly recognized by sera

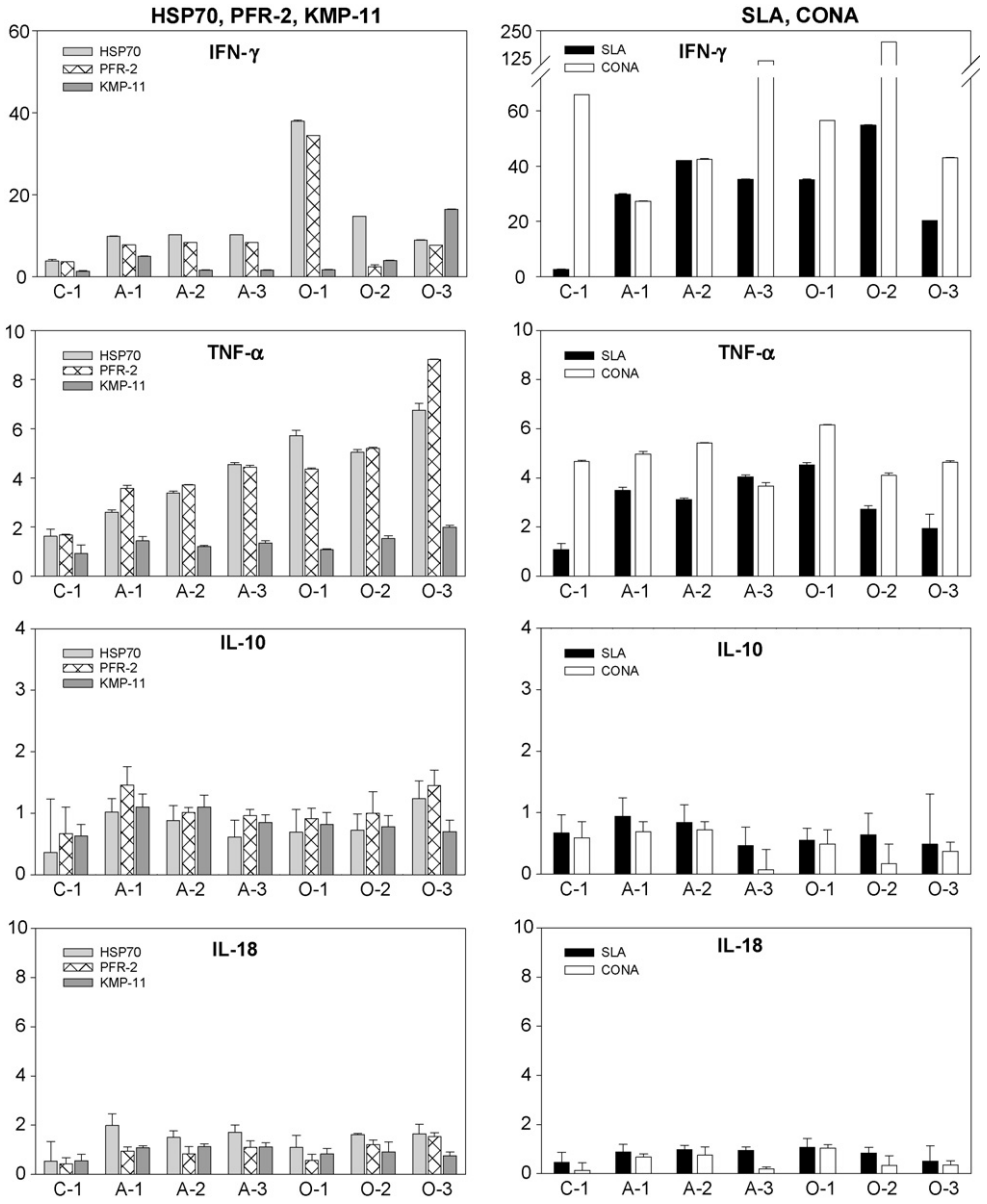

Figure 4 Cytokine gene expression in PBMC from asymptomatic and oligosymptomatic *L. infantum* infected dogs and a healthy control dog after stimulation with the leishmanial antigens. Asymptomatic and oligosymptomatic dogs showed antigen specific proliferation to SLA. qRT-PCR data were analyzed by using the $2^{-\Delta\Delta Ct}$ method. Differences in gene transcription after stimulation are expressed as *n*-fold difference relative to the calibrator (unstimulated cells). Shown are the cytokine expression (IFN-γ, TNF-α, IL-10 and IL-18) for three asymptomatic (A-1, A-2 and A-3) and three oligosymptomatic (O-4, O-5 and O-6) infected animals as well as one control (C-1). Light grey bars represent results from HSP-70 stimulation, stripped bars represent PFR-2, dark grey bars represent KMP-11, black bars represent SLA stimulation and open bars represent ConA stimulation.

from *L. infantum* infected animals, however, it was able to induce a low but specific lymphoproliferative response in *L. infantum* infected dogs. The cell stimulation with PFR-2 showed a Th1-like cytokine expression pattern, with modest IL-4 mRNA levels. The protective immune response showed against different manifestations of leishmaniasis (cutaneous and visceral) suggests that PFR-2 has potential as a leishmanial vaccine candidate. Due to the moderate Th1-like response elicited in infected dogs, we propose the inclusion of this antigen in a multicomponent leishmanial vaccine with other potent immunogens able to induce a strong Th1-like response.

Kinetoplastid membrane protein-11 (KMP-11) is an LPG-associated surface membrane protein present in all members of the family Kinetoplastidae, differentially expressed both in amastigote and promastigote forms of *Leishmania* [29,30,51]. The present study showed the presence of B and T epitope(s) in KMP-11 isolated from *L. infantum* after the humoral and cellular assays performed in dogs infected with *L. infantum*. The immunogenic properties

of this protein were previously described in dogs and hamsters infected with *L. infantum* [31,52], as well as in cured patients of visceral leishmaniasis caused by *L. donovani* who showed an important lymphoproliferative response to the antigen, with a specific production of IFN-γ and IL-4 [33,53]. It has also been described that the *L. donovani* KMP-11 DNA vaccine conferred protection against the experimental infection of hamsters with *L. donovani*, inducing increased expression of IFN-γ, TNF-α and IL-12 and the diminution of IL-10 response, promoting the elimination of *Leishmania* in the spleen and liver of these animals [34]. In the experimental model of canine leishmaniasis, the stimulation with *L. infantum* KMP-11 induced a modest increase in the IFN-γ gene expression in three animals; however, we did not find changes in the expression of TNF-α and IL-4 mRNA. The marked conservation among *Leishmania* species of KMP-11 [54] and the recognition of the B and T epitope(s) by different host of *L. infantum* (human, dogs) show the potential of KMP-11 as a vaccine candidate against visceral leishmaniasis. Despite the expression pattern induced by *L. infantum* KMP-11 in canine PBMC was preferentially Th1-like, the IFN-γ transcript abundance was only moderate. In consequence, it will be necessary to asses the protective capability of the KMP-11 as a component of a multiantigen vaccine against visceral leishmaniasis constituted by some antigens and/or adjuvant able to induce a potent Th1 response including the production of IFN-γ.

In the present work, we have described the cytokine pattern involved in a specific cellular response against *Leishmania* in canine visceral leishmaniasis. The SLA-stimulation of PBMC from dogs infected with *L. infantum* revealed the cytokines involved in the defence against the parasite. This also allowed the evaluation of the immunogenicity of several leishmanial antigens as potential vaccine candidates for canine leishmaniasis, previously to *in vivo* immunization assays. The higher lymphoproliferative response and the IFN-γ mRNA abundance induced by HSP-70, KMP-11 and PFR-2 in asymptomatic dogs suggest that these antigens may be implicated in protection and consequently are potential vaccine candidates against visceral leishmaniasis caused by *L. infantum*.

Acknowledgements

This work was supported by the Ministerio de Ciencia y Tecnologia grant AGL 2000-0284 and by grants from Fondo de Investigaciones Sanitarias (RICET-CO3/04-12 and ISCIII-RETIC RD06/0021). E. Carrillo was supported by a FPI fellowship from the Ministerio de Ciencia y Tecnologia. J. Moreno holds a ''Ramon y Cajal'' contract from MEC. The authors are grateful to Dr. B.L. Travi for critical reading and comments on the manuscript.

Conflict of interest statement: The authors disclose that they do not have any financial conflicts of interest.

References

[1] Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol 2005;35(11—12):1169—80.

[2] Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. J Clin Microbiol 2001;39(2):560—3.

[3] Travi BL, Tabares CJ, Cadena H, Ferro C, Osorio Y. Canine visceral leishmaniasis in Colombia: relationship between clinical and parasitologic status and infectivity for sand flies. Am J Trop Med Hyg 2001;64(3—4):119—24.

[4] Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis 2002;186(9):1314—20.

[5] Moreno J, Nieto J, Chamizo C, Gonzalez F, Blanco F, Barker DC, et al. The immune response and PBMC subsets in canine visceral leishmaniasis before, and after, chemotherapy. Vet Immunol Immunopathol 1999;71(3—4):181—95.

[6] Baneth G, Shaw SE. Chemotherapy of canine leishmaniosis. Vet Parasitol 2002;106(4):315—24.

[7] Pasa S, Toz SO, Voyvoda H, Ozbel Y. Clinical and serological follow-up in dogs with visceral leishmaniosis treated with allopurinol and sodium stibogluconate. Vet Parasitol 2005;128(3—4):243—9.

[8] Pennisi MG, De Majo M, Masucci M, Britti D, Vitale F, Del Maso R. Efficacy of the treatment of dogs with leishmaniosis with a combination of metronidazole and spiramycin. Vet Rec 2005;156(11):346—9.

[9] Alvar J, Molina R, San Andres M, Tesouro M, Nieto J, Vitutia M, et al. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. Ann Trop Med Parasitol 1994;88(4):371—8.

[10] Tesh RB. Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am J Trop Med Hyg 1995;52(3):287—92.

[11] Ashford DA, David JR, Freire M, David R, Sherlock I, Eulalio MC, et al. Studies on control of visceral leishmaniasis: impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. Am J Trop Med Hyg 1998;59(1):53—7.

[12] Carrillo E, Ahmed S, Goldsmith-Pestana K, Nieto J, Osorio Y, Travi B, et al. Immunogenicity of the P-8 amastigote antigen in the experimental model of canine visceral leishmaniasis. Vaccine 2007;25(8):1534—43.

[13] Fujiwara RT, Vale AM, Franca da Silva JC, da Costa RT, Quetz Jda S, Martins Filho OA, et al. Immunogenicity in dogs of three recombinant antigens (TSA LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. Vet Res 2005;36(5—6):827—38.

[14] Requena JM, Iborra S, Carrion J, Alonso C, Soto M. Recent advances in vaccines for leishmaniasis. Expert Opin Biol Ther 2004;4(9):1505—17.

[15] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, de Souza EP, Luz KG, Palatnik M, et al. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto RN). Vaccine 2000;19(9—10):1082—92.

[16] Molano I, Alonso MG, Miron C, Redondo E, Requena JM, Soto M, et al. A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum*. Vet Immunol Immunopathol 2003;92(1—2):1—13.

[17] Lemesre JL, Holzmuller P, Cavaleyra M, Goncalves RB, Hottin G, Papierok G. Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of *Leishmania infantum* promastigotes. Vaccine 2005;23(22):2825—40.

[18] Moreno J, Nieto J, Masina S, Canavate C, Cruz I, Chicharro C, et al. Immunization with H1, HASPB1 and MML *Leishmania* proteins in a vaccine trial against experimental canine leishmaniasis. Vaccine 2007;25(29):5290—300.

[19] Gradoni L, Foglia Manzillo V, Pagano A, Piantedosi D, De Luna R, Gramiccia M, et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from *Leishmania infantum* infection and to prevent disease progression in infected animals. Vaccine 2005;23(45):5245—51.

[20] Ramiro MJ, Zarate JJ, Hanke T, Rodriguez D, Rodriguez JR, Esteban M, et al. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine 2003;21(19—20):2474—84.

[21] Rafati S, Nakhaee A, Taheri T, Taslimi Y, Darabi H, Eravani D, et al. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of *L. infantum*. Vaccine 2005;23(28):3716—25.

[22] Poot J, Spreeuwenberg K, Sanderson SJ, Schijns VE, Mottram JC, Coombs GH, et al. Vaccination with a preparation based on recombinant cysteine peptidases and canine IL-12 does not protect dogs from infection with *Leishmania infantum*. Vaccine 2006;24(14):2460—8.

[23] Zugel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999;12(1):19—39.

[24] Skeiky YA, Benson DR, Guderian JA, Whittle JA, Bacelar O, Carvalho EM, et al. Immune responses of leishmaniasis patients to heat shock proteins of *Leishmania* species and humans. Infect Immun 1995;63(10):4105—14.

[25] Rico AI, Angel SO, Alonso C, Requena JM. Immunostimulatory properties of the *Leishmania infantum* heat shock proteins HSP70 and HSP83. Mol Immunol 1999;36(17):1131—9.

[26] Morell M, Thomas MC, Caballero T, Alonso C, Lopez MC. The genetic immunization with paraflagellar rod protein-2 fused to the HSP70 confers protection against late *Trypanosoma cruzi* infection. Vaccine 2006;24(49—50):7046—55.

[27] Miller MJ, Wrightsman RA, Manning JE. *Trypanosoma cruzi*: protective immunity in mice immunized with paraflagellar rod proteins is associated with a T-helper type 1 response. Exp Parasitol 1996;84(2):156—67.

[28] Saravia NG, Hazbon MH, Osorio Y, Valderrama L, Walker J, Santrich C, et al. Protective immunogenicity of the paraflagellar rod protein 2 of *Leishmania mexicana*. Vaccine 2005;23(8):984—95.

[29] Jardim A, Funk V, Caprioli RM, Olafson RW. Isolation and structural characterization of the *Leishmania donovani* kinetoplastid membrane protein-11, a major immunoreactive membrane glycoprotein. Biochem J 1995;305(Pt 1):307—13.

[30] Jardim A, Hanson S, Ullman B, McCubbin WD, Kay CM, Olafson RW. Cloning and structure-function analysis of the *Leishmania donovani* kinetoplastid membrane protein-11. Biochem J 1995;305(Pt 1):315—20.

[31] Berberich C, Requena JM, Alonso C. Cloning of genes and expression and antigenicity analysis of the *Leishmania infantum* KMP-11 protein. Exp Parasitol 1997;85(1):105—8.

[32] Kemp M, Kurtzhals JA, Christensen CB, Kharazmi A, Jardim A, Bendtzen K, et al. Production of interferon-gamma and interleukin-4 by human T cells recognizing *Leishmania* lipophosphoglycan-associated protein. Immunol Lett 1993;38(2):137—44.

[33] Kurtzhals JA, Hey AS, Jardim A, Kemp M, Schaefer KU, Odera EO, et al. Dichotomy of the human T cell response to *Leishmania* antigens II. Absent or Th2-like response to gp63 and Th1-like response to lipophosphoglycan-associated protein in cells from cured visceral leishmaniasis patients. Clin Exp Immunol 1994;96(3):416—21.

[34] Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. J Immunol 2005;174(11):7160—71.

[35] Abranches P, Silva-Pereira MC, Conceicao-Silva FM, Santos-Gomes GM, Janz JG. Canine leishmaniasis: pathological and ecological factors influencing transmission of infection. J Parasitol 1991;77(4):557—61.

[36] Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol 2002;18(9):399—405.

[37] Rico AI, Del Real G, Soto M, Quijada L, Martinez AC, Alonso C, et al. Characterization of the immunostimulatory properties of *Leishmania infantum* HSP70 by fusion to the *Escherichia coli* maltose-binding protein in normal and nu/nu BALB/c mice. Infect Immun 1998;66(1):347—52.

[38] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25(4):402—8.

[39] Young DB. Heat-shock proteins: immunity and autoimmunity. Curr Opin Immunol 1992;4(4):396—400.

[40] Strauss-Ayali D, Baneth G, Shor S, Okano F, Jaffe CL. Interleukin-12 augments a Th1-type immune response manifested as lymphocyte proliferation and interferon gamma production in *Leishmania infantum*-infected dogs. Int J Parasitol 2005;35(1):63—73.

[41] Chamizo C, Moreno J, Alvar J. Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. Vet Immunol Immunopathol 2005;103(1—2):67—75.

[42] Rafati S, Zahedifard F, Nazgouee F. Prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. Vaccine 2006;24(12):2169—75.

[43] Pinelli E, Killick-Kendrick R, Wagenaar J, Bernadina W, Del Real G, Ruitenberg. J. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. Infect Immun 1994;62(1):229—35.

[44] Quijada L, Requena JM, Soto M, Alonso C. During canine viscero-cutaneous leishmaniasis the anti-Hsp70 antibodies are specifically elicited by the parasite protein. Parasitology 1996;112(Pt 3):277—84.

[45] Quijada L, Requena JM, Soto M, Alonso C. Analysis of the antigenic properties of the *L. infantum* Hsp70: design of synthetic peptides for specific serodiagnosis of human leishmaniasis. Immunol Lett 1998;63(3):169—74.

[46] MacFarlane J, Blaxter ML, Bishop RP, Miles MA, Kelly JM. Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock protein family. Eur J Biochem 1990;190(2):377—84.

[47] Skeiky YA, Benson DR, Costa JL, Badaro R, Reed SG. Association of *Leishmania* heat shock protein 83 antigen and immunoglobulin G4 antibody titers in Brazilian patients with diffuse cutaneous leishmaniasis. Infect Immun 1997;65(12):5368—70.

[48] Rico AI, Girones N, Fresno M, Alonso C, Requena JM. The heat shock proteins, Hsp70 and Hsp83, of *Leishmania infantum* are mitogens for mouse B cells. Cell Stress Chaperones 2002;7(4):339—46.

[49] Segura EL, Paulone I, Cerisola J, Cappa SM. Experimental Chagas' disease: protective activity in relation with subcellular fractions of the parasite. J Parasitol 1976;62(1):131—3.

[50] Segura EL, Vazquez C, Bronzina A, Campos JM, Cerisola JA, Cappa SM. Antigens of the subcellular fractions of *Trypanosoma cruzi*. II. Flagellar and membrane fraction. J Protozool 1977;24(4):540—3.

[51] Berberich C, Machado G, Morales G, Carrillo G, Jimenez-Ruiz A, Alonso C. The expression of the *Leishmania infantum* KMP-11 protein is developmentally regulated and stage specific. Biochim Biophys Acta 1998;1442(2—3):230—7.

[52] Requena JM, Alonso C, Soto M. Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. Parasitol Today 2000;16(6):246—50.

[53] Kemp M, Kurtzhals JA, Kharazmi A, Theander TG. Interferon-gamma and interleukin-4 in human *Leishmania donovani* infections. Immunol Cell Biol 1993;71(Pt 6):583—7.

[54] Stebeck CE, Beecroft RP, Singh BN, Jardim A, Olafson RW, Tuckey C, et al. Kinetoplastid membrane protein-11 (KMP-11) is differentially expressed during the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid parasites. Mol Biochem Parasitol 1995;71(1):1—13.