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Immunogenicity of the P-8 amastigote antigen in the experimental model of canine visceral leishmaniasis

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Abstract

The P-8 proteoglycolipid complex (P-8 PGLC), an amastigote antigen of *L. pifanoi*, has been demonstrated to induce protection in mouse models, as well as to induce Tc1/Th1-like cellular responses in American cutaneous leishmaniasis patients. Because the immunization with P-8 PGLC in the murine model does not appear to be genetically restricted, we have studied the reactivity of the P-8 PGLC in *L. infantum* infected dogs. In this study, it is shown that PBMC from experimentally infected dogs (asymptomatic, oligosymptomatic) significantly proliferated in response to soluble leishmanial antigen (SLA) or the P-8 PGLC. Further, quantification of the gene expression induced by the stimulation with P-8 in asymptomatically infected dogs showed an up-regulation of IFN-γ and TNF-α, which were three to four-fold higher than that induced by soluble *Leishmania* antigen (SLA). While no measurable induction of IL-10 was observed, low levels of IL-4 mRNA were observed in response to both P-8 and SLA antigens. Thus, our studies establish that P-8 is recognized by infected canines and elicits a potentially curative/protective Th1-like immune response. The identification of *Leishmania* antigens that elicit appropriate immune responses across different host species (humans, canine) and disease manifestations (cutaneous or visceral) could be an advantage in generating a general vaccine for leishmaniasis.

Keywords

Leishmania; canine; cytokine; immunogenicity; P-8

1. Introduction

Zoonotic visceral leishmaniasis (ZVL), caused by *Leishmania infantum/Leishmania chagasi* is a progressive wasting disease of dogs and humans that is often fatal if untreated. The disease is endemic in parts of the Mediterranean basin, Asia, Central and South America, where it is

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widespread. ZVL disease incidence is increasing, representing a serious public health problem [1].

Dogs are the main domestic reservoirs for the parasite, which is transmitted from dogs to humans by phlebotomine sand flies (*Lutzomyia* or *Phlebotomus* spp.). The use of molecular techniques (such as the polymerase chain reaction, PCR) has determined that prevalence of infected dogs in endemic areas can be high [2]; further, there exists a high level of subclinical infection in canines in endemic areas that can, at least in part, have infective capability [3,4]. Chemotherapy is able to reduce or eliminate clinical symptoms [5–8] but does not consistently eliminate infectivity to sand flies, indicating the difficulty of achieving parasitological cure in dogs [9]. This situation might explain the failure of treatment and culling of seropositive animals as strategies to control ZVL [10]. Since dogs constitute the major source of parasites transmitted to humans, successful canine immunization could significantly reduce the incidence of human visceral leishmaniasis. This epidemiological feature has promoted the development of vaccines against canine leishmaniasis as an important tool and a cost-effective strategy for controlling visceral leishmaniasis caused by *L. infantum/L. chagasi* [11].

The use of a laboratory canine model of leishmaniasis allows the longitudinal study of the immune response to infection and has been used to evaluate both infection treatment and vaccine efficacy. The canine model has helped to improve understanding of the natural history of leishmaniasis and the underlying events occurring during the prepatent/asymptomatic stage of the disease [12]. Experimentally infected animals can also be used to study the immunogenic capability of defined leishmanial antigens. Vaccines against canine leishmaniasis must be safe and should induce strong and long-lasting cell mediated immunity [13]. Many *Leishmania* vaccine candidates have been identified in murine models [14], but conclusions obtained in this model might not be directly applicable to dogs. To date, only a small number of *Leishmania* proteins have been investigated in the canine model of visceral leishmaniasis. The fucose-mannose ligand [15], protein Q [16], purified excreted/secreted antigens from *L. infantum* [17], H1 and HASPB1 [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.

However, further vaccine studies in the canine reservoir, examining the immunogenicity and protective capacity of different antigens, and the identification of adequate adjuvants are still required; also, the efficacy of these vaccines in blocking transmission should be considered. Among the many *Leishmania* antigens isolated and characterized, molecules that are specific to or up-regulated in the amastigote stage are relevant for study since this stage is the progressive form found in the infected mammalian host. The P-8 antigen is a proteoglycolipid-complex associated with the external surface membrane of the amastigote [23]. Immunization with P-8 has been shown to induce significant cross- protection against infection with either *L. amazonensis* or *L. pifanoi* in mice with different H-2 haplotypes [24,25]. Further, when the P-8 proteoglycolipid complex was tested *in vitro* using PBMCs from American cutaneous leishmaniasis patients infected with *L. (Viannia) braziliensis*, it was found to induce Tc1/Th1-like responsiveness in CD8+ and CD4+ T cells, respectively [25–27].

Further studies are required to evaluate the potential of the P-8 PGLC as vaccine candidate against canine visceral leishmaniasis. In this paper we report the antigenicity of P-8 PGLC in the experimental canine model of visceral leishmaniasis. We have evaluated the lymphoproliferative and cytokine responses of PBMCs and determined serum levels of P-8 specific subclass antibodies in these animals.

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2. Materials and Methods

2.1. Animals

Two different groups of dogs were used in this study, which was conducted at two sites (Colombia, Spain) and utilized either outbred or inbred animals, respectively.

Group 1 Mixed-breed, 4–5 month old female dogs were obtained from the Centro de Zoonosis of Cali, Colombia. All these animals were collected in areas not endemic for visceral leishmaniasis. Animals were maintained in CIDEIM's kennel near Cali where neither *Leishmania* transmission nor sand fly activity has been recorded. Dogs were housed and handled according to local and federal regulations, following international and Colombian guidelines (Law 84/89). The research protocols were approved by the animal care and use committee at CIDEIM. Prior to the experimental infection the animals were quarantined, subjected to treatment for common intestinal parasites (Triantelm®, Intervet; Rondel®, Virbac labs.; Ivomec®, Merial) and vaccinated against frequent dog pathogens (Galaxy DH2PPiL, Wyeth-Fort Dodge Labs; Novicac Rabia, Intervet). Dogs were negative for anti-leishmanial antibody by ELISA.

The *Leishmania chagasi* strain (MCAN/COL/98/CATIRE) used for all experimental infections was isolated previously from a polysymptomatic dog. Metacyclic promastigotes (10^4–10^5) obtained from experimentally infected *Lutzomyia longipalpis* were inoculated either intravenously or intradermally in the ear, which lead to different clinical presentations varying from asymptomatic to polysymptomatic dogs. Dogs representative of each clinical group (asymptomatic, oligosymptomatic and polysymptomatic) [28,29] were examined in terms of their lymphoproliferative responses to concanavalin A (ConA), soluble leishmanial antigen (SLA) and P-8. In group 1, 24 dogs were studied: 3 asymptomatic, 8 oligosymptomatic, 6 polysymptomatic and 7 non-infected control dogs.

Group 2 consisted of beagle dogs ranging in age from 24–30 months, purchased to a local breeder. These animals were kept at the facilities of the National Center for Microbiology in Majadahonda (Madrid). All dogs received routine vaccinations. These dogs were experimentally infected by intravenous (iv) inoculation with 10^8 *L. infantum* promastigotes (MCAN/ES/98/LLM-722). Prior to experimental infection, both the antibody and cellular immune responses to soluble leishmanial antigen (SLA) were confirmed to be negative in all animals. After infection, dogs were monitored for 1.5 years, and a complete characterization of the animals was carried out, including haematological, parasitological and immunological aspects. The animals showed a variable evolution of disease and presented with a range of different clinical conditions: asymptomatic, oligosymptomatic or polysymptomatic, as previously defined [28,29]. 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 14 dogs representative of each clinical group: 5 asymptomatic, 4 oligosymptomatic and 5 polysymptomatic dogs. In addition, 2 non-infected) dogs were employed as controls. Control animals were negative for anti-leishmanial antibody, lymphoproliferative response to SLA and parasitological studies performed.

It should be noted that the species employed at the two sites, *L. infantum* and *L. chagasi,* are considered indistinguishable [30] and cause similar disease in all animal models (mouse, dog, hamster). The approach of this study was to compare the immune responses of animals in different defined clinical states, as determined using standardized observational methods for canine visceral leishmaniasis [28,29] in both inbred and outbred canine subjects and to determine if the responses were consistent across breeds based on clinical status.

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2.2 P-8 purification

The P-8 antigen was purified as described previously [23,25]. Briefly, surface membranes of axenically cultured *L. pifanoi* amastigotes were isolated using nitrogen cavitation and differential centrifugation. Membrane proteins were solubilized with 1% decanoyl-N-methylglucamide (Mega-10, Sigma). The solubilized membranes were reduced and alkylated and then fractionated on Sephadex G-25 (Pharmacia, Piscataway, NJ) gel exclusion chromatography to remove the excess of reagents. The sample was then diluted with PBS and subjected to P-8 immunoaffinity chromatography. The various chromatography columns used in the purification of P-8 PGLC were performed with solutions made using endotoxin-free water; further, column eluents are assessed for endotoxin using a LAL-assay before and after each use. The fractions eluted from the affinity column were assessed for protein by measuring the absorbance at 280 nm and 320 nm; protein fractions were pooled, concentrated and then stored at −70°C. P-8 preparations had <0.05ng/mL of endotoxin using a standard assay for LPS.

2.3. ELISA tests

Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 250 ng of P-8 or 1000 ng of SLA in carbonate buffer (15 mM Na_2CO_3, 28mM $NaHCO_3$, pH 9.6) and blocked with 200 μl of blocking buffer (PBS containing 1 BSA% and 0.1% Tween 20, pH 7.4) for 1 hour at room temperature. Plates were washed three times using PBS (pH 7.4) containing 0.01% Tween 20; then 100 μL of sera diluted 1:100 in dilution buffer (PBS containing 0.1 BSA% and 0.1% Tween 20) were incubated for 30 minutes. Plates were subsequently washed and then incubated for 30 minutes with either horseradish peroxidase (HRP)-conjugated sheep anti-canine IgG (Bethyl Laboratories, Montgomery TX; 1:2500), anti- canine IgG1 (Bethyl Laboratories; 1:800), or anti- canine IgG2 (Bethyl Laboratories; 1:2500). Immune complexes were revealed with 2,2′-azino-bis (3-ethylbenzthioline-6-sulfonic acid) diluted in phosphate-citrate buffer (ABTS; (Sigma-Aldrich, St. Louis MO, USA); absorbance values (405 nm) were read using a microelisa reader (Benchmark, Biorad, USA). Results are expressed as mean values of duplicate samples.

2.4. Cell isolation

PBMC were isolated from heparinized blood samples using standard Ficoll-hypaque gradient centrifugation (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 (0.15 M NaCl, 0.05 M Na_2PO4, 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 fetal calf serum).

2.5. Cell-proliferation assay

Lymphocyte proliferation was evaluated in 96 well plates using 2×10^5 PBMCs stimulated for 6 days with either P-8 PGLC (5 μg/mL) or SLA (10 μg/mL) or for 3 days with 0.2 μg of concanavalin A (ConA) in a final volume of 200μl per well. Cells were pulsed for the final 8 h of culture with 1 μCi of methyl-^{3}H thymidine and counted in a scintillation counter. Results were expressed as stimulation index (net counts per min of stimulated cells/net counts per min of unstimulated cells).

2.6. PBMC stimulation and RNA extraction

PBMC (5×10^6 cells/well) from infected animals (3 asymptomatic, 4 oligosymptomatic and 4 polysymptomatic dogs) and controls (2 non-infected) were incubated with either complete media (unstimulated), 10 μg/ml SLA, 5 μg/ml P-8 or 10 μg/ml ConA in a 6-well plate (Nunc,

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Roskilde, Denmark) 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 per μl with nuclease-free H_2O and stored at −80ºC until use. RNA was free of genomic DNA as determined by PCR.

2.7. Cytokine mRNA quantification by real-time QRT-PCR

Due to the lack of specific antibodies for various canine cytokines, comparative studies of cytokine levels utilized the determination of mRNA rather than protein levels. Although variation between cytokines (in the efficiency of transcription, translation and post-transcriptional processing of cytokine mRNA) does not allow a direct correlation between mRNA and protein levels, for comparisons for a single cytokine (between groups), it would be expected that relative mRNA levels would, in fact, reflect overall relative protein levels. Quantitative Real-Time-PCR analyses were performed by using an Applied Biosystems 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 [31]. Each 25 μl reaction mixture contained 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.

When the response to SLA was examined in different groups of infected dogs, the amount of the target RNA relative to the control gene β-actin, is presented as the $2^{-\Delta CT}$ value. Data presented are the averaged mRNA levels of PBMC from three asymptomatic, four oligosymptomatic, four polysymptomatic and two control non-infected dogs. To examine the effect of specific antigen stimulation (P-8 or SLA) in asymptomatic infected animals, the cytokine expression of individual animals (V-25, V-38 and V-27) is shown. For these determinations, 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 ΔΔCt). 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 but IL-4 mRNA gene expression was measurable after stimulation with SLA, P-8 or ConA. Therefore, for calculation of ΔΔCt in IL-4 gene expression, a Ct value of 35 was assigned to the undetected unstimulated PBMCs.

2.8. 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 $P \leq 0.05$.

3. Results

3.1. Antibody responses to leishmanial antigens

The sera from *Leishmania* experimentally infected dogs (Group 2: asymptomatic, oligosymptomatic and polysymptomatic) collected at 2, 6, 11 and 16 months post-infection, were examined. Serum antibody levels to P-8 and SLA measured by ELISA are shown in Figure 1. In addition, 2 control non-infected dogs (data not shown) were examined; these

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animals were negative for anti-leishmanial antibody, as well as in their lymphoproliferative responses to leishmanial antigens.

SLA-specific IgG antibodies were found in all dogs screened. However, polysymptomatic and oligosymptomatic dogs, in general, showed higher levels than asymptomatic animals; these results may reflect the relative parasite burdens present. Interestingly, high levels of IgG1 antibodies against SLA (Figure 1) were found only in polysymptomatic animals (2 of 3) and appeared to correlate with the progression of disease. In addition, the levels of IgG2 antibodies to SLA trended to be lower in asymptomatic dogs; however, overall the IgG1/IgG2 ratios were higher for the polysymptomatic animals. Although the correlation of IgG subclass responses has been controversial [32–37], these data are consistent with previous studies [35,36]. The pattern of P-8-specific IgG antibody response (Figure 1) with time post-infection was similar to that observed to SLA in the dogs studied; the IgG2 subclass predominated in the sera of the infected animals. Notably although not statistically significant, asymptomatic dogs appeared to develop lower specific P-8- IgG2 antibody responses. Overall, these results indicated an ongoing response to both SLA and the P-8 PGLC during infection.

3.2. Lymphoproliferative response to leishmanial antigens

The antibody responses to P-8 as well as SLA lead to the question of the nature of this response at the cellular level. Hence, the proliferative responses of dogs at 6, 8 and 10 months post-infection were examined and related to their clinical status (asymptomatic, oligosymptomatic and polysymptomatic); results were comparable across the time points. As shown in Figure 2, at 10 months post-infection, PBMC from asymptomatic and oligosymptomatic infected dogs showed antigen specific proliferation to SLA and P-8. In contrast, lymphocyte proliferation in presence of SLA and P-8 was at basal levels in most of the polysymptomatic dogs. Stimulation with ConA produced high lymphoproliferative responses in both asymptomatic and oligosymptomatic infected dogs; however, as they progressed to the polysymptomatic stage, this response decreased along with the response to leishmanial antigens (SLA). As previously observed, the pattern of cellular response to leishmanial antigens appears to be increasingly impaired with progression of illness in canine visceral leishmaniasis [13,38].

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

The study of the cytokine pattern elicited after 24 hours in SLA- stimulated PBMC from dogs with different status of the illness showed that cytokine expression of asymptomatic and oligosymptomatic dogs were, in general, similar. Asymptomatic (P= 0.05) and oligosymptomatic (P= 0.021) dogs significantly up-regulated IFN-γ in comparison to unstimulated PBMC (Figure 3). An up-regulation of TNF-α mRNA in the SLA- stimulated PBMC also occurred in asymptomatic and oligosymptomatic dogs; however, this was only significant in the oligosymptomatic group (P= 0.021). After stimulation with SLA, asymptomatic and oligosymptomatic animals showed a tendency to decrease the IL-10 transcripts below basal levels of the control cells; however, this was not significant. The expression of IL-4 by unstimulated PBMC was below the detection limit. Further, although antigen stimulation produced detectable IL-4 in PBMCs from infected animals; the levels of IL-4 did not differ significantly among clinical groups (data not shown). In the case of IL-18, elevated levels were observed in polysymptomatic animals; however these differences did not appear to be statistically significant (Figure 3).

3.4. Expression of cytokine transcripts in P-8 antigen-stimulated PBMC from L. infantum experimentally infected and control dogs

To investigate whether the *L. pifanoi* P-8 antigen could stimulate cells from *L. infantum* infected dogs to produce specific cytokines, we examined the induction of mRNA for cytokines

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known to be relevant to the pathogenesis or control of visceral leishmaniasis. The capability of P-8 to induce a specific cytokine profile was studied in asymptomatic dogs (Group 2) and compared to the cytokine profile induced by SLA, as asymptomatically infected dogs presented with a distinct but preferential Th1-like response. This provided an internal control (standard) for this response and allowed a comparison of the mixture of leishmanial antigens to that of the isolated P-8 proteoglycolipid complex. PBMC from three asymptomatic dogs were stimulated with SLA or P-8 antigen for 24 hours and the levels of cytokine mRNA were determined.

The stimulation with leishmanial antigens produced an up-regulation of the IFN-γ mRNA abundance in PBMC from asymptomatic animals (Figure 4). The incubation with P-8 induced 168.9 to 442.6- fold increase in the IFN-γ gene expression compared to unstimulated cells. However, the IFN-γ transcript abundance of SLA- stimulated cells increased 34.8 to 138.1-fold (Figure 3). Therefore, *L. pifanoi* P-8 antigen induced a 3 to 4-fold higher level of IFN-γ expression than the *L. infantum* soluble antigen (SLA) in the asymptomatic *L. infantum* infected dogs.

P-8 antigen also produced an up-regulation of the TNF-α gene expression in PBMC from asymptomatic dogs (4.6–11.2-fold increase) (Figure 4). This enhancement of expression was again higher than that observed for SLA stimulation of these PBMC (1.9–3.3- fold increase). Surprisingly, healthy (non-infected) dogs presented an unspecific mRNA production after P-8 stimulation (4.4 to 5.0- fold increase), whereas no TNF-α expression was observed in response to incubation with SLA (1.1- fold increase in both C-1 and C-2).

In general IL-10 and IL-18 mRNA transcript abundances in PBMCs stimulated with either SLA or P-8 were similar to those found for unstimulated cells. IL-4 gene expression levels (data not shown) after stimulation with P-8 showed 7.4–65.8- fold increases compared to unstimulated PBMCs; these were comparable to those found for SLA stimulation (14.8–61.8-fold increase).

4. Discussion

The ability of vaccine candidate molecules to elicit an immune response in infected hosts is indicative of antigen presentation during infection and might be considered to be a minimal requirement for any vaccine candidate molecule. If such molecules also elicit a Th1-like cytokine response (IFN-γ, TNF-α) during an ongoing infection, it might be indicative of protection. In the present study, P-8 PGLC has been found to elicit such a response. This is similar to observations for other vaccine candidates which induce the expansion of Th1-type T-cells producing interferon (IFN)-γ cytokine in vaccinated dogs [17] and are protective against infection.

The stimulation with the leishmanial antigens P-8 PGLC and SLA produced an up-regulation of the IFN-γ mRNA abundance in PBMC from asymptomatic animals. The increase in IFN-γ transcripts in SLA- stimulated PBMC, contrasts with the lack of IFN-γ induction in polysymptomatic dogs. Experimentally *L. infantum* infected beagle dogs that exhibited mild clinical signs (oligosymptomatic) showed strong PBMC proliferation, IFN-γ production and mRNA expression in response to stimulation with *L. infantum* antigen (SLA) [39]. While PBMC from naturally infected polysymptomatic dogs still proliferate in response to SLA, this is minimal and IFN-γ production in response to *Leishmania* antigen was completely abolished [39]. In the canine model, the expression of IFN-γ from PBMCs correlates with disease resistance/asymptomatic status in non-vaccinated animals [17,40]; further, IFN-γ is observed to increase and correlate with protection in vaccinated dogs [17,22,41]. In humans, IFN-γ has been shown to be a mediator of resistance to the parasites because of its ability to induce killing

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of the parasite by macrophages; further, IFN-γ levels increase after treatment [42]. These results are consistent with murine model studies where IFN-γ has been shown to be associated with control of cutaneous [43] and visceral leishmaniasis [44].

The effect on IL-4 gene expression after stimulation with P-8 and SLA was low in asymptomatic dogs. In the current study, we have found IL-4 mRNA transcription after SLA stimulation in all the stages of the infection. Although previous reports have found that early detection of IL-4 appeared to be correlated with disease progression [13,38], the role of this cytokine in canine VL remains controversial [38]. Further studies are necessary in order to determine the role of IL-4 in susceptibility. In asymptomatic, experimentally infected dogs we have found that values of IL-10 transcription after P-8 or SLA stimulation were near to those of unstimulated dogs. IL-10 has been related to progressive disease in human visceral leishmaniasis [45] and was shown to play a role in susceptibility to VL in hamster and murine models [46]. However in dogs experimentally infected with *L. infantum*, IL-10 transcripts were have not been generally observed except occasionally late in infection [47]. Furthermore, IL-10 mRNA accumulation in infected tissues of naturally infected dogs, including those with severe disease, was comparable with that of uninfected control dogs [38]. Thus, IL-10 does not seem to have a predominant negative immunoregulatory role in canine visceral leishmaniasis, as has been previously described in Indian kalaazar [48,49] or the murine model [50].

Abundance of IL-18 mRNA transcripts was similar in stimulated and unstimulated cells, suggesting that this cytokine may not to be necessary for the development of a protective cellular response in canine leishmaniasis. IL-18 alone cannot induce Th1 differentiation, but does facilitate/accelerate it [51]. Although IL-18 was initially identified as a potent IFN-γ-inducing factor in T cells and NK cells [52], recently it has been shown to enhance both innate immunity and promote both Th1- and Th2- driven immune responses, leading to protective immunity in murine VL [53–55]. However, in canine leishmaniasis, a negative relationship between IL-18 expression and clinical status was found in bone marrow aspirates from naturally infected dogs [38]. In the current study, no clear associations could be established between dog resistance or susceptibility to VL and IL-18 expression levels.

P-8 antigen elicited a higher expression of TNF-α in PBMC from asymptomatic dogs compared to SLA. Surprisingly, healthy non-infected dogs also produced TNF-α mRNA after P-8 stimulation. However, it has been observed that P-8 PGLC is capable of inducing TNF-α and IL-1 (but not IL-10, IL-8) from uninfected murine macrophages (Whitaker, Colmenares and McMahon-Pratt, unpublished data). Consequently, although the precise cell population has not been determined here, it is possible that a similar induction within canine PBMCs has occurred. As observed in the current study, higher TNF-α levels have been observed during asymptomatic infection in the canine model [40,56,57]. These higher levels of TNF-α are consistent with murine leishmaniasis model studies of both cutaneous and visceral diseases [58,59], where TNF-α has been shown to enhance the effects of IFN-γ in mediating parasite killing. These results suggest that TNF-α is important in the resolution of canine visceral leishmaniasis and may be useful for evaluation of potential vaccine candidate molecules.

While it is generally accepted that cellular, rather than humoral immunity, plays an important role in host defense against leishmaniasis a few studies have shown that antibodies are instrumental in providing resistance to many intracellular pathogens [60]. The actual contribution of antibodies to the protective response in leishmaniasis is still a question hotly debated. In murine leishmaniasis a negative regulatory role at the level of the macrophage has been associated with IL-10 production [61]; however, recent studies have suggested a potentially positive role for the modulation of dendritic cell function [62]. Further, specific subclasses of immunoglobulin (IgG2a, IgG1) are considered to correlate with Th1-like and Th2-like responses. It is known that IgG2a elicitation is regulated by IL-12 induced production

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of IFN-γ [63], and IgG1 by IL-4 [64]. However, this dichotomy is not absolute and it has been observed that IL-12 can induce enhanced IgG1 production [65–67], suggesting the possibility that IgG1 and IgG2a could work in tandem rather than acting antagonistically. Given the lack of regulation by IL-10 in the canine model, the role of antibody responses in mounting successful protective immune response against visceral leishmaniasis requires further investigation. The challenge ahead in terms of vaccine development is to understand how the various parts of the immune system work collectively [68] in the canine model.

In this study, we have examined the antigenicity of the antigen P-8 in canine visceral leishmaniasis. The higher lymphoproliferative response in asymptomatic dogs in comparison to symptomatic dogs and level of IFN-γ expression induced by P-8 in asymptomatic dogs, suggest that this antigen may be involved in protection and thus, represents a potential vaccine candidate for the control of canine leishmaniasis.

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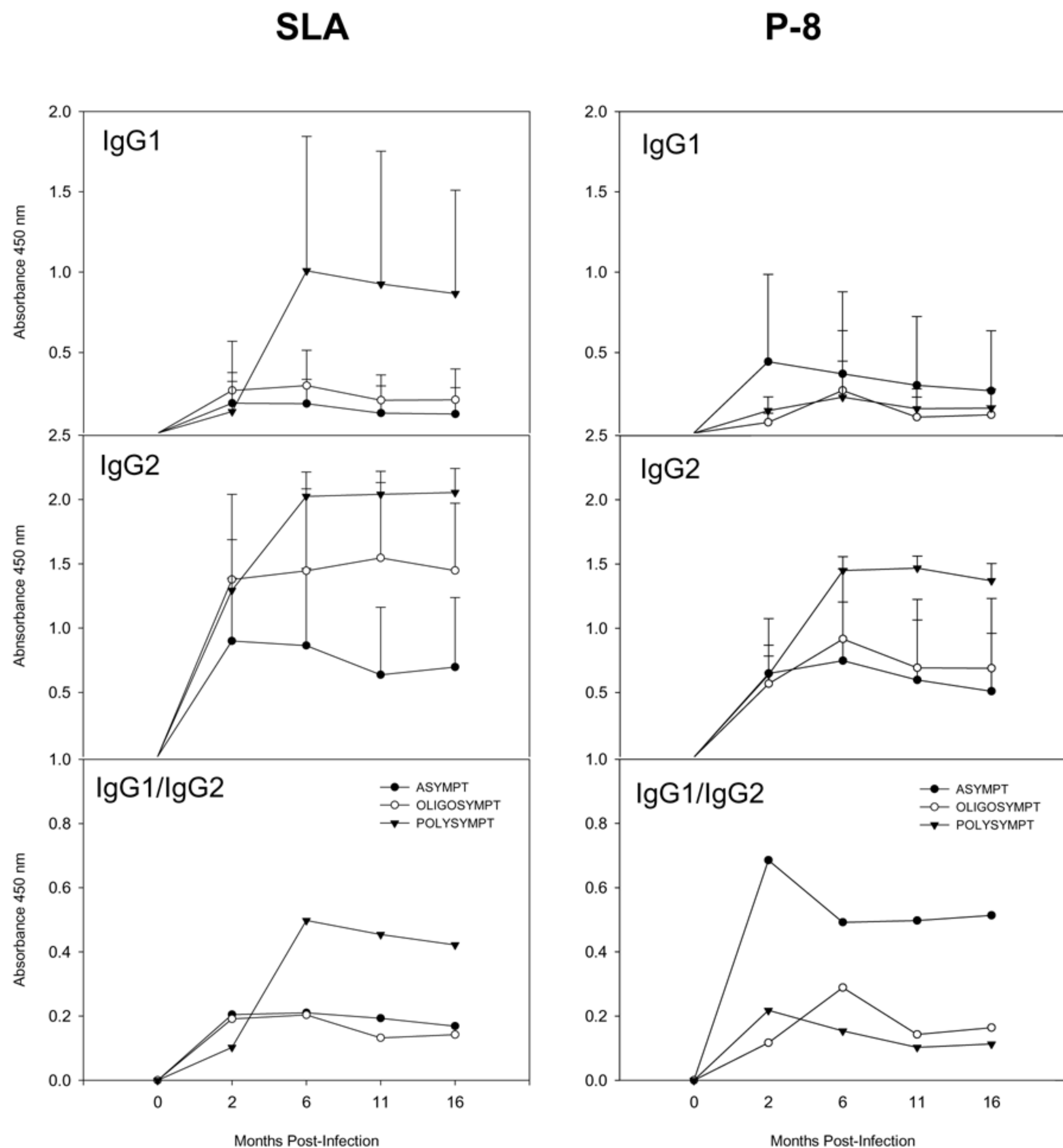

Figure 1.
Antibody responses in infected dogs (Group 2) to soluble leishmanianial antigens (SLA) and the P-8 proteoglycolipid complex (P-8 PGLC). Shown are the antibody responses A) IgG1; B) IgG2 and C) Ratio: IgG1/IgG2 of: ▼ — ▼**,** polysymptomatic; ○— ○, oligosymptomatic; and ●— ● asymptomatic infected dogs at the indicated times post-infection. The antibody responses were examined in 5 asymptomatic, 3 oligosymptomatic and 3 polysymptomatic dogs (P-8) and for 5 asymptomatic, 4 oligosymptomatic and 5 polysymptomatic dogs for SLA antigen.

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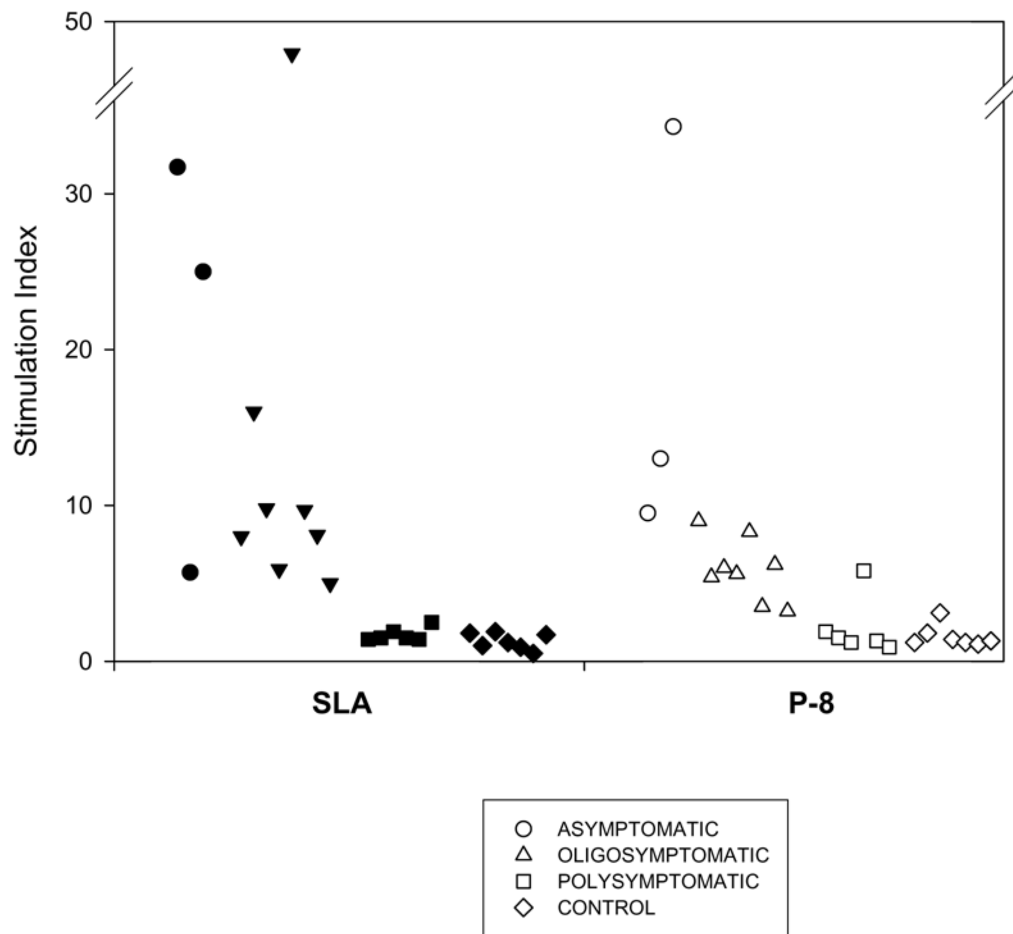

Figure 2.

Proliferative responses of infected dogs with different clinical outcomes. Shown are the proliferative responses at 10 month post-infection of PBMCs from polysymptomatic (6; ■, □), oligosymptomatic (8; ▲, △) and asymptomatic (3; ●, ○) infected dogs and control (7; ◆, ◇) dogs at 10 months post-infection to either soluble leishmanial antigens (SLA) or the P-8 proteoglycolipid complex. The responses observed at 10 months post-infection were similar to those observed for these animals at both 6 and 8 months post-infection.

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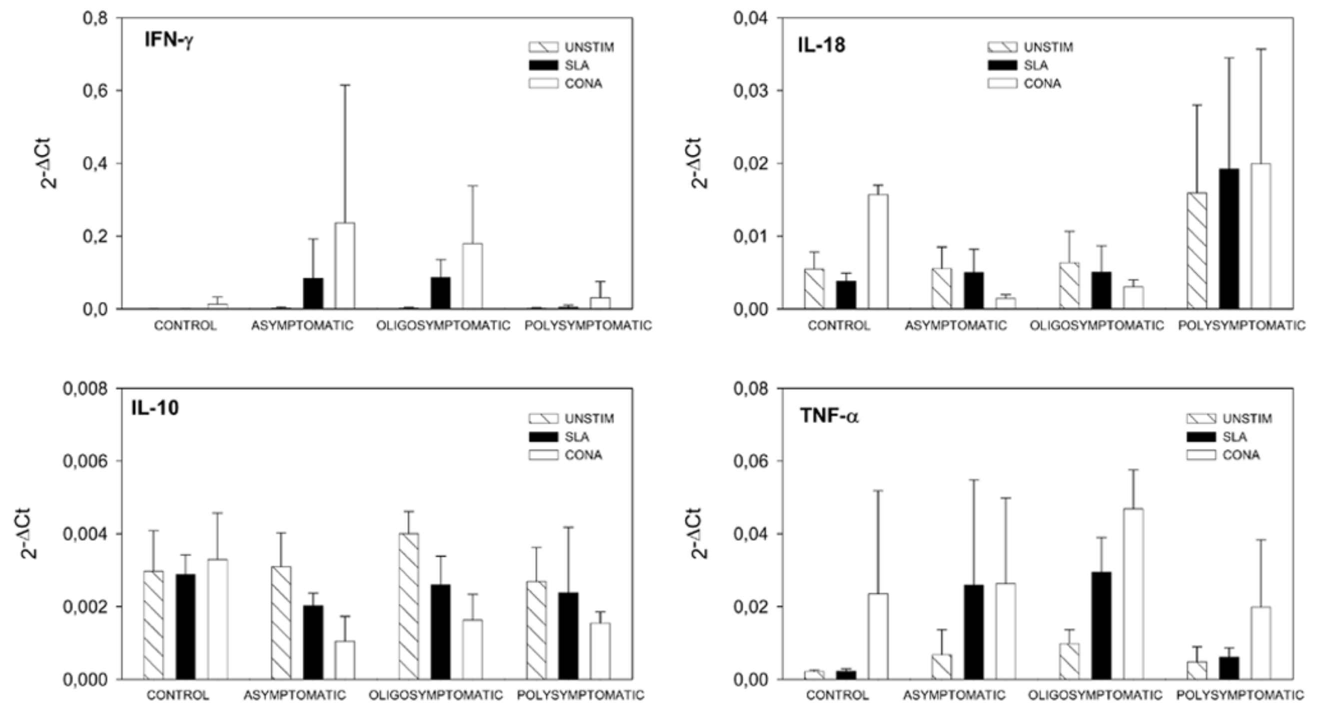

Figure 3.

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. RNA isolation and QRT-PCR were performed after 24 hours of stimulation as described in Material and Methods. QRT-PCR data were analysed by using the $2^{-\Delta Ct}$ method. The average of the mRNA levels of PBMC from three asymptomatic, four oligosymptomatic, four polysymptomatic and two control non-infected dogs are presented. Striped bars represent unstimulated PBMCs, black bars represent SLA and open bars represent ConA. A) IFNγ; B) TNF-α; C) IL-10; D) IL-18. Cytokine responses were examined in: 3 asymptomatic, 4 oligosymptomatic, 4 polysymptomatic dogs and 2 control dogs.

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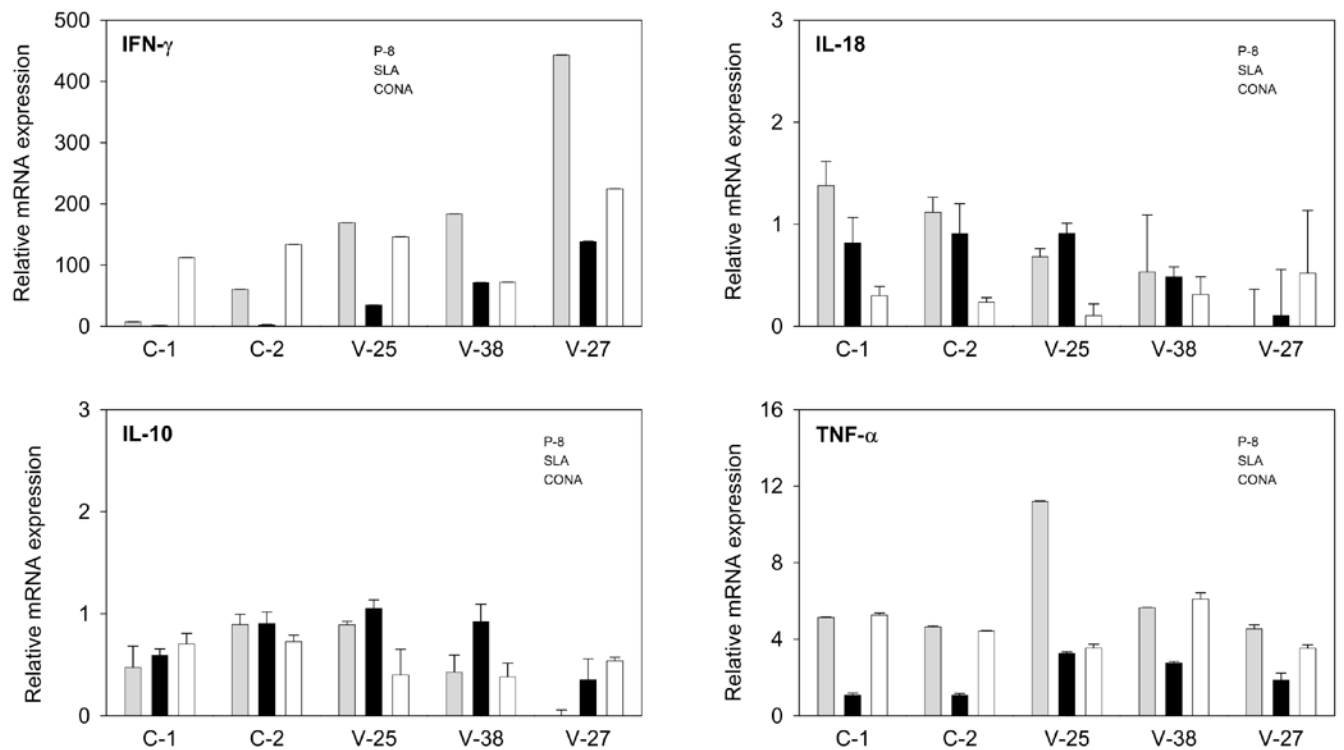

Figure 4.

Cytokine gene expression in PBMC from asymptomatic *L. infantum* infected dogs and healthy control dogs (Group 2) after stimulation with P-8 antigen from *L. pifanoi*. QRT-PCR data were analysed 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; IL-18) for each infected animal (V-25, V-38 and V-27) as well as controls (C-1, C-2). Grey bars represent results from P-8 stimulation, black bars represent SLA stimulation and open bars represent ConA stimulation. Standard deviations are indicated and range from 0.0001 to 0.300.