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Protective immunity against challenge with *Leishmania* (*Leishmania*) *chagasi* in beagle dogs vaccinated with recombinant A2 protein

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ABSTRACT

In this study, we investigated in dogs the immunogenicity and protective immunity against *Leishmania* (*Leishmania*) *chagasi* infection induced by vaccination with a formulation containing the recombinant A2 protein, an amastigote specific antigen, and saponin. Vaccinated animals produced significantly increased levels of total IgG and IgG2, but not IgG1 anti-A2 antibodies, and remained negative in conventional leishmaniasis serodiagnostic methods. Significantly increased IFN-γ and low IL-10 levels were detected in vaccinated animals before and after challenge, as compared to control animals. Importantly, while the symptoms onset appeared as early as three months after infection in most control dogs, 14 months after challenge, 5 out of 7 vaccinated dogs remained asymptomatic. Therefore, immunization with rA2 antigen was immunogenic and induced partial protection in dogs, and allowed the serological differentiation between vaccinated and infected animals, an important requirement for a canine visceral leishmaniasis (CVL) vaccine.

1. Introduction

Leishmaniasis is an infectious disease with an overall prevalence of 12 million cases and 350 million people at risk of infection [1]. *Leishmania* parasites are transmitted through the bite of an infected sand fly to vertebrate hosts, leading to a spectrum of clinical manifestations, including visceral leishmaniasis [1]. *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *chagasi*, which are now considered synonymous species [2], and *Leishmania* (*Leishmania*) *donovani* are the major etiologic agents of visceral leishmaniasis (VL), a fatal infection if diagnosis and treatment are not promptly established. Dogs are highly susceptible to infection and considered the major reservoir for *L.* (*L.*) *infantum* and *L.* (*L.*) *chagasi*, in different geographical regions of the globe [3]. Canine visceral leishmaniasis (CVL) is assuming increasing importance in countries around the Mediterranean Basin and in the Americas. Research has recently revealed much new information, but advances in treatment and control have been disappointing. Treatment of infected dogs is of limited effectiveness and not recommended in endemic regions, since dogs that respond to treatment may still be a source of parasites [3,4].

The epidemiological control of VL depends of actions against the vectors (sand flies) and reservoirs, mainly the domestic dogs. Therefore, the development of a protective vaccine against CVL is an alternative approach for interrupting the VL domestic cycle [3,5]. Ideally, this vaccine should reduce symptoms, tissue parasitism as well as vector transmission rates. In addition, it is recommended that it allows for the serological differentiation of vaccinated and infected dogs, by means of serological tests using promatigote

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antigens, i.e. indirect immunofluorescence (IFI) or ELISA, since culling of sero-positive dogs is a major VL control measure adopted in endemic areas in Americas.

The A2 antigen has been identified in *L.* (*L.*) *donovani* and represents the first amastigote-specific virulence factor identified in *Leishmania*. A2 genes are present within a multi-gene family that code for A2 proteins ranging from 45 to 110 kDa, which are required for *Leishmania* survival in mammalian hosts [6–8]. A2 antigens, administered as recombinant protein or DNA, are protective against *L.* (*L.*) *donovani*, *Leishmania* (*Leishmania*) *amazonensis* and *L.* (*L.*) *chagasi* infections in mice [9–12]. Anti-A2 antibodies have been detected in sera samples from patients with active VL, confirming that A2 proteins are expressed during active infection [13,14]. Anti-A2 antibodies are also detected in sera of infected dogs [14], including symptomatic and asymptomatic animals, though higher numbers of asymptomatic animals are reactive as compared with the symptomatic dogs [15]. Moreover, A2 antigen was one of the antigens identified through a *L.* (*L.*) *chagasi* T-cell antigens screening [16], indicating the presence in A2 antigen of T cell epitopes, an important requirement for induction of protection against leishmaniasis.

In this work, we have investigated the protective effect of vaccination with the recombinant A2 (rA2) protein against *L.* (*L.*) *chagasi* infection in dogs. Our findings demonstrated that immunization with rA2 induced type I immune responses, protected a significant number of dogs from developing symptoms and delayed symptoms on those that developed CVL. Finally, vaccination with rA2 did not convert serological tests using promatigote antigens, allowing for discrimination between vaccinated and infected animals, an important requirement for a public campaign control of CVL.

2. Materials and methods

2.1. Animals

The study included twenty-one 3–9-months-old beagle dogs, purchased from dog breeders located in non-endemic areas of Brazil. After a quarantine period, only healthy and well-fed animals under constant scrutiny for health problems by veterinarian, presenting normal hematological and biochemical parameters and that had received their routine vaccinations against leptospirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and parvovirosis were included in this study. Dogs were also treated with anti-helmintic drugs. The dogs were maintained according to the International Guiding Principles for Biomedical Research Involving Animals. The experimental design and all the conditions of animal maintenance and handling were approved by the Brazilian Public Animal Health and Agriculture authorities (MAPA 21028.007698/2003-15). Animals were maintained under conditions designed to exclude any possible contaminating *Leishmania* infections, after approval of experimental design and conditions by the local public health authorities. Kennel was sprayed with pyrethroid insecticide and all its extension was protected throughout appropriated and security stainless steel gauze as recommended by Brazilian Ministry of Health. During all the experiments, veterinarians followed animals and all the invasive procedures were performed following the rules of ethical procedures in animal experimentation and biosafety. All of them were negative for the presence of *Leishmania* antibodies as detected by indirect immunofluorescence (IFI titles lower than 1:40 dilutions) and ELISA. Assays were performed using negative and positive control sera. In ELISA, cut off values were determined from average optical densities plus two standard deviations, obtained from negative sera.

2.2. Immunization

Dogs ($n = 14$) were immunized subcutaneously on days 0, 21 and 42 with a vaccine, consisting of 100 μg of A2 recombinant protein (rA2), purified as previously described [14], and of 250 μg of Saponin (Riedel). Of those, 7 animals were challenged, constituting the group VI and 7 remained uninfected, composing the group VNI. Control animals received either PBS ($n = 4$) or Adjuvant ($n = 3$), composing the groups PBS and Infected and Adjuvant and Infected.

2.3. Parasites, total protein extract (LcPA) preparation and infection

Promastigotes of *L.* (*L.*) *chagasi* (MCAN/BR/2000/BH400) were grown at 23 °C in α-MEM medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 200 U of penicillin/ml (Sigma), 100 μg of streptomycin/ml (Sigma) at pH 7.4. Total protein extracts (LcPA) of *L.* (*L.*) *chagasi* promastigotes (LcPA) were prepared from stationary phase promastigotes, submitted to 5 cycles of freezing (liquid N_2) and thawing (42 °C), followed by ultrasonication (Ultrasonic processor, GEX600), with cycles of 10 s for 2 min at 35 MHz. The extracts were then submitted to centrifugation at 8000 × *g* for 20 min at 4 °C. The supernatant was collected and stored at −70 °C.

For challenge infection, 5×10^7 late-log-phase promastigotes of *L.* (*L.*) *chagasi* were injected intravenously into each dog, four weeks after the last vaccine dose.

2.4. ELISA for specific IgG, IgG1 and IgG2 isotypes

Levels of specific immunoglobulin IgG, IgG1 and IgG2 isotypes were measured by ELISA before vaccination, after each vaccine dose and at each three months over a period of 14 months after vaccination. Briefly, 96-well plates (Costar®) were sensitized with either rA2 (250 ng/well) or total protein extracts (LcPA) of *L.* (*L.*) *chagasi* promastigotes (1 μg/well), in coating buffer pH 9.6 (Na_2CO_3 0.015 M, $NaHCO_3$ 0.03 M) overnight at 4 °C. Plates were blocked with PBS/2% casein at 37 °C for 1 h and treated successively with 1:100 dilutions of canine serum samples for 2 h at 37 °C. Peroxidase-labeled antibodies specific to canine IgG, IgG1 or IgG2 isotypes (Sigma) were diluted at 1:5000 or 1:6000, respectively, and added for 2 h at 37 °C. The plates were washed six times with PBS/Tween 20 0.05% and incubated with H_2O_2 and *o*-phenylenediamine for reaction development. Reactions were stopped by the addition of 20 μl of H_2SO_4 2N (Merck). Optical densities were determinated at 492 nm in ELISA reader (BioRad, Model 2550, CA, USA).

For evaluation of specificity of anti-A2 total IgG and IgG2 antibodies elicited in vaccinated animals, 96-well plates (Costar®) were sensitized with 8 μg/well of an A2 derived peptide (VGPQSVGPLSVGPQSVGPLS), at 37 °C for 1 h and then overnight at 4 °C. Sera of vaccinated animals were diluted 1:20 and peroxidase-labeled antibodies specific to canine total IgG or IgG2 isotypes were diluted 1:6000.

2.5. Peripheral blood mononuclear cell isolation and cytokine production assay

Levels of gamma interferon (IFN-γ) and IL-10 were assessed in vaccinated and control animals one month after the three vaccine doses, before infection and also seven months after challenge infection. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood, homogenized 1:1 with PBS at room temperature, layered over Ficoll (Histopaque®; Sigma) and submitted to centrifugation at 800 × *g*, for 20 min at 18 °C. Red blood cells were lysed by addition of cold sterile water for 45 s, followed by

the addition 3× PBS to stop cellular lyses. Cells were submitted to centrifugation at 600 × *g*, for 15 min at 4 °C. The white blood cells ring was collected and ressuspended in DMEM medium (Dulbecco's Modified Eagle's Medium; Sigma), supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamina, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) (Sigma), 50 μM 2-mercaptoethanol, 200 UI/ml penicillin and 100 μg/ml streptomycin (all from Sigma) at pH 7.2. 1 ml of cell suspension (2×10^6 ml^{-1}) was plated in duplicated 24-well culture plates (Nunc). Isolated PBMC were incubated for 48 h in the presence of 5 μg/ml of ConA (Sigma), 10 μg/ml of antigen A2, 50 μg/ml of LcPA or in the absence of exogenous stimuli at 37 °C and 5% CO_2. After incubation, the supernatant was collected and stored at −70 °C. Levels of IFN-γ and IL-10 in supernatants were assessed by sandwich ELISA (Duoset ELISA Canine IFN-γ and Duoset ELISA canine IL-10; R&D Systems). A titration curve was performed to determine the best supernatant dilutions for cytokine assays. For IFN-γ measurements, culture supernatants were diluted 1:5. Detection limits are 62.5–2000 pg/ml for the canine IFN-γ and 15.6–500 pg/ml for IL-10, according to the manufacturer.

2.6. Clinical and laboratorial evaluation of dogs

Biochemical and haematological analysis were preformed in animals monthly and considered by veterinarians for clinical evaluation of the animals. Whole blood cell counting were performed by a semi-automated blood cell counter (Abaccus Júnior, Diatron, Austria) and differential leukocyte counting were analyzed in blood smear stained with May Grünwald-Giemsa®, according to Jain [17]. Albumin, alpha-(α), beta-(β) and gamma-(γ) globulin fractions and the ratio albumin/globulin were evaluated in dog's sera and references values given by Amusategui et al. [18], were used to calculate protein concentrations. Serum levels of alanine-amino transferase (ALT) and aspartate aminotransferase (AST) and creatinine and urea were measured to evaluate liver and renal functions, respectively. References values to interpret the parameters of the liver and kidney functions were from Kaneko et al. [19]. All the biochemical analysis were performed by the Cobas® equipment using the diagnostic kits from LabTeste® (Brazil). Independent clinical veterinarians carried out blindly the examination of the dogs for endpoint determination of CVL. Animals were clinically classified, according to Mancianti et al. [20], and Lima et al. [21], as follows: (1) symptomatic dogs exhibited classical signs of the disease such as cutaneous alterations (alopecia, dry exfoliative dermatitis or ulcers), onychogryphosis, keratoconjunctivitis, cachexia and anemia; (2) oligosymptomatic dogs exhibited some clinical signs of the disease and/or lesions such as lymphadenopathy, moderate weight loss and/or dull brittle hair accompanied by cutaneous lesions; (3) asymptomatic dogs showed none of the above clinical symptoms.

2.7. DNA extraction

DNA was extracted either from blood samples collected in FTA® cards (Whatman International Ltd.) or from ear skin biopsy specimens collected from each dog, 6 and 9 months after infection. Skin biopsy specimens, measuring approximately 1.0 mm^3, were mounted in paraffin blocks. For DNA extraction from blood samples, punches obtained from FTA® cards were washed twice with 200 μl FTA Purification Reagent and twice with 200 μl TE buffer (Tris 10 mM, EDTA 1 mM; Sigma), as recommended by the manufacturer (Whatman International Ltd.). Skin biopsy slices from paraffin-mounted samples were obtained and washed in ethanol (Merck) and then xylol (Merck). Biopsy fragments were incubated with proteinase K (Life Technologies) at a final concentration of 100 μg/ml, for 3 h at 56 °C, followed by heat inactivation at 100 °C for 15 min. DNA was extracted using a Wizard TM Genomic Purification Kit (Promega), according to the manufacturer's instructions.

2.8. Polymerase chain reaction (PCR)

PCR reactions performed with primers derived from a repetitive sequence of the *L.* (*L.*) *infantum* genome and that are specific for *L.* (*L.*) *infantum*, *L.* (*L.*) *chagasi*, and *L.* (*L.*) *donovani* (5′-ACG AGG TCA GCT CCA CTCC-3′ and 5′-CTG CAA CGC CTG TGT CTA CG-3′), according to Piarroux et al. [22]. The reaction mixture consisted of 1× DNA polymerase buffer (Invitrogen) containing 1.5 mM of $MgCl_2$, 5 mM KCl, 75 mM Tris–HCl, pH 9.0, 2.0 mM $(NH_4)_2SO_4$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 μM of each primer (Promega) and Taq polymerase (Invitrogen) (1.5 units), in a final volume of 20 μl. The conditions for PCR amplification were as follows: 94 °C for 5 min, 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. In all assays, a negative control without DNA was included. PCR samples were submitted to electrophoresis on 6% polyacrylamide gel and silver-stained.

2.9. Culture of bone marrow aspirated fluid

Bone marrow samples were collected 6 and 9 months after challenge infection from each dog. Samples were mixed with 3 ml of culture medium (10% heat inactivated fetal bovine serum; Sigma®) in "Novy-MacNeal-Nicolle" NNN medium. The mixture was then incubated for 7 days at 23 °C and was examined by microscopy. When no *Leishmania* cells were found, 1 ml of the culture samples was sub-cultured onto the same medium for another 7 days at 23 °C. This procedure was repeated once more before confirming a negative result.

2.10. Statistical analysis

All data comparisons were tested for significance by using Kruskal–Wallis or accurate Fisher tests. Differences were considered statistically significant when p values < 0.05.

3. Results

3.1. Vaccine and Leishmania-specific serological responses

Levels of anti rA2 or anti-LcPA antibodies were examined in sera samples collected before vaccination, after each vaccine dose and over a period of one year after vaccination. Levels of anti-A2 specific total IgG (Fig. 1A) and IgG2 (Fig. 1B) antibodies increased after each vaccine dose and infection in the groups of animals vaccinated with rA2 and the higher levels were observed 1 month after infection and at the end of the study, but not in the control-infected animals that received PBS or only adjuvant. Interestingly, significantly increased levels of anti-A2 specific IgG2 antibodies ($p = 0.0001$) were detected in vaccinated groups shortly after vaccination (after the second dose). Moreover, as shown in Fig. 1C, anti-A2 IgG2 antibodies present in sera of vaccinated animals recognized an A2-derived peptide, when compared to sera of animals before vaccine doses ($p = 0.002$), attesting for the specificity of the antibody response elicited by vaccination. Similar results were obtained for anti-A2 total IgG antibodies (data not shown). In contrast, no significant alterations in anti-A2 IgG1 (Fig. 1D) antibodies were detected either in VNI (vaccinated and non-infected dogs) and VI (vaccinated and infected) groups or I (infected) and ADI (adjuvant and infected) control groups, suggesting the development of Th1 specific immune responses in vaccinated animals.

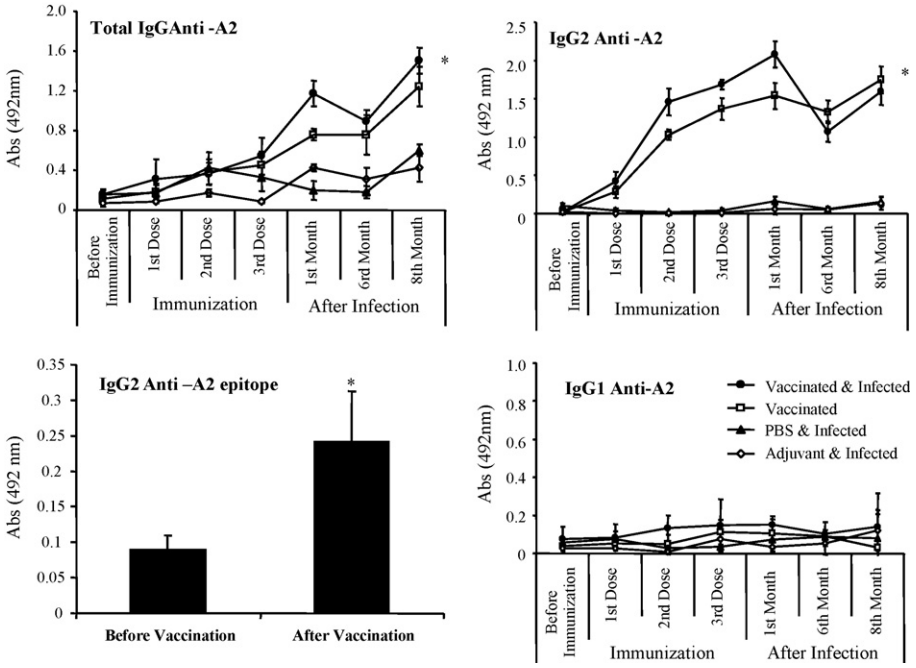

Fig. 1. Vaccine specific serological responses. Panels (A, B and D) show the evolution of levels of anti-A2 IgG, IgG2 and IgG1 antibodies, respectively, before and after vaccination and after challenge, as detected by ELISA. Plates were coated with 250 ng/well of rA2 and sera samples were diluted 1:100. Each point represents average ± standard error. Panel (C) shows levels of anti-A2 IgG2 antibodies, as detected by ELISA using an A2-derived peptide (8 μg/well) and sera samples diluted 1:20. Each bar represents average ± standard deviation of optical densities. The asterisks indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

During vaccination, no significant increases in anti-LcPA total IgG antibodies (Fig. 2A) were observed in any of the study groups. Increased levels of anti-LcPA total IgG antibodies ($p = 0.01$) were detected only after 3 months p.i. in animals of the infected groups (values above cut off appeared in the first month p.i. in animals of the I group and after 3 months p.i. in animals of the VI and ADI groups). Six months p.i., significant differences ($p = 0.01$) were observed for levels of anti-LcPA total IgG antibodies either between vaccinated and vaccinated-infected, vaccinated and PBS-infected or vaccinated and adjuvant-infected groups. A similar pattern ($p = 0.03$) was observed for levels of anti-LcPA IgG2 antibodies (Fig. 2B). In contrast, increased levels of anti-LcPA IgG1 (Fig. 2C)

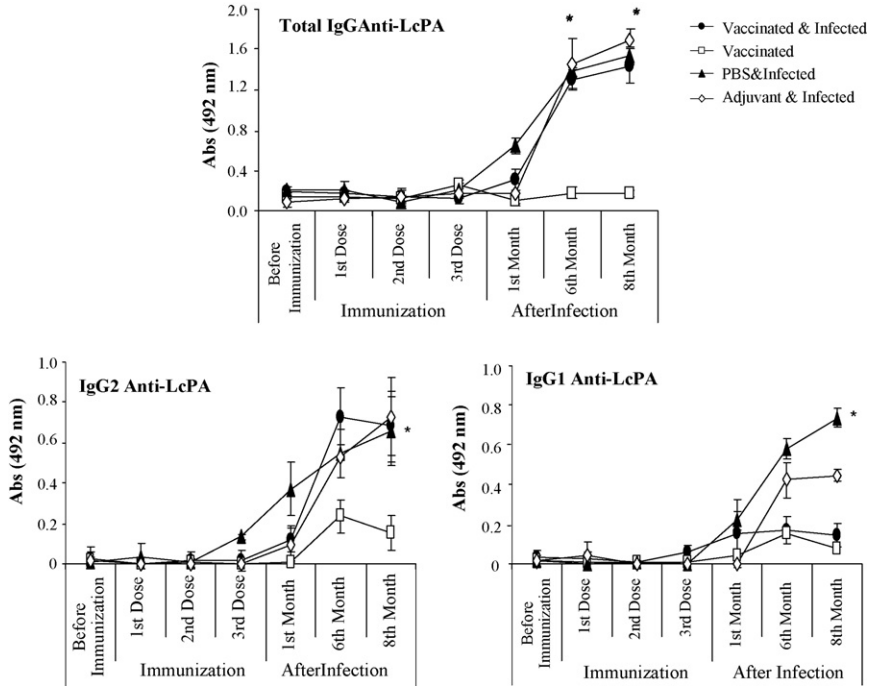

Fig. 2. Leishmania-specific serological responses. Panels (A–C) show the evolution of levels of anti-LcPA IgG, IgG2 and IgG1 antibodies, respectively, before and after vaccination and after challenge, as detected by ELISA. Plates were coated with 1 μg/well of LcPA and sera samples were diluted 1:100. Each point represents average ± standard error. The asterisks indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

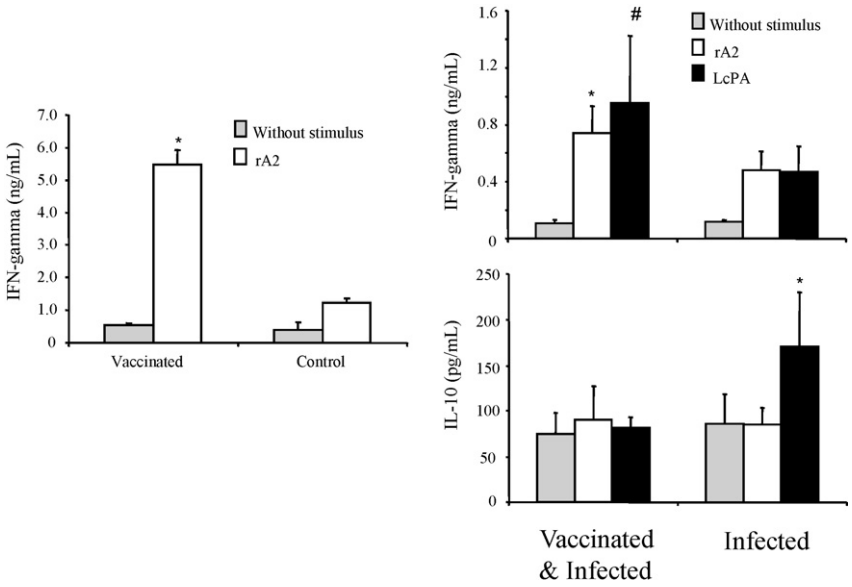

Fig. 3. Cytokine levels detected in culture supernatants of canine PBMC. Panel (A) shows IFN-γ levels detected after immunization and before infection in PBMC culture supernatants stimulated with rA2. Panel (B) shows levels of IFN-γ detected in PBMC culture supernatants produced in response to rA2 or LcPA, seven months after infection. Panel (C) shows levels of IL-10 in response to rA2 or LcPA seven months after infection. The asteriscs and # indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

antibodies were detected only in animals of the adjuvant-infected ($p = 0.025$) and PBS-infected groups ($p = 0.03$). Thus, animals that received rA2 and remained negative for anti-LcPA antibodies after vaccination, while those that were vaccinated but not infected remained negative during the follow-up period. These findings indicate that vaccination with rA2 allows the serological distinction between vaccinated and infected dogs.

3.2. IFN-γ and IL-10 productions by peripheral blood mononuclear cell (PBMC) of immunized dogs

We determined whether immunization with rA2 resulted in increased IFN-γ or IL-10 production in response to rA2 or LcPA stimulation. As shown in Fig. 3A, PBMC from dogs vaccinated with rA2, before infection but after three vaccine doses, secreted significantly higher levels of IFN-γ ($p = 0.003$) when stimulated with A2 than PBMC collected from control infected dogs (I and ADI groups). Before challenge, IL-10 was not detected in vaccinated or in control animals in response to rA2 or LcPA. Seven months after infection, significantly higher levels of IFN-γ, as compared to control animals, were also produced by PBMC from dogs vaccinated with rA2, when stimulated either with rA2 ($p = 0.025$) or LcPA ($p = 0.003$) (Fig. 3B), indicating that these animals displayed not only a sustained IFN-γ production after vaccination, but responded with an increased IFN-γ production to total parasite antigens after infection. Additionally, the production of IL-10 (Fig. 3C) was not significantly higher in the rA2-vaccinated dogs as compared to control animals, following stimulation with rA2. In contrast, seven months after infection (Fig. 3C), stimulation with LcPA resulted in a significantly increased IL-10 production ($p = 0.03$) by PBMC of control-infected animals, as compared to the vaccinated-infected group.

3.3. Biochemical, haematological, clinical and parasitological findings

The main clinical and parasitological features presented by all animals are summarized in Table 1. Clinical signs of VL appeared earlier (3–6 months p.i.) in control-infected animals (I) as compared to the Vaccinated and Infected (VI) group (1 year p.i.). In addition, 71.5% of the control-infected dogs were symptomatic, displaying more intense VL signs such as profuse blood diarrhea, intense loss of weight and cachexia. Some animals in this group presented a progressive form of the disease, showing multiple and severe symptoms that required, in some cases, that the animal be killed ($n = 3$) at nine months after infection. In contrast, in the VI group only 28.5% of the dogs showed signs of the active disease. In fact, they presented a moderate loss of weight, alopecia and mild lymphadenopathy, symptoms that appeared 12 months p.i. The remaining VI animals were considered asymptomatic. Asymptomatic animals in this group remained in this condition to the end of the follow-up period (14 months after challenge).

Experimental infection also induced alterations in biochemical and haematological parameters. As shown in Table 2, significant differences were observed only in the control infected animals (I) for average leukocyte counts ($p = 0.005$), albumin/globulin ratios ($p = 0.022$) and AST levels ($p = 0.018$) when pre-challenge average values were compared to the post-challenge determinations. No significant differences were detected for ALT, urea and creatinine levels and platelets or erythrocytes counting by comparing pre- and post-challenge values in both groups (not shown).

Parasitological evaluation consisted in culture of bone marrow aspirates and PCR of *Leishmania* DNA extracted from ear skin biopsy and blood samples, performed six and nine months after challenge. As shown in Table 1, nine months after challenge cultures resulted positive from bone marrows collected from all (100%) the dogs of the control infected groups, while 57.14% of the Vaccinated and Infected (VI) animals had positive results. *Leishmania* DNA was more frequently found in blood from the control infected dogs (71.5%) than in the rA2-vaccinated animals (28.5%). A positive correlation was thus observed between dogs developing clinical findings and presence of *Leishmania* DNA in peripheral blood. The ratio of PCR positive animals was significantly higher among animals that were Oligo/Symptomatic than in those that remained asymptomatic ($p = 0.048$; accurate Test of Fisher). Vaccinated asymptomatic dogs did not present *Leishmania* parasites in

Table 1
Clinical and parasitological evaluation of animals

Animal	Group	Bone marrow[a]	Peripheral blood[a]	Symptoms
1	VI	Positive	Positive	Asymptomatic
2	VI	Positive	Negative	[b]Symptomatic: loss of weight, alopecia of the left ear
3	VI	Positive	Positive	[b]Symptomatic: moderate loss of weight, alopecia, lymphadenopatia
4	VI	Negative	Negative	Asymptomatic
5	VI	Negative	Negative	Asymptomatic
6	VI	Positive	Negative	Asymptomatic
7	VI	Negative	Negative	Asymptomatic
Total		4/7	2/7	2/7
1	I	Positive	Negative	Asymptomatic
2	I	Positive	Positive	[c]Symptomatic: intense loss of weight, profuse bloody diarrhea, cachexia
3	I	Positive	Negative	Asymptomatic
4	I	Positive	Positive	[c]Symptomatic: alopecia, intense loss of weight, diarrhea
5	I	Positive	Positive	[c]Symptomatic: skin lesions, onychogryphosis, intense loss of weight, alopecia, cachexia
6	I	Positive	Positive	[c]Sympotmatic: intense loss of weight, profuse diarrhea, cachexia, purulent eye secretion
7	I	Positive	Positive	[c]Symptomatic: intense loss of weight, profuse diarrhoea, onychogryphosis, cachexia
Total		7/7	5/7	5/7

VI, Vaccinated and Infected dogs; I, dogs that received PBS or Adjuvant and were infected.
[a] Parasites were detected by culture of bone marrow aspirates or by of peripheral blood collected 9 months p.i.
[b] Symptoms appeared 1 year p.i.
[c] Symptoms appeared 3–6 months p.i.

skin, as detected either by PCR or by specific immunocytochemical analysis (data not shown).

4. Discussion

In the present investigation, we tested, in dogs, a vaccine formulation against CVL consisting of the recombinant protein A2 in combination with saponin. Saponin has been extensively used in other vaccine formulations and was shown to preferentially induce type 1 immune responses in animal models [23,24]. In high concentrations, however, saponin may induce adverse reactions such as local pain. In this study, animals tolerated quite well the vaccine doses, with no signs of local pain, abscess, ulceration or fever, may be due to the low saponin concentration used.

Clinical follow-up of the animals during the study allowed the classification of dogs as asymptomatic or symptomatic. In terms of clinical manifestations, the percentages of animals scored asymptomatic by the end of the study were higher in the rA2 immunized group than in control groups immunized with adjuvant only or PBS, resulting in 42.86% of efficacy. These results indicate that rA2 was able to induce partial protection against *Leishmania* infection, although due to the low number of animals per group, it was difficult to establish whether such differences were statistically significant.

The outcome of CVL following natural and experimental infection is highly variable [25]. A proportion of infected dogs develop symptomatic, ultimately fatal infection, while other dogs remain asymptomatic. Progression of clinical CVL is associated with establishment of large numbers of parasites in different tissues, although *Leishmania* DNA found in bone marrow might be significantly higher than that measured in blood samples, in both experimental and natural infections [26]. A sequential parasite dissemination pattern, in which lymph reticular organs harbored higher numbers of parasites at earlier stages of infection than the skin, has been also reported [27,28]. Although, in the present study not all animals in the control groups developed clinical signs of CVL, bone marrow cultures were positive for all of them. A high proportion of these animals were also positive for the presence of *Leishmania* DNA in peripheral blood. Although the parasitological methods may differ in sensitivity, both of them indicated a higher proportion of parasitized animals in control groups (PBS/Adjuvant) than in rA2-vaccinated dogs. It is worth noting that three serial passages were performed before bone marrow cultures were considered negative. Rodríguez-Cortés et al. [28] reported that *Leishmania* DNA was more frequently found in blood from experimentally infected dogs developing clinic-pathological findings than in blood from asymptomatic dogs and that the intensity of *Leishmania* infection in target organs and clinical status might be positively correlated, as in naturally infected dogs [29,30]. In agreement, a significant positive correlation ($p = 0.008$) was observed in the present study between dogs developing clinical findings and presence of *Leishmania* DNA in peripheral blood.

The evaluation of immune responses suggested that rA2-immunized animals mounted a type 1 immune response. In the humoral response, the first observed Ig was A2-specific IgG2 antibodies and no anti-A2 IgG1 antibodies were detected. This humoral response correlated with increased levels of IFN-γ in response to rA2 before and in response to both rA2 and total parasite antigens, after infection. In contrast, detectable levels of IgG1 antibodies and IL-10 in response to total parasite antigens were observed only

Table 2
Leukocyte counts and albumin/globulin ratios in experimental groups

Group	Leukocyte counts (average ± standard deviations)		Albumin/globulin ratio (average ± standard deviations)		AST levels (average ± standard deviations)	
	Pre-challenge	Post-challenge[a]	Pre-challenge	Post-challenge[a]	Pre-challenge	Post-challenge[a]
VI	10.994 ± 1.750	8.489 ± 2.625	1.70 ± 0.33	1.25 ± 0.40	42.42 ± 6.29	49.85 ± 14.80
I	12.968 ± 3.108	6.945 ± 2.588*	1.86 ± 0.59	1.12 ± 0.44*	31.28 ± 0.59	52.82 ± 16.50*

VI, Vaccinated and Infected dogs; I, dogs that received PBS or Adjuvant and were infected.
[a] Nine months post-challenge.
* Indicates significant differences ($p < 0.05$) when compared to pre-challenge determinations.

in control-infected animals. Although immunological and parasitological parameters associated with asymptomatic infection or active disease in CVL are only partially defined, resistance in CVL has been associated with the development of a *Leishmania*-specific, cell-mediated immune response (CMI) and increased levels of IFN-γ, whereas active disease has been associated with high antibody levels and suppressed CMI [28,31–33]. Recently, increased IL-10 expression has been associated with increase in parasitic loads and progression of the disease [34,35].

In this study, detection of anti-LcPA IgG1 antibodies correlated with appearance of symptoms in control-infected groups, suggesting an association between this Ig isotype and active disease. Several studies have attempted to link *Leishmania*-specific IgG1 and IgG2 levels with clinical status and disease progression [36–38], leading contradictory results. This is likely due to genetic heterogeneity of dog populations and in timing course of infection in naturally infected animals. On the other hand, in the present study, under experimental conditions, only the control-infected animals, many of them presenting severe symptoms, produced IgG1, suggesting an association between this isotype and the development of the disease.

It has been proposed that adoption of multiple and simultaneous actions to control vector populations and reservoir infection are required to significantly decrease VL incidence [5]. Culling of sero-positive dogs, although a matter of intense debate is a major VL control measure adopted in endemic areas in Americas. In this complex scenario, a CVL vaccine is certainly an important tool, but must fit some requirements, such as the discrimination between vaccinated and infected dogs by means of inexpensive serological tests. One of the major findings of the present study indicated that vaccinated dogs remained negative for anti-LcPA antibodies during the vaccination period, thus allowing the distinction between vaccinated and infected dogs. This is a relevant issue regarding the interface among VL control measures adopted in Brazil, although additional data, obtained after vaccination of a larger number of dogs in natural populations, are required to confirm this finding. It would be also of interest if vaccination could lead to reduction of zoonotic transmission. Considering that symptomatic dogs are more infectious to sand flies than the oligosymptomatic and asymptomatic animals [27,39,40] and the reduction of symptomatic animals as demonstrated for the rA2-vaccinated animals, it is plausible that vaccination with rA2 would impact favorably, reducing the parasite burden in the reservoir and transmission in endemic areas. It is worth noting that parasites were not detected neither by PCR nor immunocytochemical analysis in the skin of rA2-vaccinated animals. However, additional studies are required to clarify this issue.

A major difficulty associated with phase II vaccine trials against CVL is the lack of an adequate infection model. Important variations in the course of infection following either infection with different parasite doses, different infective stages of the parasites or different routes of infections have been evidenced [25]. Increased numbers of symptomatic dogs are generally observed after infection with high parasite doses ($>10^7$ promastigotes via endovenous injection), resulting in a very rigorous infection model as compared to natural infection conditions [25]. In the present study, a high dose was used of a highly infective parasite strain, which was isolated from a symptomatic dog. This challenge infection conditions proved to be a good method to induce severe symptoms of CVL in 5 out of 7 control animals (PBS and adjuvant inoculated animals). On the other hand, considering previous attempts of vaccination against CVL, the prevention of severe disease in the majority of animals is clearly difficult to achieve. However, the highly infective challenge conditions may have overpowered a protective response present in the vaccinated dogs. Thus, the partial protection obtained in the present study confirms the capacity of recombinant protein A2 to limit parasite replication and prevent severe disease even under high dose experimental challenge infection. These findings strongly support further evaluation of efficiency of vaccination with rA2 against CVL in natural infection conditions.

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