Immunological comparison of DNA vaccination using two delivery systems against canine leishmaniasis

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Visceral leishmaniasis (VL) is a fatal disease caused by the intracellular protozoan parasite Leishmania infantum. Dogs are the primary reservoirs of this parasite, and vaccination of dogs could be an effective method to reduce its transfer to humans. In order to develop a vaccine against VL (apart from the choice of immunogenic candidate antigens), it is necessary to use an appropriate delivery system to promote a proper antigen-specific immune response. In this study, we compared two vaccine delivery systems, namely electroporation and cationic solid–lipid nanoparticle (cSLN) formulation, to administer a DNA vaccine containing the Leishmania donovani A2 antigen, and L. infantum cysteine proteinases of type I (CPA) and II (CPB) without its unusual C-terminal extension. The protective potencies of these two vaccine delivery systems were evaluated against L. infantum challenge in outbred dogs. Our results show that the administration of pcDNA-A2-CPA-CPB-GFP vaccine as a prime-boost by either electroporation or cSLN formulation protects the dogs against L. infantum infection. Partial protection in vaccinated dogs is associated with significantly (p < 0.05) higher levels of IgG2, IFN-γ, and TNF-α and with low levels of IgG1 and IL-10 as compared to the control group. Protection was also correlated with a low parasite burden and a strong delayed-type hypersensitivity (DTH) response. This study demonstrates that both electroporation and cSLN formulation can be used as efficient vaccine delivery systems against visceral leishmaniasis.

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1. Introduction

Leishmaniasis has been classified as one of the most neglected diseases (McDowell and Rafati, 2014). The three major clinical forms of this disease in humans are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis (VL). There are nearly 20 commonly recognized species of Leishmania that are known to cause CL or VL in humans (Desjeux, 2004). The Leishmania donovani complex (L. donovani, Leishmania infantum, and Leishmania chagasi) is responsible for more severe clinical manifestations than CL (Mauricio et al., 2000), and leads to death, if left untreated. The numbers of leishmaniasis cases are increasing worldwide. Approximately 57,000 human deaths result annually from VL (Reithinger and Davies, 2002). VL is emerging as an important opportunistic infection among people with HIV-1 infection (Desjeux and Alvar, 2003; Alvar et al., 2008).

A variety of vaccination strategies against Leishmania have been attempted so far in mice, dogs, and humans (Cober and Reed, 2005). Of them, DNA-based vaccines have practical advantages, especially because they are generally less costly to produce than peptide or protein vaccines, can be produced on a large scale with high purity, and are stable under a wide variety of environmental conditions (Gurunathan et al., 2000).

The L. donovani A2 antigen, a gene family expressed specifically in the amastigote stage (Charest and Matlashewski, 1994) and associated with virucerilization (Zhang and Matlashewski, 2001), has been tested previously as a candidate vaccine against Leishmania. Immunization with A2 antigen as a recombinant protein or a DNA
vaccine significantly protected BALB/c mice, and more recently dogs, against *L. donovani* and *Leishmania amazonensis* infections (Ghosh et al., 2001; Coelho et al., 2003; Zanin et al., 2007; Fernandes et al., 2008; Resende et al., 2008). The saponin–adjuvanted recombinant A2 antigen, called Leish–TeC, was licensed for prophylaxis against canine leishmaniasis and has been used in Brazil for partial protection in the high-dose *L. infantum* beagle dog model (Fernandes et al., 2008). In addition, recombinant *Leishmania tarantolae* expressing A2 antigen proved to be an effective live vaccine against *L. infantum* infection in mice (Mizbani et al., 2009). Among the other *L. infantum* antigens, candidate proteinases of type I (CPB) and II (CPA) have been examined in experimental vaccinations in both mouse and dog models (Rafati et al., 2005, 2006).

Our previous study in dogs showed that DNA vaccination using *cpa* and *cpb* genes followed by administration of recombinant CPA and CPB proteins could induce partial protection (Rafati et al., 2005).

In general, DNA delivery methods can be classified into viral- and nonviral-based systems (Escors and Breckpot, 2010). Nonviral-based systems are subdivided into physical and chemical types. Electroporation (EP) is a physical delivery system involving the application of short electric pulses to the vaccination site, resulting in the formation of transient pores in the plasma membrane of the adjacent cells (Trollet et al., 2006; Cukjati et al., 2007). This allows macromolecules such as nucleic acids to enter the cytoplasm (Becker and Kuznetsov, 2007). The cationic solid–liquid nanoparticle (cSLN) formulation as a chemical delivery system has more advantages than other carrier systems, such as high stability in body fluids and tissues, ability to release drugs for sustained periods, biodegradability, ease of manufacture (Joshi and Müller, 2009), and low cost (Wissing et al., 2004; Shidhaye et al., 2008). Our previous study showed that the use of cSLN formulation as a delivery system could protect CPA, CPB, and CPB–CTE genes from extracellular enzymatic degradation, and also displayed remarkable low cytotoxicity (Doroud et al., 2010). Moreover, this delivery system together with pcDNA encoding CPA and CPB could partially protect BALB/c mice against cutaneous *Leishmania* infection (Doroud et al., 2011b). Our recent study in mice showed that both electroporation and cSLN formulation were able to deliver the DNA vaccine containing the *L. donovani* A2 antigen with *L. infantum* cysteine proteinases to promote a strong T helper (Th1) immune response and protect against *L. infantum* infection (Saljoughian et al., 2013).

In this study, we evaluated the potency of the pcDNA-A2-CPA-CPB-CTE GTP trifusion gene delivered by either a physical method (electroporation) or a chemical method (cSLN formulation) as a candidate vaccine against *L. infantum* infection in dogs, and also assessed its ability to induce protective immunity after a 20-month follow-up.

2. Materials and methods

2.1. Ethical consideration

All experiments in dogs, including maintenance, handling program, and blood and bone marrow (BM) sample collection, were approved by and found to be in accordance with the guidelines of the Animal Care and Ethics Committee at Pasteur Institute of Iran (Grant ID 564 dated 2011) and Veterinary Board of Tehran Medical School (700/4038 dated 2011) based on the specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medical Education (MOHME) of Iran (2005).

2.2. Plasmid construction and purification

pcDNA 3.1(-) (Invitrogen, Grand Island, NY, USA) vector containing *A2-CPA-CPB-CTE GTP* genes was transformed into the DH5α *Escherichia coli* strain available from our previous study (Saljoughian et al., 2013). The A2–CPA–CPB trifusion expression was confirmed in COS-7 mammalian cells before the commencement of vaccination (data not shown). Large Luria-Bertani (LB) culture was prepared, the pcDNA-A2-CPA–CPB–*CTE GTP* vaccine was purified by ion-exchange chromatography with QIAGEN EndoFree Mega Kit (Hilden, Germany), and each gene fragment was confirmed by polymerase chain reaction (PCR) (Saljoughian et al., 2013). The total concentration and purity of pcDNA were determined by a NanoDrop ND-1000 spectrophotometer.

2.3. Animal studies

This study included 30 outbred dogs (18 males and 12 females, aging 6 months to 4 years, and weighing 18 ± 4 kg), from non-endemic parts of Iran. The animals were housed individually in conventional kennels at the School of Veterinary Medicine, Tehran University, and fed with standard commercial diet (Nutripet, Iran). The animals were regularly maintained for 3–4 months in the animal facility. All the animals were treated for intestinal helminth infections and were immunized against distemper (DHP, produced by NOBIVAC, Intervet), canine parvovirus (CPV strain 154), canine adenovirus (CAV 2 strain Manhattan LPV3), and rabies (BHK, produced by Pasteur Institute of Iran) before study. All dogs responded negatively for the presence of *Leishmania* DNA by PCR (primers: RV1 and RV2 that targeted the region of kinetoplast minicircle DNA of *L. infantum*) (Lachaude et al., 2002) of peripheral blood mononuclear cells (PBMCs) and serum anti-*Leishmania*-specific immunoglobulin G (IgG) antibody by enzyme-linked immunosorbent assay (ELISA). ELISA was performed with some modifications (serum 1/100 diluted in phosphate-buffered saline (PBS)/BSA 1%, 100 μL substrate system (KPL, ABTS), and the OD was measured at 450 nm) as described earlier (Rafati et al., 2005). The well-being of the animals was determined regularly by veterinarians, and all the invasive procedures were performed in accordance with the rules of ethical procedures in animal experimentation and biosafety.

2.4. Vaccine administration and parasite challenge

The dogs were divided into three groups of 10 (according to their weight, sex, and age), denoted as G1, G2, and G3. The first group (G1) was immunized subcutaneously (SC) with pcDNA-A2-CPA-CPB–*CTE GTP* vaccine (200 μg/dog) via cSLN formulation, a chemical delivery previously described by Doroud et al. (2011a). The second group (G2) was immunized SC with the same amount of DNA via electroporation, with a field strength of 80 V/cm (constant), 10 pulses of 30 ms each using ECM 830 Electroporator (BTX, Holliston, MA, USA). The control group (G3) was immunized with PBS. Our previous studies in dog and mouse models showed that pcDNA did not significantly stimulate the immune response; hence, in this study, we did not consider the empty pcDNA as control group (Rafati et al., 2005; Saljoughian et al., 2013). All groups were immunized similarly for their booster immunization. All groups were tested by intravenous injection of 4 × 10^7 parasites/dog *L. infantum* (MCAN/ES/98/LM-877) in stationary-phase promastigotes (kindly provided by the World Health Organization (WHO) collaborating center for leishmaniasis, Servicio de Parasitologia, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain) 3 weeks after the booster vaccination.

2.5. Humoral response assay

Sera of the dogs were tested for the presence of IgG1 and IgG2a against either rA2, rCPs, or *Leishmania* (F/T) at six different periods (T0: before challenge at day 41; T2: 2 months after challenge at day 60; T6: 6 months after challenge at day 180; T11: 11 months
after challenge at day 330; T14: 14 months after challenge at day 420; and T17: 17 months after challenge at day 510). The rA2, rCPA, and rCPB of L. infantum were expressed separately in pET23a, and purified as described earlier (Rafati et al., 2003; Mizbani et al., 2009). Each well of 96-well plates (Greiner) was coated with a mixture of rA2, rCPA, and rCPB (10 μg/ml of each) or L. infantum (F/T) (10 μg/ml). The ELISA was performed as described in Section 2.3.

2.6. Cytokine assay

The IFN-γ, TNF-α and IL-10 levels were determined before challenge (T0) and two (T2), six (T6), 11 (T11), 14 (T14), and 17 (T17) months after challenge. PBMCs were isolated from heparinized blood by centrifugation over Ficol (Histopaque-1077; Sigma) for 30 min at 974 × g at room temperature as described earlier (Rafati et al., 2005). PBMCs were adjusted to 3 × 10^6/ml and cultured in duplicate within 48-well culture plates. Then, isolated PBMCs were incubated for 96 h with 10 μg/ml of PHA, 10 μg/ml each of rA2, rCPA, and rCPB as a mixture, and 20 μg/ml of L. infantum (F/T), or without antigens at 37 °C and 5% CO2. After incubation, the supernatant was collected for assessing production of IL-10 and TNF-α after 24 h and IFN-γ after 96 h and stored at −70 °C until analysis. Levels of IFN-γ, IL-10, and TNF-α in supernatants were assayed by sandwich ELISA (Duoset ELISA canine IFN-γ, Duoset ELISA canine TNF-α, and Duoset ELISA canine IL-10; R&D Systems). Detection limits were 17.5–2000 pg/ml for the canine IFN-γ and IL-10, and 8.75–1000 pg/ml for TNF-α, according to the manufacturer instruction.

2.7. Delayed-type hypersensitivity

The delayed-type hypersensitivity (DTH) was performed at months 11 and 16 post infection. L. infantum parasites were grown at 26 °C in complete Schneider’s Drosophila medium supplemented with 20% heat-inactivated fetal calf serum and gentamicin (0.1%). The stationary-phase promastigotes of L. infantum were harvested and washed thrice with sterile PBS1x (pH 7.2). Following centrifugation, the pellet was resuspended at the appropriate concentration (3 × 10^6/ml) in 0.4% phenol-saline (WHO, 1996). Immunized and control dogs were injected intradermally (id) with 100 μl of inactivated suspension of stationary-phase promastigotes of L. infantum in the right shaved groin and 100 μl of a diluent in the left shaved groin. Assessment of the severity of intradermal reaction was performed 48 h after antigen injection. Induration areas were marked, and each time the values of the saline control were subtracted from the reaction due to the Leishmania antigen. An induration of >5 mm in diameter was considered positive.

2.8. BM aspiration and parasite quantification by real-time PCR

BM samples were taken from BM aspirates of all dogs at 18 months post infection. The animals were anesthetized by a mixture of medetomidine hydrochloride (Dominator) and ketamine. A biopsy of BM was taken aseptically from the iliac bone through a 6-mm × 25-mm Klima needle into a 20-ml syringe containing 0.5% ethylenediaminetetraacetic acid (EDTA). Each sample was divided into three parts for quantifying parasite burden using cytology, immunocytochemistry (ICC), and real-time PCR examinations. A quantitative real-time PCR technique was used to determine the parasite load in BM samples. BM aspirates of 1 ml were collected in the EDTA tubes and stored at −20 °C. DNA was extracted from 200 μl of BM using DNeasy Blood & Tissue Kits (Qiagen) according to the manufacturer’s protocols. Quantity and purity of the extracted DNA were measured in 2-μl sample aliquots by optical absorbance at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK). The primers targeting the region of kinetoplastid minicircle DNA of L. infantum RV1 (F: 5′-CTTTTCTGTCGCCCGGTAGG-3′) and RV2 (R: 5′-CCACCTGGGCCATTTTACACCA-3′) were used (Lachaud et al., 2002). Quantification of Leishmania DNA was performed using an absolute method: by comparing Ct values with those from a standard curve generated using serial dilutions of L. infantum DNA from 1 × 10^6 to 0.1 parasites/ml, and each dilution was done in duplicate. Applied Biosystems 7500 Real-Time PCR System was used for quantification of parasites in BM. An aliquot of 200 ng of DNA was subjected to the reaction containing 5 pmol of each forward and reverse primers, 12.5 μl Qiagen QuantiFast SYBR Green Master Mix in the total volume of 25 μl. All reactions were performed in duplicate. Experimental conditions for PCR amplification were as follows: 95 °C for 10 min; 40 cycles consisting of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 40 s. Specific amplification of the target region was confirmed by gel electrophoresis of the PCR products.

2.9. Cytology and ICC

Multiple aspirated smears were made on slides, and were both air-dried and alcohol-fixed and stained by Wright–Giemsa method. The monoclonal antibody, WHO LXXXVII-2ES-AB (D2: specific for L. donovani/L. infantum), was used as the primary antibody as described earlier (Oryan et al., 2013). In order to quench endogenous peroxidases, the slides were rehydrated and treated with 3% hydrogen peroxide solution for 10 min at room temperature.

The antigen retrieval was performed by boiling the slides in a target retrieval solution (citrate buffer) using a microwave oven twice at 750 W (100% power for 10 min and then 20% power for 20 min). The primary antibody was used for 1 h (diluted 1:200). The detection system used was EnVision+ (DakoCytomation), and was developed with diaminobenzidine (DakoCytomation). Hematoxylin and 3,3′-diaminobenzidine-hydrogen peroxide were applied as the counterstain and chromogen, respectively. Cytological and immunocytochemical smears of all the cases were reviewed blindly by two pathologists with 1000× and 400× magnification. The modified scoring method was used for Leishman body burden, as described earlier (Shirian et al., 2014). The samples were considered negative if amastigotes were not found in 1000× magnification (oil immersion field [OIF]) in the whole slide smear.

2.10. Endpoint culture of spleen tissues

All the dogs were euthanized by intravenous injection of thiopental sodium 33% (5 ml/kg) 20 months post infection. A piece of spleen was removed under aseptic condition and cultured in 2 ml of Schneider’s Drosophila medium supplemented with 20% heat-inactivated fetal calf serum and gentamicin (0.1%) at 26 °C for 1 month (Binhazim et al., 1992). The subcultures were also made in Schneider’s Drosophila medium and examined daily for the presence of promastigotes under an inverted microscope at 40× magnification.

2.11. Clinical and biochemical evaluations

For additional evaluation, the dogs were monitored for development of clinical signs of VL approximately every 3 months. The dogs were weighed, and their general health status was assessed by a veterinarian at each interval. Finally, all the dogs were clinically classified, according to the presence/absence of infection signs, as subpatent (clinically healthy and DNA-positive), patent subclinical (clinically healthy, DNA-positive, and culture of spleen-positive), and clinically ill when they showed one or more clinical signs of canine leishmaniasis, including lymphadenopathy, alopecia,
weight loss, DNA-positive, and culture of spleen-positive (Solano-Gallego et al. 2009, 2011). Biochemical analyses were performed for all the dogs at 18 months after challenge. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, alkaline phosphatase (ALP), albumin (Alb), and total proteins were determined by a biochemistry serum analyzer (Technicon RA-1000, USA). The clinical biochemical abnormalities were graded (+3 < biochemical parameters with abnormalities) and ++ (>3 biochemical parameters with abnormalities).

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (Graphpad Software Inc. 2007, San Diego, CA, USA) as well as SPSS version 18. Nonparametric tests were used because humoral and cellular immune responses, DTH responses, and parasite load were not normally distributed. Data were expressed as mean ± SD (humoral and cytokine) and median (DTH responses and parasite load). Mann–Whitney test was used for comparing different parameters between groups, and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Vaccination regimens and follow-up

A total of 30 outbred dogs were examined in three groups using prime-boost protocols. The route of vaccination, dose interval, and vaccine formulation are summarized in Table 1. During the study, one dog from each group died for reasons other than canine leishmaniasis (e.g., gastric dilatation volvulus (GVD), uremia, and chronic kidney disease). In addition, one dog each from G1 (17 months post infection) and G3 (20 months post infection) were scarified because of VL.

<table>
<thead>
<tr>
<th>Group</th>
<th>Priming (day 0)</th>
<th>Booster (day 21)</th>
<th>Delivery system</th>
<th>Challenge (day 42)</th>
<th>Modality</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (10 dogs)</td>
<td>pcDNA-A2-CPA-CPB-CTE-GFP (200 μg/dog)</td>
<td>pcDNA-A2-CPA-CPB-CTE-GFP (200 μg/dog)</td>
<td>Solid–lipid nanoparticles (SLN) formulation Electroportation</td>
<td>Leishmania infantum</td>
<td>DNA/DNA</td>
</tr>
<tr>
<td>G2 (10 dogs)</td>
<td>pcDNA-A2-CPA-CPB-CTE-GFP (200 μg/dog)</td>
<td>pcDNA-A2-CPA-CPB-CTE-GFP (200 μg/dog)</td>
<td>–</td>
<td>Leishmania infantum</td>
<td>DNA/DNA</td>
</tr>
<tr>
<td>G3 (10 dogs)</td>
<td>PBS</td>
<td>PBS</td>
<td>–</td>
<td>Leishmania infantum</td>
<td>Control</td>
</tr>
</tbody>
</table>

In addition, significant differences were observed between G2 and G3 at T0, T6, and T14 ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively) (Fig. 1D).

Fig. 1E shows the ratio of IgG2 to IgG1 with respect to anti-L. infantum (F/T) antibody. The value of the ratio was found to be significantly higher at T6–T17 in G1 ($p < 0.05$, $p < 0.05$, and $p < 0.001$, respectively) and only at T14 and T17 in G2 ($p < 0.05$ and $p < 0.001$, respectively) in comparison with G3. The IgG2/IgG1 ratio in G1 against the mixture of rA2, rCPA, and rCPB was significantly higher than that of G3 at T6–T17 ($p < 0.05$, $p < 0.05$, and $p < 0.05$, respectively), and a similar pattern was observed between G2 and G3 at T0–T14 ($p < 0.05$, $p < 0.05$, and $p < 0.005$, respectively) (Fig. 1F).

3.3. Immunization with pcDNA-A2-CPA-CPB-CTE-GFP vaccine delivered by a physical and/or chemical delivery system induces a mixed Th1/Th2 response

After challenge, the level of IFN-γ against L. infantum (F/T) antigen significantly increased at T2–T17 in G1, in comparison with G3 ($p < 0.05$, $p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively). There were significantly higher levels of IFN-γ at T6, T14, and T17 between G2 and G3 ($p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively), as shown in Fig. 2A. We observed significant levels of IFN-γ production in response to the mixture of rA2, rCPA, and rCPB at T0, T2, T11, and T17 in G1, in comparison with G3 ($p < 0.05$, for all the mentioned periods). In addition, there were significant differences of IFN-γ only at T14 and T17 ($p < 0.05$) between G2 and G3, as shown in Fig. 2B. In G3, there was a significant increase in IL-10 production after stimulation with L. infantum (F/T), and significant levels were observed at T2 and T6 ($p < 0.05$, at both time points) in comparison with G1 (Fig. 2C). We observed a significant difference between G2 and G3 only at T14 ($p < 0.05$) (Fig. 2C). The level of IL-10 against the mixture of rA2, rCPA, and rCPB was significantly higher in G3 than in G1 at T2 and T6 ($p < 0.05$, at both time points) and G2 at T0 and T11 ($p < 0.05$, at both time points) (Fig. 2D).

The ratio of IFN-γ to IL-10 for dogs within each group is demonstrated in Fig. 2E. There was a significant increase in this ratio at T2–T17 between G1 and G3 against L. infantum (F/T) ($p < 0.01$, $p < 0.01$, $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively). Almost similar patterns were observed between G2 and G3, as shown in Fig. 2E. For the mixture of rA2, rCPA, and rCPB, this ratio was significantly different at T0–T17 between G1 and G3 ($p < 0.01$, $p < 0.001$, $p < 0.01$, $p < 0.05$, $p < 0.05$, and $p < 0.05$, respectively) and at T0, T2, T11, and T17 between G2 and G3 ($p < 0.01$, $p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) (Fig. 2F).

As another indicator of Th1 response, we observed significantly higher levels of TNF-α production against L. infantum (F/T) at T2 and T17 for G1 and at T2, T6, T11, and T17 for G2, in comparison with the control group (G3) ($p < 0.05$ and $p < 0.01$) and ($p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively), as shown in Fig. 2G. For the mixture of rA2, rCPA, and rCPB, there were only significantly higher levels of TNF-α at T6 between G1 and G3 ($p < 0.05$) (Fig. 2H).
3.4. DTH response

The results of our DTH study showed that all but two dogs in G2 (11 months post challenge) had a positive DTH response, and G1 showed a significantly higher DTH response compared with G3 ($p < 0.05$) at both periods (Fig. 3A and 3B). There was no difference in DTH responses between G2 and G3. It is interesting to note that two of the dogs in G3 did not develop a DTH response at 16 months post infection. In addition, at 16 months post infection, one dog in G2 had a negative DTH response (Fig. 3B).

3.5. Quantification of parasite load by real-time PCR

G2 had significantly lower number of parasites in BM than G3 ($p < 0.05$) (Fig. 4). Because almost all but two dogs in G1 had lower parasite density than G3, there was no significant difference in the quantity of parasites between the two groups.

3.6. Cytological and immunocytochemical findings

Quantified parasite density in aspirated cytological and immunocytochemical smears for *Leishmania* was classified by two
independent observers as shown in Fig. 5 (panels A–D indicate the infection grades I–IV, and panel E represents the negative control). The density of amastigotes in the vaccinated G1 group varied cytologically and immunocytochemically from negative to grades I, III, and IV. Although no parasite was detected in one dog of the vaccinated G1 group, five dogs with grade I and one dog with grade III infections were seen. In addition, the severe loading of the parasites (grade IV) was seen in one dog in this group, while grades I
and II were only verified in the vaccinated G2 group. Mild loading of the parasite (grade I) was detected in seven dogs of the group, and the remaining two dogs were of grade II. The density of amastigotes in G3 or the unvaccinated group varied from grades I to IV. The severe parasite loading grades of III and IV were seen in two and three dogs, respectively. Only two moderate (grade II) and two mild (grade I) infections were diagnosed in the control group (G3), as shown in Table S1.

### Table 2
Clinical status of dogs and percentage of each group within each category.

<table>
<thead>
<tr>
<th>Bone marrow PCR*</th>
<th>Spleen culture*</th>
<th>Clinical biochemistry abnormality</th>
<th>Clinical biochemistry abnormality</th>
<th>Weight loss</th>
<th>G1 (%)</th>
<th>G2 (%)</th>
<th>G3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subpatent</td>
<td></td>
<td>None</td>
<td>None</td>
<td>66</td>
<td>11</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Patent subclinical</td>
<td></td>
<td>*</td>
<td>Minor*</td>
<td>11</td>
<td>33</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Clinically ill</td>
<td></td>
<td>*</td>
<td>Intensive*</td>
<td>22</td>
<td>56</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* Leishmania infantum was detected by PCR in the bone marrow aspirates or by the culture of spleen collected at 18 and 20 months post infection, respectively.

### 3.7. Clinical and laboratory findings

Clinical signs of VL appeared earlier in the control group (G3) as compared to the vaccinated groups (G1 and G2). In addition, 55% of the dogs in the control group were clinically ill, whereas 22% and 11% of their counterparts in the vaccinated groups (G1 and G2, respectively) were clinically ill. The remaining dogs in each group were clinically healthy, and were classified as patent subclinical and subpatent, as shown in Table 2 and Fig. S1. The evaluation of biochemical parameters related to protein alterations showed a significant difference in AST, Alb, ALP, urea, creatinine, and total protein concentration between the vaccinated groups and the PBS control group (p < 0.05). No significant difference was detected in ALT levels between the groups.

### 4. Discussion

In this study, we compared the potency and immunogenicity of pDNA vaccine encoding A2-CPA-CPB-CteGFP as a trifusion gene with two delivery systems (physical/electroporation and chemical/cSLN formulation) for inducing protective immunity against infectious L. infantum in dogs. As previously shown, green fluorescent protein (GFP) is not immunogenic and cannot interfere with the immune response against specific antigens; therefore, in this study, we also used GFP as a reporter gene in vaccine vehicle (Ploemen et al., 2011). We thereby evaluated both cellular and humoral immunity associated with post-challenge protection against L. infantum. We demonstrated that vaccination with pDNA-A2-CPA-CPB-CteGFP by electroporation or cSLN formulation induced an antibody response that reacted with L. infantum. After the challenge, although the level of IgG1 increased in all groups, it
was significantly higher in the control group (G3) than in the vaccinated groups (G1 and G2). We observed significant differences of anti-\(L. \text{infantum}\) IgG2 levels between G1 and G3 at 6 (T6), 11 (T11) and 17 (T17) months post challenge, and only at T17, there was there a significant difference between G2 and G3. Previous studies have reported that severity and susceptibility of canine leishmaniasis are associated with IgG1 subtype, whereas protection and resistance are associated with increased levels of IgG2 (Borja-Cabrera et al., 2004; Fujiwara et al., 2005; Rafati et al., 2005; Santos et al., 2007). Furthermore, a previous study showed that dogs infected naturally with \(L. \text{infantum}\) induced both IgG1 and IgG2 antibodies, in which IgG1 and IgG2 were associated with progressive disease and subclinical infection, respectively (Deplazes et al., 1995). Although our results are in agreement with these studies, there are some conflicting reports showing high levels of IgG2 antibody in subclinical infected dogs (Leandro et al., 2001; Iniesta et al., 2005; Barbieri, 2006; Reis et al., 2006; Day, 2007; Bergeron et al., 2014). We also calculated the IgG2/IgG1 ratio to evaluate the Th1/Th2 polarization of the immune response (Lemesre et al., 2005; Rafati et al., 2005). Our results showed that the IgG2/IgG1 ratio was significantly higher between G1 and G3 from 6 to 17 months post challenge and at T14 and T17 between G2 and G3. A study by de Oliveira (2003) reported that the IgG2/IgG1 ratio in vaccinated and protected dogs was >1, whereas in dogs with canine leishmaniasis progressing to disease, it was <1. In this regard, the IgG2/IgG1 ratio in most of our vaccinated dogs was >1, and in most dogs in the control PBS group (G3), it was <1. A study of human and canine leishmaniasis showed that cell-mediated immunity is more important than antibodies for protection (Hoerauf et al., 1994).

The level of IFN-\(\gamma\) in G1 against \(L. \text{infantum} \ F/T\) antigen significantly increased at T2–T17 in comparison with the control group (G3), and significant differences were also observed at T6, T14, and T17 between G2 and G3. Studies in experimentally infected dogs have shown that protective immunity is associated with the production of IFN-\(\gamma\) by PBMC besides the induction of parasite-specific cytotoxic T cells (Moreno and Alvar, 2002). The predominant role of IFN-\(\gamma\) in the activation of macrophages and the stimulation of its leishmanicidal activity is to control parasite dissemination (Chamizo et al., 2005; Manna et al., 2006; Carrillo et al., 2007). Our results are in agreement with those of other studies, and suggest that vaccinated groups (G1 and G2) polarize the immune response toward a Th1 response, and high levels of IFN-\(\gamma\) can activate macrophages to kill \(Leishmania\) amastigotes. IL-10 is as important as IFN-\(\gamma\) in determining whether a vaccine has induced protective immunity (Khalil et al., 2000). Our previous study showed that high levels of IL-10 are associated with an increase in parasitic loads and a progression of the disease (Rafati et al., 2005). In addition, many VL patients produce high levels of IL-10 that can inhibit the activity of antiparasitic pro-inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) (Gautam et al., 2011). Here, the control group (G3) showed a remarkable release of IL-10 after stimulation with \(L. \text{infantum} \ F/T\) at T14, in comparison with G2 group, and also at T2 and T6 between G3 and G1. It has been shown recently that clinically ill animals with high parasitism are associated with a predominant
accumulation of IL-10 (Lage et al., 2007). The ratio of IFN-γ/IL-10 levels may be an indicator of vaccine success (Stober et al., 2005). A correlation between increase of IFN-γ/IL-10 ratio and elimination of L. infantum has been demonstrated (Rafati et al., 2005). Our results showed that there was a significant IFN-γ/IL-10 ratio at T2–T7 between G1 and G3 against L. infantum (F/T), and a similar pattern was observed between G2 and G3. Resistance to canine leishmaniasis has been associated with the activation of Th1 cells producing IFN-γ, IL-2, and TNF-α (Pinelli et al., 1994, 1995), and patent subclinical dogs presented higher levels of TNF-α than clinically ill dogs (Pinelli et al., 1994). In this study, significantly higher levels of TNF-α against L. infantum (F/T) were observed at T2 and T17 in G1, and at T2, T16, T11, and T17 in G2, in comparison with the control group (G3).

The DTH test is one of the most informative and practical immunological surrogate markers used in clinical trials (Khalil et al., 2000); several studies have also identified a positive DTH as a marker of vaccine-induced immunity in dogs (Molano et al., 2003; Rafati et al., 2005). In addition, patent subclinical dogs that were infected naturally with L. infantum had a stronger DTH response, and did not progress to an active visceral disease compared with the dogs that progressed to active VL (Pinelli et al., 1994; Solano-Gallego et al., 2001). In our study, all the dogs in G1 were positive for DTH response and the induration size in this group was significantly higher than those in G3 at 11 and 16 months post challenge. The DTH response in G2 was also higher than that of G3, but the difference was not significant.

BM is an important lymphoid organ in the clinical analyses of dogs (Momo et al., 2014). It has been demonstrated that there is a strong correlation between parasite density of BM and spleen with clinical status (Reis et al., 2006). Here, parasite density of BM in all dogs was evaluated by reliable methods such as direct detection (cytology and ICC) and real-time PCR. On the basis of the results of direct detection, particularly ICC, and real-time PCR, the highest density of amastigotes was seen in G3, followed by G1 and G2. Our results show that both the vaccinated groups had the lowest quantity of parasites compared with the control group. It should be noted that although G1 had a higher DTH response than G2, no significant difference in parasite burden was observed between the two groups. Although the clinical status of G2 was better than G1, the difference was not significant. Altogether, both G1 and G2 showed similar cellular and humoral responses.

In conclusion, the results presented here demonstrate that DNA vaccination could induce cellular immunity when delivered by electroporation or cSLN formulation. Relatively higher levels of specific IFN-γ and lower levels of IL-10 induce partial protection in both vaccinated regimens. This study used experimental needle challenge with L. infantum. Further confirmation is needed using sand-fly challenge.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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References
