



Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs

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ABSTRACT

Zoonotic visceral leishmaniasis, caused by the intracellular protozoan parasite *Leishmania infantum*, is a neglected tropical disease that is often fatal when untreated. Dogs are considered the main reservoir of *L. infantum* in zoonotic VL as the presence of infected dogs may increase the risk for human infection. Canine visceral leishmaniasis (CVL) is a major veterinary and public health problem in Southern Europe, Middle East and South America. Control of animal reservoirs relies on elimination of seropositive dogs in endemic areas. However, treatment of infected dogs is not considered a favorable approach as this can lead to emergence of drug resistance since the same drugs are used to treat human infections. Therefore, vaccination against CVL remains the best alternative in control of the animal reservoirs. In this study, we present data on the immunogenicity profile of a live attenuated parasite *LdCen*^{−/−} in a canine infection model and compared it to that of Leishmune®, a commercially available recombinant vaccine. The immunogenicity of the *LdCen*^{−/−} parasites was evaluated by antibody secretion, production of intracytoplasmic and secreted cytokines, activation and proliferation of T cells. Vaccination with *LdCen*^{−/−} resulted in high immunogenicity as revealed by the higher IgGTotal, IgG1, and IgG2 production and higher lymphoproliferative response. Further, *LdCen*^{−/−} vaccinated dogs showed higher frequencies of activated CD4+ and CD8+ T cells, IFN-γ production by CD8+ T cells, increased secretion of TNF-α and IL-12/IL-23p40 and decreased secretion of IL-4. These results contribute to the understanding of immunogenicity elicited by live attenuated *L. donovani* parasites and, consequently, to the development of effective vaccines against visceral leishmaniasis.

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1. Introduction

Visceral leishmaniasis (VL) is a tropical disease with an annual incidence of ~500,000 cases and causes more than 50,000 deaths each year [1]. The zoonotic visceral leishmaniasis is caused by the obligate intracellular protozoan parasite *Leishmania infantum* (syn. *L. chagasi*). Dogs are considered the main reservoir of *L. infantum* in

zoonotic VL [2] and the presence of infected dogs may increase the risk for human infection [3]. Canine visceral leishmaniasis (CVL) is a major veterinary and public health problem not only in endemic areas but also in Northern Europe, the United States and Canada, where autochthonous cases or outbreaks of disease are occasionally reported [4–6].

Control of VL relies on early diagnosis, control of the vector population and outbreaks in domestic reservoirs and treatment of infected individuals [7]. These measures can eliminate [8] or drastically reduce the transmission [9] when used over a long period [10]. The availability of new diagnostic kits [1], efficient vector control including insecticide spraying and collars impregnated with deltamethrin for use in animals [11] and new drugs such as miltefosine [1] has enabled better control of VL [1,12]. On the other hand,

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despite the decreased incidence of human and canine VL cases following elimination of seropositive dogs in endemic areas, this control strategy provides only a transient effect and is not accepted widely [13]. Although treatment of dogs usually results in clinical improvement, relapses are frequent and in most cases does not lead to parasitological cure or inhibition of infectivity to the sand fly vector [14]. Further, treatment of infected dogs is not the most effective approach as the same drug is used to treat human infections and possibly hasten the emergence of drug-resistance [7,13]. Therefore, vaccination represents the most efficacious method for the control of zoonotic VL. Widespread application of an effective vaccine in dogs would also dramatically decrease transmission of *L. infantum* to humans [15].

Previous attempts at vaccination based on killed *Leishmania* parasites or defined parasite antigens resulted in a limited and short-term protection [16]. Live-attenuated vaccines, on the other hand, allow the host immune system to interact with a broad repertoire of antigens considered to be essential in the development of protective immunity and importantly cause no pathology [17–19]. Several methods have been used to attenuate *Leishmania* parasites for vaccination [20]. Specifically, our labs have previously evaluated the protective immunity of an attenuated *L. donovani* strain from which centrin has been deleted (*LdCen*^{−/−}) in a BALB/C mouse model [18]. Deletion of centrin in *L. donovani* specifically affects the cytokinesis and leads to multinucleated cells and eventual cell death of amastigote forms while the growth of promastigote forms is unaffected [21]. Tests to evaluate the potential of *LdCen*^{−/−} parasites as vaccine candidates have demonstrated the safety, immunogenicity and protection against infection with wild type *L. donovani* in mice and hamster models [18]. More importantly, the protection induced by *LdCen*^{−/−} parasites was found to be long lasting in these models suggesting that it may be a leading vaccine candidate for CVL. In the present study, we report the immunogenicity profile of *LdCen*^{−/−} parasites in dogs, measured by antibody secretion, production of intracellular and secreted cytokines as well as T cell activation, proliferation and phenotypic markers. These results were compared to those obtained from either placebo (saline) treated or Leishmune® (a commercially available vaccine) immunized dogs.

2. Material and methods

2.1. Parasites and soluble antigen (SLA) preparation

The *L. donovani* centrin1-deleted (*LdCen*^{−/−}) parasites were used [21]. The parasite cultures were maintained as previously described [21]. *L. infantum* promastigote forms (MHOM/BR/1972/BH46) were grown as described [22]. *L. infantum* stationary-phase promastigotes were harvested, washed three times in PBS and sonicated. The sonicated material was centrifuged at 18,500 rpm for 90 min at 4 °C. The supernatant was dialyzed against PBS for 24 h and filtered through 0.22 µm filters and stored at −80 °C. Protein quantification was performed using Pierce® BCA Protein Assay Kit, as described by the manufacturer.

2.2. Animals and vaccination protocol

This study was approved by the Ethical Committee for the Use of Experimental Animals of the Federal University of Minas Gerais, Brazil (CETEA#122/09) and performed according to the guidelines set by the Brazilian Animal Experimental College (COBEA). All animals were treated for intestinal parasitic infections, immunized against parvovirus, leptospirosis, distemper, parainfluenza and hepatitis. Eighteen healthy beagle dogs, 8 months of age were

divided into three groups [3 males and 3 females per group]. *LdCen*^{−/−} group received subcutaneously 1×10^7 *LdCen*^{−/−} promastigotes at stationary phase. Leishmune® group received three subcutaneous doses of vaccine (1 mL each) with an interval of 21 days between each dose, as recommended by the manufacturer (Pfizer Animal Health, Brazil). Control group received PBS alone. The immunological parameters were measured 15 days after the dose of *LdCen*^{−/−}.

2.3. Flow cytometric analysis of phenotypic profile and intracytoplasmic cytokine production

Flow cytometry of the *ex vivo* and *in vitro*-stimulated cells was performed as previously described [23]. For *ex vivo* analysis, peripheral blood was collected in Vacutainer tubes containing EDTA (Becton Dickinson, USA); erythrocytes were lysed using 2 mL of FACS Lysing Solution (BD Biosciences, USA). The remaining cells were permeabilized with saponin buffer (Sigma, USA) for 15 min. 2 µL of undiluted monoclonal antibodies corresponding to the following cell surface or cytokine markers were added to the tubes: CD3-FITC (clone CA17.2A12), CD4-FITC or Alexa Fluor® 647 (YKIX302.9), CD8-Alexa Fluor® 647 (YCATE55.9), CD21-Alexa Fluor® 647 (CA2.1D6), CD14-PE (TÜK4), MHC-II-FITC (YKIX334.2), CD11/18-FITC (YKIX490.6.4), IFN-γ-PE (CC302) and IL-4-PE (CC303) (AbD Serotec, USA). Cells were incubated in the dark for 30 min at RT, washed with PBS twice and fixed in 200 µL of fixative solution (BD Biosciences, USA).

For the analysis of cultures, whole blood was collected in heparinized Vacutainer tubes, incubated at a dilution of 1:10 in RPMI-1640 media supplemented with 1.6% L-glutamine, 3% antibiotic-antimycotic solution (Sigma, USA), 5% of heat inactivated FBS for 22 h at 37 °C and 5% CO₂ and pulsed with SLA [10 µg/well]. During the last 4 h of culture, Brefeldin A (Sigma, USA) (10 µg/mL) was added [23]. Cells were stained as described above. Acquisitions were performed in a FACScan flow cytometer. Data were collected on 1×10^5 lymphocytes (gated by forward and side scatter) and analyzed using CellQuest Pro software.

2.4. In vitro proliferative response of lymphocytes

PBMCs were isolated from heparinized blood by density gradient centrifugation as described [24]. Cell culture experiments were performed in triplicate using 5×10^5 PBMC per well, in a final volume of 1000 µL complete RPMI-1640 medium. Cells were stimulated with 25 µg/well of PHA (Sigma, USA) or 10 µg/well of *L. infantum* SLA. After 72 h incubation, supernatants were collected for cytokine detection. Cell proliferation analysis was performed on PBMC labeled with BrdU essentially as described [25].

2.5. Antibody responses

Antigen-specific IgG_{Total} titers and IgG₁ and IgG₂ levels were measured by indirect ELISA [26]. Briefly, 96 well micro titer plates (Nalge Intl., USA) were coated overnight with 5 µg/mL of SLA. For IgG_{Total} analysis, sera were added at 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200 dilutions. The IgG ELISA result is expressed in titers (dilutions). A positive reaction in a dilution $\geq 1:200$ is considered. For IgG₁ and IgG₂, sera were added at a 1:100 dilution. Peroxidase-conjugated Rabbit anti-dog IgG_{Total}, IgG₁ and IgG₂ antibodies were added at a 1:5000 dilution for 1 h. The substrate, o-phenylenediamine (Sigma, USA) was added and absorbance was measured on SpectraMac-240/PC microplate reader (Molecular Devices, USA) at 492 nm.

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