Vaccine 31 (2013) 1785–1792

Contents lists available at SciVerse ScienceDirect

Vaccine

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

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

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ARTICLE INFO

Article history:
Received 8 December 2012
Received in revised form 17 January 2013
Accepted 25 January 2013
Available online 8 February 2013

Keywords:
Canine visceral leishmaniasis
Vaccine
Attenuated parasite

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.

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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0264-410X
http://dx.doi.org/10.1016/j.vaccine.2013.01.048

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 ere 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% CO_2 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 IgG_1 and IgG_2 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 ≥1:200 is considered. For IgG_1 and IgG_2, sera were added at a 1:100 dilution. Peroxidase-conjugated Rabbit anti-dog IgG_{Total}, IgG_1 and IgG_2 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.

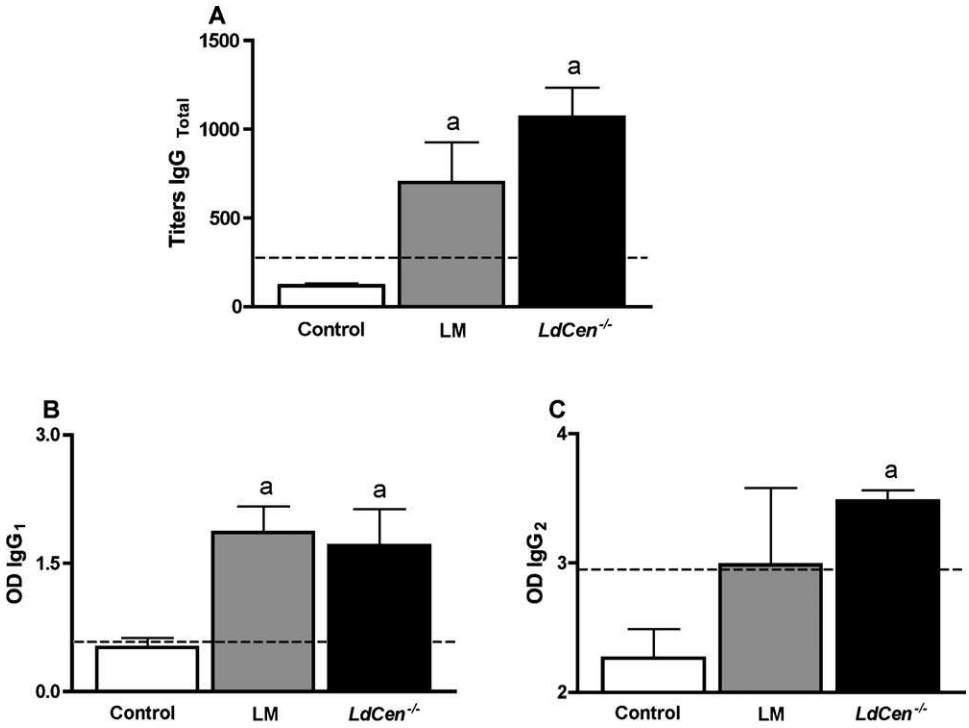

Fig. 1. Antibody production after immunization with Leishmune® and *LdCen*$^{-/-}$. (A) Anti-*L. infantum* IgG_{total} titers. Levels of IgG1 (B) and IgG2 (C) of PBS (Control) and vaccinated dogs (Leishmune® and *LdCen*$^{-/-}$). The line represents cut-off. [a,b]Significant differences at $p < 0.05$ compared with Control (PBS) and Leishmune®, respectively.

2.6. Cytokines production

The cytokines IL-4, TNF-α and IL-12/IL-23p40 were measured in cell supernatants by a sandwich ELISA (R&D Systems, USA) following manufacturer's instructions. Cytokine concentrations were calculated from the standard curve using 5-parameter curve fitting software (SOFTmax®Pro-5.3, Molecular Devices, USA). The lower detection limits were: 31.3 pg/mL for IL-12/IL-23p40 and IL-4; 7.8 pg/mL for TNF-α.

2.7. Biochemical and hematological analyses

Levels of albumin, AST, ALT, total bilirubin, calcium, total cholesterol, creatinine, urea, alkaline phosphatase, phosphorus, gamma GT, glucose, total proteins, and whole blood count were evaluated in the three groups before (day 0) and after (15 days after last dose) immunization.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism-v5.0 software (GraphPad Software Inc, USA). Non-parametric Kruskal–Wallis test followed by Dunns test was used to compare data from three groups (*LdCen*$^{-/-}$, Leishmune® and PBS). Differences were considered significant when a p value ≤ 0.05 was obtained.

3. Results

3.1. Vaccination with LdCen$^{-/-}$ induces a strong B cell response

The B cell responses were assessed to measure the ability of *LdCen*$^{-/-}$ and Leishmune® (LM) vaccines to induce antibodies. Results showed that all vaccinated animals developed anti-*L. infantum* IgG. Post immunization titers of IgG_{Total} in *LdCen*$^{-/-}$ or Leishmune® groups were significantly higher ($p = 0.02$ and $p = 0.0002$, respectively) than those observed in the PBS group ($p < 0.0001$) (Fig. 1A). Also, increased levels of IgG_1 were detected in Leishmune® and *LdCen*$^{-/-}$ groups compared to PBS controls ($p = 0.002$ and $p = 0.02$, respectively) (Fig. 1B). Of note, levels of IgG_2 were significantly higher in *LdCen*$^{-/-}$ vaccinated dogs compared to PBS and Leishmune® groups ($p = 0.02$ and $p = 0.0005$, respectively) (Fig. 1C).

3.2. Proliferative response of T cells and B cells

To investigate if *LdCen*$^{-/-}$ vaccination induces specific T and B cell proliferation, we performed cell proliferation assays. The results were presented as stimulation index. Results showed that vaccination with *LdCen*$^{-/-}$ induced an enhanced CD4+ T cell proliferation in response to SLA compared to Leishmune® group ($p = 0.03$) (Fig. 2A). Importantly, immunization with *LdCen*$^{-/-}$ induced a much higher proliferation of CD8+ T cells compared to PBS and Leishmune® groups ($p = 0.04$ for all) (Fig. 2B). Furthermore, induction of proliferative response of B cells in *LdCen*$^{-/-}$ group was significantly higher compared to Leishmune® group ($p = 0.03$) (Fig. 2C).

3.3. Immunization with LdCen$^{-/-}$ induces higher activation of CD8+ T cells

After ascertaining the proliferative responses of the T cells, we examined the activation profile of the T cells by analyzing surface expression of MHC-II and CD11/18 markers on CD4+ and CD8+ T cells. *Ex vivo* analysis of T lymphocyte subsets revealed that *LdCen*$^{-/-}$ vaccination induced significantly lower MHC-II expression by CD4+ T cells compared to PBS group ($p < 0.03$) (Fig. 3A). In contrast, an elevated MHC-II expression was observed in CD8+ T cells obtained from *LdCen*$^{-/-}$ group compared to Leishmune® and PBS groups ($p < 0.0001$ and $p = 0.01$, respectively) (Fig. 3B).

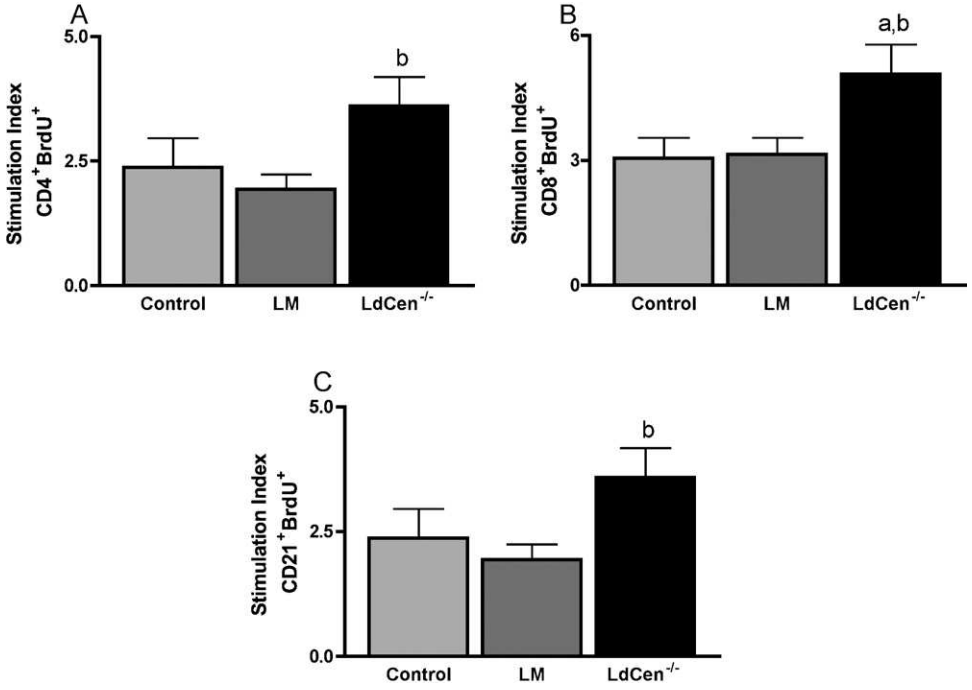

Fig. 2. Specific cell proliferative response in control (PBS), and vaccinated dogs (Leishmune® and *LdCen*$^{-/-}$) groups. Proliferation of CD4+ (A), CD8+ (B) and B cells (C) were assessed by incorporation of BrdU after stimulation of peripheral blood mononuclear cells with soluble *L. infantum* antigen. Results are expressed as stimulation index (Ratio between stimulated cultures and non-stimulated cultures). [a,b]Significant differences at $p < 0.05$ compared with Control and Leishmune®, respectively.

In addition, vaccination with *LdCen*$^{-/-}$ or Leishmune® induced increased expression of CD11/18 by CD4+ and CD8+ T cell populations ($p < 0.001$) compared to non-vaccinated controls (Fig. 3C and D). No major differences were detected in the expression of MHC-II in CD4+ T cells or CD11/18 in CD4+ and CD8+ populations between stimulated and non-stimulated cells (data not shown).

3.4. *LdCen*$^{-/-}$ vaccine induces a strong IFN-γ response in CD8+ T cells

Intracytoplasmic detection of IFN-γ and IL-4 within CD4+ and CD8+ T lymphocytes was performed immediately after blood collection and in whole blood cultures stimulated with SLA for

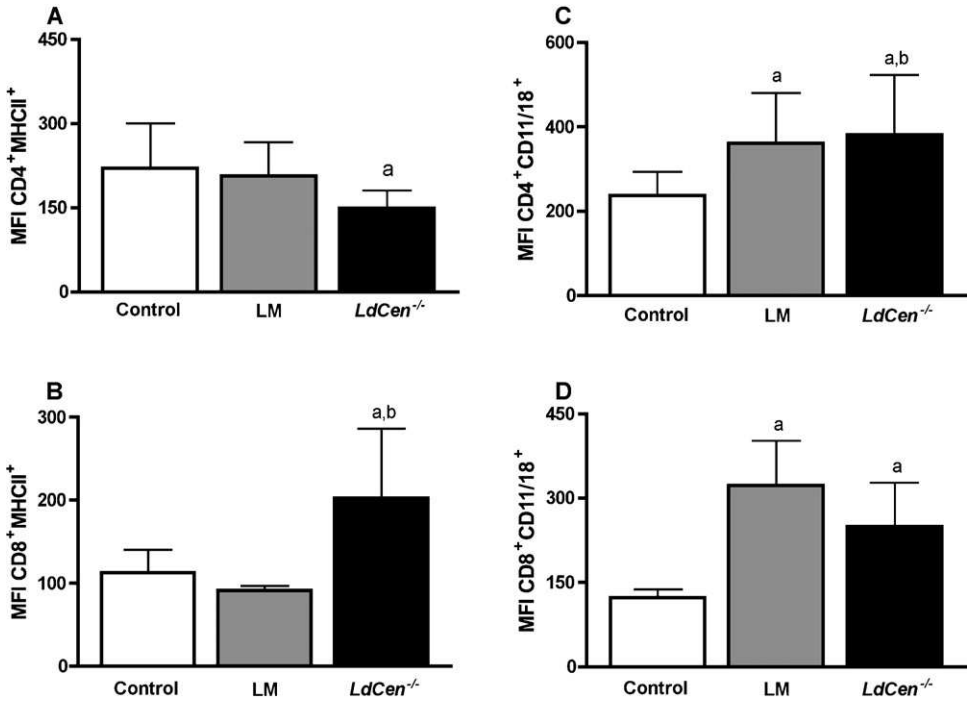

Fig. 3. *Ex vivo* profile of T cell activation in control (PBS) and vaccinated dogs (Leishmune® and *LdCen*$^{-/-}$) groups. Results are expressed as mean fluorescence intensity (MFI) of MHC II (A and B) or CD11/18 (C and D) by T CD4+ (A and C) or T CD8+ (B and D) cells. [a,b]Significant differences at $p < 0.05$ compared with Control and Leishmune®, respectively.

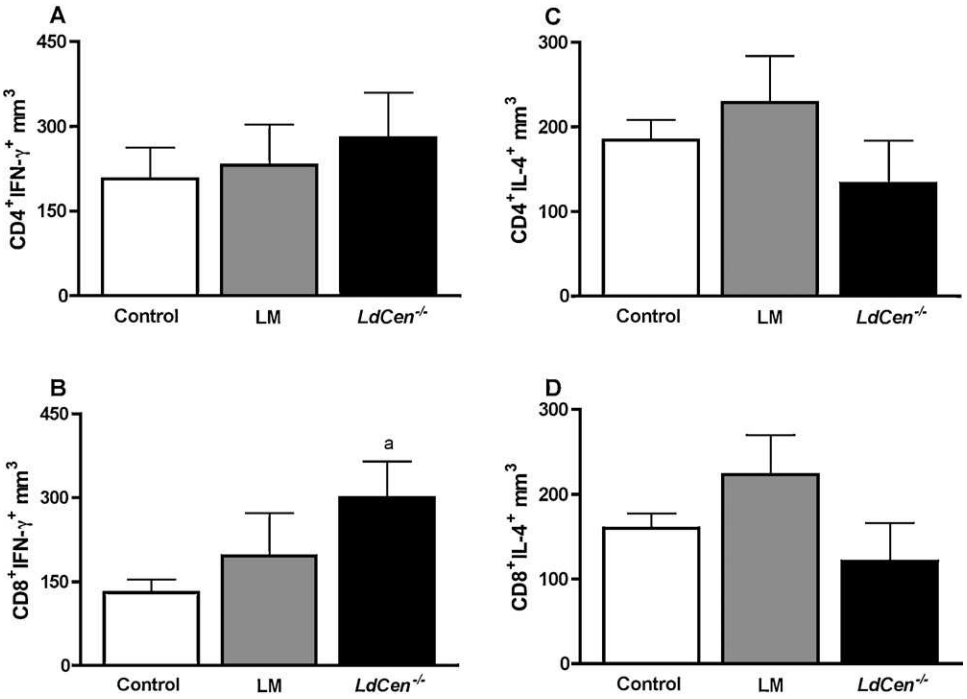

Fig. 4. *Ex vivo* intracytoplasmic cytokine expression within CD4+ and CD8+ T cells in control (PBS) and vaccinated dogs (Leishmune® and *LdCen*$^{-/-}$). Results are expressed as absolute number (cells/mm^3) of IFN-γ (A and B) or IL-4 (C and D) production by T CD4+ (A and C) or T CD8+ (B and D) cells. [a,b]Significant differences at $p < 0.05$ compared with PBS and Leishmune®, respectively.

24 h. This analysis revealed that circulating lymphocytes from *LdCen*$^{-/-}$ vaccinated group displayed decreased IL-4 levels in CD4+ ($p = 0.054$) and CD8+ ($p = 0.891$) subpopulations even though this difference was not statistically significant (Fig. 4C and D). Importantly, *LdCen*$^{-/-}$ vaccination induced higher IFN-γ production in CD8+ T cells ($p = 0.0103$) (Fig. 4B) and not in CD4+ T cells (Fig. 4A). No statistical differences were observed in stimulation index between different groups analyzed for expression of IL-4 and IFN-γ within CD4+ and CD8+ T cell subpopulations (data not shown).

3.5. *LdCen*$^{-/-}$ vaccination elicits a pro-inflammatory cytokine production

TNF-α, IL-12/IL-23p40 and IL-4 were evaluated in SLA stimulated PBMC culture supernatants 15 days post immunization. The results were presented as delta stimulation. *LdCen*$^{-/-}$ group showed significantly higher levels of TNF-α ($p < 0.005$, Fig. 5A) and IL-12p40 ($p < 0.0019$, Fig. 5B) in PBMCs. Similar enhanced production of TNF-α, but not IL-12 was observed in Leishmune® group ($p < 0.005$) (Fig. 5A). The production of IL-4 was significantly reduced in *LdCen*$^{-/-}$ vaccinated animals compared to PBS and Leishmune® vaccinated dogs ($p < 0.05$ for all, respectively) (Fig. 5C). IL-10 production was not observed in supernatant from all the groups (data not shown).

3.6. Phenotypic profile of circulating cells (monocytes, B cells and CD4+ and CD8+ T cells) and hematological and biochemical analysis

Vaccination with LM or *LdCen*$^{-/-}$ parasites did not alter the frequencies of CD3+CD4+, CD3+CD8+, CD21+ and CD14+ when compared to T0 (before vaccination) (Supplementary Table 1) in the *ex vivo* analysis. Hematological and biochemical analysis showed that vaccination with LM or *LdCen*$^{-/-}$ parasite did not result in changes in the entire parameters analyzed Supplementary Table 2).

4. Discussion

Developing vaccines against protozoan parasitic infections remains a challenge. Major impediments to developing effective vaccines include discovery of good vaccine candidates, systematic evaluation of the vaccines in mouse models to establish immune correlates of protection and importantly, to validate the results obtained in mouse models in outbred species [27–30]. Genetically modified attenuated parasites, which mimic the wild type infection but do not cause overt disease confer safety and better protection over recombinant antigens and thus represent the best approach [17]. We are reporting on a vaccine formulation against CVL consisting of centrin deleted *L. donovani* parasite (*LdCen*$^{-/-}$) [21]. This deletion specifically affects the proliferation of the amastigote stage that replicates inside macrophages. This attribute contributes to the safety of *LdCen*$^{-/-}$ that has been demonstrated in mouse and hamster models [18]. In the current study, we investigated the immunogenicity of the *LdCen*$^{-/-}$ parasites in dogs and compared it with a commercially available canine *Leishmania* vaccine. Our results showed that *LdCen*$^{-/-}$ vaccination is highly immunogenic in dogs as evidenced by induction of anti-*Leishmania* IgG_{Total}, IgG_1 and IgG_2. Antibody responses are commonly found in *Leishmania* vaccines [18,24,31–33] including killed *Leishmania* [32,34], recombinant antigens [26,27,31,35,36], and live attenuated parasites [18,37]. Remarkably, *LdCen*$^{-/-}$ showed higher antibodies titers than Leishmune® in dogs suggesting that live attenuated parasites allow for the delivery of a broad range of antigens unlike a limited set of antigens that can be delivered *via* recombinant vaccines. However, protection from *Leishmania* infection is mediated primarily by cytotoxic T cells [38,39]. Previous reports demonstrated that T cell proliferation in response to *Leishmania* antigens is an important biomarker of immunogenicity of a vaccine in mice and in dogs [24,40]. Consistent with these reports, our results showed

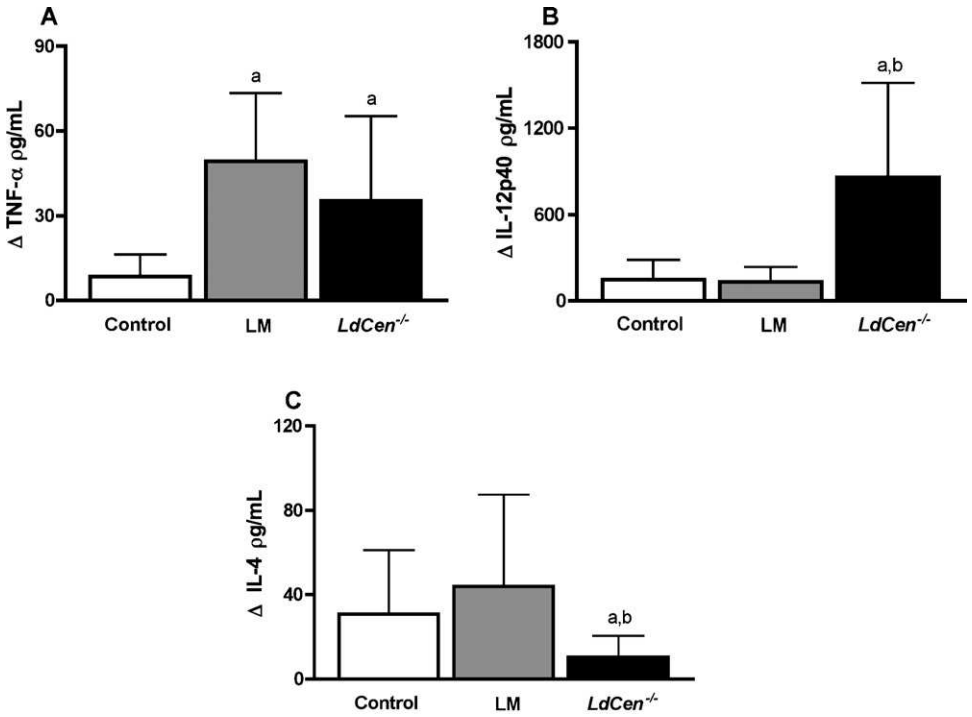

Fig. 5. *In vitro* cytokine production in control (PBS) and vaccinated dogs (Leishmune® and *LdCen*$^{-/-}$). Production of TNF-α (A), IL-12p40 (B) and IL-4 (C) was determined after stimulation of peripheral blood mononuclear cells with soluble *L. infantum* antigen. Results are expressed as stimulation delta (SD), calculated by the difference of cytokine production between stimulated and non-stimulated cultures. Bars represent mean and standard deviation. [a,b]Significant differences at $p < 0.05$ compared with Control and Leishmune®, respectively.

that vaccination with *LdCen*$^{-/-}$ parasites induces a higher T and B cell proliferation upon stimulation with *Leishmania* antigen. Such proliferation was also observed in mice vaccinated with LiSIR2$^{+/-}$ parasites [19] or *Leishmania* crude extract with adjuvants in mice [40] or dogs [24,32,34].

To determine the activation status of the T cells subsets, we evaluated the expression of MHC-II and CD11/18. Our data showed a higher expression of MHC-II and CD11/18 in CD8+ lymphocytes in the *LdCen*$^{-/-}$ vaccinated dogs suggesting that the vaccine induced circulating activated T cells. Increased expression of MHC-II represents an antigenic priming-related immunological event and this was correlated with protection in asymptomatic dogs [22,32]. Consistently, a higher T cell activation was also observed in dogs after immunization [35,41].

In order to assess the production of intracytoplasmic cytokines after immunization, we analyzed the production of IFN-γ and IL-4 by CD4+ and CD8+ T cells. Our *ex vivo* analysis showed that *LdCen*$^{-/-}$ immunized dogs displayed higher frequencies of CD8+ T cells expressing IFN-γ, as was observed in immunized mice [18]. CD8+ T cells play a critical role in the control of *L. donovani* infections [42] by contributing to the formation of granulomas in the liver of *L. donovani* infected mice [43]. Further, adoptive transfer of antigen-specific CD8+ T cells resulted in 90% reduction in the splenic parasite burden during chronic VL [44]. Our results showing the activation of CD8+ T cells and secretion of IFN-γ in cells isolated from *LdCen*$^{-/-}$ group but not from the Leishmune® group suggest that in dogs the protection may be predominantly associated with CD8+ T cell functions. In contrast, immunization of BALB/C mice with *LdCen*$^{-/-}$ resulted in robust induction of IFN-γ positive and TNF^{+} CD4+ as well as CD8+ T cells [18]. Thus the limited induction of CD4+ T cells and higher frequencies of CD8+ T cells expressing IFN-γ in *LdCen*$^{-/-}$ immunized dogs observed in the current study might represent a unique attribute of dog immunity further underscoring the importance of understanding immune correlates of protection in different animal models in pre-human vaccination trials. Infection with a wild type *L. donovani* results in defective antigen-specific CD8+ T cell responses [45] due to impaired antigen processing and presentation [42]. Therefore, the activation of antigen-specific CD8+ T cells observed in *LdCen*$^{-/-}$ immunized dogs might represent a partial restoration of antigen-presentation and thus CD8+ T cell activation.

In addition, decreased expression of IL-4 by CD4+ and CD8+ T cells was demonstrated, suggesting that *LdCen*$^{-/-}$ altered the Type 1/Type 2 balance towards a protective response, and other vaccine candidates may also influence the Type 1/Type 2 balance [46,47]. However, Leishmune® did not dramatically alter IFN-γ or IL-4 production. *LdCen*$^{-/-}$ vaccination also induced higher production of TNF-α and IL-12/IL-23p40 in the supernatants of cultures and a decreased production of IL-4 supporting the concept that this vaccine induces a Type 1 response. Similar results were detected in splenocytes from mice vaccinated with different recombinant antigens [47,48]. It has been shown that resistance to leishmaniasis is associated with a predominant IFN-γ and IL-12 production [49,50], as well as vaccines against leishmaniasis that promote IFN-γ and IL-12 are considered to be associated with protective responses [51,52]. On the other hand, the immune response mediated by Th2 cells is associated with susceptibility, allowing the parasite persistence and progression of the infection [53].

Interestingly, no differences in phenotypic profile of monocytes, B cells and CD4+ and CD8+ T cells were observed before and after vaccination. Furthermore, hematological and biochemical analysis did not show any differences between the groups, which suggests that the immunization with Leishmune® and *LdCen*$^{-/-}$ did not induce any alteration of the number of circulation peripheral blood cells and also, did not promote alteration in the homeostasis of hepatic and renal physiological parameters. These results indicate the safety of such vaccines for use in dogs.

Previous work has shown that *LdCen*$^{-/-}$ live attenuated vaccine was immunogenic and protective against VL in mice and hamsters [18]. The current work is a step further in demonstrating that

LdCen$^{-/-}$ vaccine induces strong antibody production, Type 1 polarization and Type 2 inhibition in dogs, a model more relevant to the disease because of its important role as a reservoir host. It is described that a vaccine against leishmaniasis required a Type 1 response to protect after challenge, as it was demonstrated by other authors in dog model [34,54]. Furthermore, it has been shown that CD8+ T cells actively participate in the control of VL and their presence correlates with protection [42]. This study is a first step to show the potential of genetically modified live attenuated *Leishmania donovani* vaccine for clinical veterinary use making it highly attractive for field trials against canine VL. Further studies for evaluating the protection of the *LdCen*$^{-/-}$ vaccine against visceral canine leishmaniasis after challenge are in progress.

Acknowledgements

We thank Michele Silva de Matos, Weder Gomes de Oliveira, Jefferson Bernardes and Virgilio Gandra Martins for the technical support. This work was financially supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais/FAPEMIG (Grant#CBB APQ-01202-09 and PPM-00296-11], Brazilian National Research Council (CNPq), Programa PAPES V/FIOCRUZ, Centro de Pesquisas René Rachou (CPqRR/FIOCRUZ) and Pró-Reitoria de Pesquisa/Universidade Federal de Minas Gerais and by CBER intramural funds. JAF, RCO, DB and RTF are supported by CNPq fellowships. The authors would like also to thank Dr. Robert Duncan and Dr. Ranadhir Dey for internal review of the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.01.048.

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