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Antigenicity of a whole parasite vaccine as promising candidate against canine leishmaniasis

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Abstract

Human visceral leishmaniasis, one of the most important zoonoses, is caused by the protozoa *Leishmania chagasi* (syn. *L. infantum*) and is present as a fatal disease common in South America and Europe where dogs and wild canids are the main reservoirs. A vaccine against visceral leishmaniasis would be an important tool in the control of this disease in dogs. Although the current strategies for vaccination against leishmaniasis are based on the use of recombinant antigens, killed vaccines are still attractive in terms of stability of their biochemical composition and antigenicity, cost, and safety. Here we evaluate the immunogenicity of a whole parasite vaccine as a promising candidate against canine leishmaniasis, demonstrated by cellular reactivity, changes in the cellular profile of the peripheral blood and by the differential production of immunoglobulins. Our results showed that immunization elicited mainly a strong cellular reactivity and increase in T-lymphocytes, particularly the subpopulation $CD8^+$ that would be related to the control of tissue parasitism. In addition, a higher production of anti-*Leishmania* total IgG, characterized by mixed isotypes profile (IgG1 and IgG2), was demonstrated.

Keywords: Vaccine; Canine visceral leishmaniasis; Whole parasite vaccine; Dog

1. Introduction

Human visceral leishmaniasis (HVL), one of the most important zoonoses, is caused by the protozoa *Leishmania* (*Leishmania*) *chagasi* (syn. *Leishmania. infantum*) and is present as a fatal disease common in South America and Europe where dogs and wild canids are the main reservoirs (Deane and Deane, 1962). In Brazil, the prevalence for CVL in other endemic areas has shown to vary from 5% to 35% (Evans et al., 1990; Nunes et al., 1991).

According to Hommel et al. (1995) a vaccine against visceral leishmaniasis would be an important tool in the control of CVL, and would also dramatically decrease the pressure of infection of *L. chagasi*/*L. infantum* over

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humans. However, an effective vaccine against kala azar is not yet available and only few reports in the literature deal with a vaccine against canine visceral leishmaniasis (Mayrink et al., 1996; Lasri et al., 1999; Panaro et al., 2001; Silva et al., 2001; Borja-Cabrera et al., 2002; Molano et al., 2003; Fujiwara et al., 2005b; Lemesre et al., 2005; Giunchetti et al., 2007c). Although the current strategy for vaccination against leishmaniasis is based on the use of recombinant antigens, whole parasite vaccines are still attractive in terms of stability of their biochemical composition and antigenicity, cost, safety and have been used in vaccine trials against leishmaniasis (Ravindran and Ali, 2004). In dogs, these vaccines demonstrated up to 90% of protection in Phases I and II clinical trials in Brazil, using merthyolated sound-disrupted promastigotes of *L. braziliensis* formulated BCG (Bacillus Calmete Guérin) (Mayrink et al., 1996). The use of these whole parasite vaccines was usually accompanied by strong cellular reactivity, as observed by lymphocyte proliferation (Mayrink et al., 1996). Indeed, Lasri et al. (1999) demonstrated the vaccination of dogs with autoclaved *L. major* promastigotes (ALM) and BCG as adjuvant elicited a strong cell proliferation to leishmanial antigens compared to the others groups. Cellular and humoral immune responses of dogs to a candidate vaccine, composed of *L. braziliensis* promastigote protein plus saponin as an adjuvant, presented strong antigenicity related to the increases of anti-*Leishmania* IgG isotypes, higher levels of *L. chagasi* antigen-specific $CD8^+$ T-lymphocytes as well as intense cell proliferation and increased nitric oxide production after *in vitro* stimulation by *L. chagasi* soluble antigens (Giunchetti et al., 2007c). These studies related promising results in crude antigens vaccines (Mayrink et al., 1996; Lasri et al., 1999; Panaro et al., 2001; Giunchetti et al., 2007c). Considering the facilities to manufacture crude antigens vaccines, the lower production cost and the promising results, this approach might prove to be useful in controlling leishmaniasis in endemic areas, particularly under developed countries.

Here we evaluate the immunogenicity of a whole parasite vaccine as a promising candidate against canine leishmaniasis, demonstrated by cellular reactivity and changes in the cellular profile of the peripheral blood and by the differential production of specific immunoglobulins.

2. Materials and methods

2.1. Animals and vaccine study design

Two groups of dogs were used in this study (whole parasite vaccine – WPV – and Placebo groups). The dogs were parasite naive and were selected from a colony of beagles, bred and maintained under conditions designed to exclude any possible contaminating *Leishmania* infections. The dogs were between 8 and 12 months old, well-fed animals under constant supervision by a veterinarian and had all received their routine vaccinations against parvovirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and leptospirosis (Recombitek®, Merial Inc., USA). All animals also received a single intranasal dose against adenovirosis-2, parainfluenza and *Bordetella bronchiseptica* (Bronch Shield III®, Fort Dodge, USA). The dogs were also treated with anti-helminthic drugs (Endal Plus®, Schering Plough, Brazil) and with anti-ectoparasites (Frontline®, Merial Inc., USA), and were quarantined for approximately four weeks before beginning the vaccine trial. The animals were divided into two groups. One group, namely WPV ($n = 5$), received a whole parasite vaccine (mix of *Leishmania amazonensis* – IFLA/BR/1967/PH8 – and *Leishmania braziliensis* – MCAN/BR/1972/C348 – crude extracts) formulated with nonlive lyophilized BCG (FAP, Rio de Janeiro, Brazil) as the adjuvant. The second group, Placebo ($n = 5$), received BCG alone (three decreasing doses – 400 μg of BCG in the first dose, 300 μg in the second and 200 μg in the last dose). All vaccines were administered subcutaneously in three immunizations at intervals of four weeks. Peripheral blood samples from the jugular vein of the animals were taken before the immunizations (time 0), 15 days after each dose of immunization (time 15: 15 days after first immunization or time 1; time 45: 15 days after second immunization or time 2; time 75: 15 days after third immunization or time 3) for the immune evaluations. This work was approved by the Ethical Committee of Animal Research of the Federal University of Minas Gerais, Belo Horizonte, Brazil (Protocol No. 008/02).

2.2. Cell proliferation

Lymphoproliferation assay was performed using 20 ml of heparinized blood, which was collected and added on 10 ml of the Ficoll–Hypaque density gradient (Histopaque 1.077 – Sigma Co., EUA) followed by centrifugation at 450*g*, for 40 min, at room temperature (RT). Peripheral blood mononuclear cells (PBMC) were separated from blood, resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY, USA), homogenized and washed twice in RPMI 1640 medium under centrifugation at 450*g*, for 10 min, at RT. The PBMC was homogenized and resuspended in 10^7 cells/ml in RPMI 1640. The lymphoproliferation assay was performed in 96 well flat bottom tissue culture plates (Nunc, Denmark) in 150 μl with RPMI medium (supplemented with streptomycin (100 mg/ml), penicillin (100 U/ml), L-glutamine (2 mM), β-mercaptoethanol (5×10^{-5} M) and 10% heat-inactivated FCS). The cells were added to triplicate wells using 25 μl of PBMC (2.5×10^5 cells/well) with 25 μl of *L. chagasi* antigen (10 μg/ml) obtained according to Reis et al. (2006a,c) and 15 μl of PBMC (1.5×10^5 cells/well) for the mitogenic stimulus with 25 μl of ConA (Concanavalin A – Sigma Chemical Co., EUA; 2 μl/well). Both stimuli were compared to unstimulated control with 25 μl of the RPMI 1640 medium plus 25 μl of PBMC with 2.5×10^5 cells/well. Incubation was carried out in a humidified 5% CO_2 atmosphere at 37 °C for three (ConA-stimulated cultures) and five (*L. chagasi*-stimulated cultures) days. Six hours prior to

termination of culture, 1 μCi of 3 H-thymidine (Sigma Chemical Co., USA) was added to each well and harvested onto glass fibre filters. Radioactive incorporation was determined by liquid scintillation spectrometry. Proliferation responses were expressed as mean counts of triplicate wells per minute and performed stimulation delta (SD = mean proliferation of stimulated cultures by *L. chagasi* antigen – mean proliferation of unstimulated cultures).

2.3. Immunophenotyping

Immunofluorescence assay was performed according to Fujiwara et al. (2005a) and Reis et al. (2005). In brief, peripheral blood (5 ml) was collected into EDTA containing tubes and then lysed with FACS lysing solution (FACS lysing solution – FLS – Becton Dickinson, Moutain View, CA). Cell suspension was incubated with monoclonal antibodies (mAb) rat anti-canine Thy-1 (Rat-IgG2b: clone YKIX337.217), CD4 (Rat-IgG2a: clone YKIX302.9), CD8 (Rat-IgG1: clone YCATE55.9), fluorescein isothiocyanate conjugated (FITC) CD21 to human B lymphocytes and CD45RA (Rat-IgG2b: clone YKIX753.22.2). Polyclonal anti-rat IgG FITC conjugated was used as secondary antibody to develop the non-fluorescent antibodies. The cells were washed with PBS and analyzed on a FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) using the CellQuest™ software (Becton Dickinson, USA). Ten thousand ungated events were collected from each sample. Gating was based on forward- and side-scatter light characteristics. The results for Thy-1$^+$, CD4$^+$, CD8$^+$, and CD21$^+$ were expressed as percentual values of cells to each marker represented by the percentage of positive lymphocytes to each surface marker staining. Semi-quantitative analyses of cell surface markers presenting unimodal distribution (CD45RA) were also stable for immunophenotyping after fixation, with no changes on fluorescence intensity distribution as determined by mean fluorescence channel (MFC). Unlabeled mAbs and polyclonal anti-rat IgG FITC conjugated used as secondary antibody in this study were purchased from SEROTEC (Oxford, UK) and anti-CD21 was purchased from Immunotech Co. (Marselle, France).

2.4. Humoral immune response

Antibodies were determined by conventional enzyme-linked immunosorbent assay (ELISA) for the immunogenicity evaluation with soluble lysate of both *L. amazonensis* (MHOM/BR/1960/BH6) (SLaA) and *L. chagasi* (MHOM/BR/1972/BH46) (SLcA) used according to Reis et al. (2006a,c). The antigens were coated onto 96-well microplates (Maxi-Sorp™, Nalge Nunc Intl., USA) at a concentration of 10 μg/well for SLcA. The sera were added at a dilution of 1:80 followed by washes and addition of peroxidase conjugated goat anti-dog IgG1 or sheep anti-dog IgG and IgG2 (Bethyl Laboratories Inc., Montgomery, TX, USA). The wells were then washed and substrate and chromogen (*O*-Phenylenediamine, Sigma–Aldrich Co., USA) were added and the absorbance was read on an automatic ELISA microplate reader (Multiskan® MCC 340, Labsystems, Helsinki, Finland) at 492 nm. The conjugate anti-IgG1 was used at a dilution of 1:1000 and the conjugates anti-IgG and IgG2 were used at 1:8000 and 1:16000 dilutions, respectively.

2.5. Statistical analysis

Statistical analysis was performed using the Prism 3.0 software package (Prism Software, Irvine, CA, USA). The normality test Kolmogorov–Smirnoff was performed and it demonstrated the normality of the data. The repeated measures one-way analysis of variance (ANOVA) and Tukey post test were used for the longitudinal study among the different times tested in both WPV and placebo groups. Student's *t*-test was used for determining the differences between the WPV and placebo groups. The differences were considered significant when the probabilities of equality, *P* values, were <0.05.

3. Results

3.1. Marked increase in in vitro cell reactivity, mainly after first immunization is a hallmark of WPV

In order to evaluate cell proliferation in the presence of the *L. chagasi* antigen as a potential vaccine against etiological agent of visceral leishmaniasis, cell reactivity was analyzed (Fig. 1). WPV showed significant improvement ($P < 0.05$) in stimulation delta (SD) after first and second immunizations. Longitudinal analyses considering

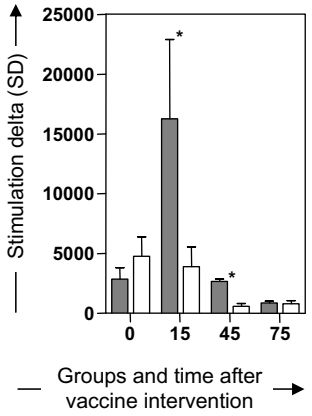

Fig. 1. Specific cell proliferation response in whole parasite vaccine (WPV – grey bars) and Placebo (white bars) from peripheral blood mononuclear cells after stimulation with soluble *L. chagasi* antigen. Results are expressed as stimulation delta (SD) and the bars represent the mean for each group plus standard deviation. The *x*-axis represents the subcutaneous doses at different times (0: before the first dose; 15: 15 days after the first dose on day 15; 45: 15 days after the second dose on day 45; 75: 15 days after the third dose on day 75). *Significant differences at $P < 0.05$ are indicated in comparison to WPV and Placebo groups.

immunizations for WPV revealed significant increase ($P < 0.05$) only in time 1 compared to time 3.

3.2. Enhanced frequency in Thy-1$^+$ T-lymphocytes, mainly CD8$^+$ T-cells as well as a decrease in CD4$^+$ T-cells, CD21$^+$ B-cells and expression in lymphocytes CD45RA represent the major immunophenotic features in WPV

Immunophenotypic profile of peripheral blood leukocytes is presented in Fig. 2. The evaluation for T-lymphocytes and subsets (Thy-1$^+$, CD4$^+$ and CD8$^+$) showed a distinct profile. We observed that animals immunized with WPV presented a significant increase ($P < 0.05$) in T-lymphocytes Thy-1$^+$ after second immunization compared to the Placebo group (Fig. 2a). In this context, the main subset that contributes to the augmentation of T-lymphocytes Thy-1$^+$ was the CD8$^+$ subpopulation that presented significant increase ($P < 0.05$) after first and second immunizations (Fig. 2c). Interestingly, when T-lymphocytes CD4$^+$ were evaluated in WPV, we observed a significant ($P < 0.05$) reduction after third immunization (Fig. 2b). Similarly, the evaluation in B-lymphocytes CD21$^+$ showed a significant decrease ($P < 0.05$) in dogs that received WPV after the last dose of vaccine (Fig. 2d).

Expression of lymphocytes CD45RA by peripheral blood lymphocytes was evaluated through semi-quantitative analyses in order to evaluate whether immunizations may be associated with a distinct pattern of these constitutive cell surface markers involved directly in lymphocyte activation. Significant reduction ($P < 0.05$) in the expression of lymphocytes CD45RA after first immunization was observed in WPV (Fig. 2e). Moreover, the expression of lymphocytes CD45RA in the WPV group presented a significant decrease ($P < 0.05$) along the subsequent experimental times.

3.3. Despite the high levels of total IgG anti-Leishmania, WPV demonstrates a mixed IgG1/IgG2 response

The reactivity of seric anti-*Leishmania* immunoglobulins is shown in Fig. 3. Our results demonstrated that both anti-*L. amazonensis* (Fig. 3a) and anti-*L. chagasi* (Fig. 3b) total IgG presented increase after second and third immunizations in WPV. Furthermore, WPV elicited higher levels ($P < 0.05$) of anti-*Leishmania* IgG1 after second and third immunizations (Fig. 3c). Moreover, the longitudinal evaluation of anti-*L. chagasi*/*L. amazonensis* and anti-*Leishmania* IgG1 revealed that only those dogs immunized

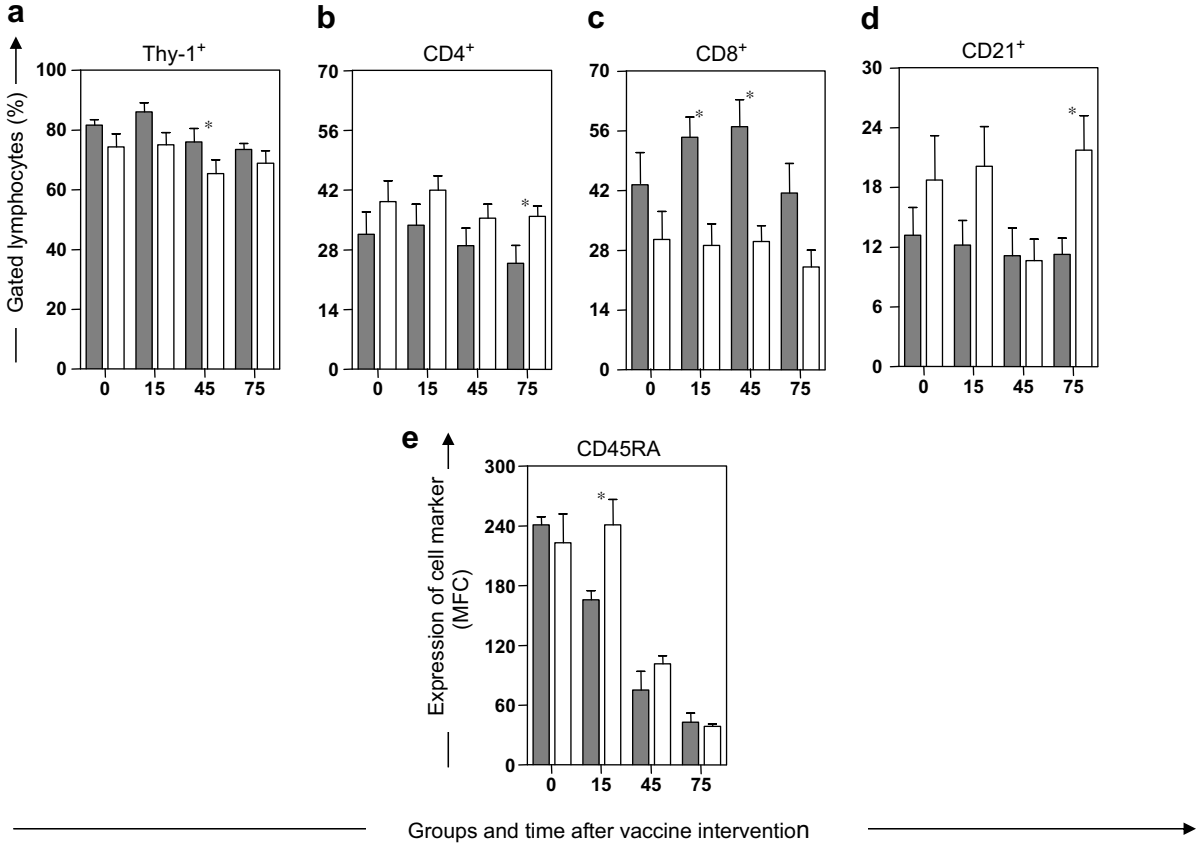

Fig. 2. Immunophenotypic profile of peripheral blood leukocytes in beagles that received whole parasite vaccine (WPV – grey bars) and Placebo (white bars). Results are expressed as percentage cell counts of Thy-1$^+$ (a), CD4$^+$ (b), CD8$^+$ (c), and CD21$^+$ (d) lymphocytes. The results are expressed as average of CD45RA expression (e) reported as mean fluorescence channel (MFC) plus standard deviation. The *x*-axis represents the subcutaneous doses at different times (0: before the first dose; 15: 15 days after the first dose on day 15; 45: 15 days after the second dose on day 45; 75: 15 days after the third dose on day 75). *Significant differences at $P < 0.05$ are indicated in comparison to WPV and Placebo groups.

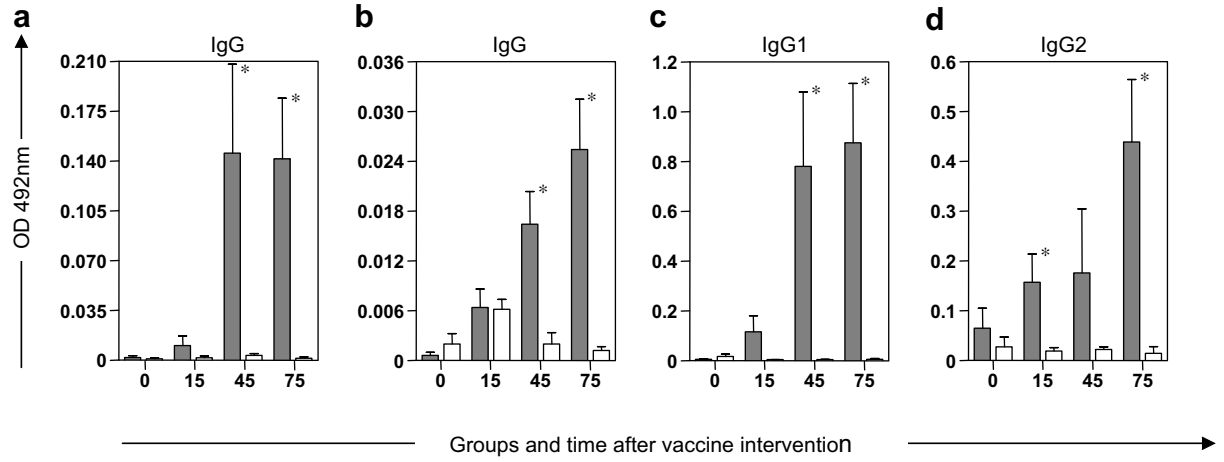

Fig. 3. Comparative levels of serum specific antibody production between whole parasite vaccine (WPV – grey bars) and Placebo (white bars). The results are expressed as the mean average plus standard deviation of absorbance in sera from each group. The *x*-axis represents the subcutaneous doses at different times (0: before the first dose; 15: 15 days after the first dose on day 15; 45: 15 days after the second dose on day 45; 75: 15 days after the third dose on day 75). The *y*-axis represents the ELISA absorbance values at 492 nm of the sera samples diluted a ratio of 1:80. a: anti-*L. amazonensis* total IgG; b: anti-*L. chagasi* total IgG; c: anti-*L. chagasi* IgG1; d: anti-*L. chagasi* IgG2. *Significant differences at $P < 0.05$ are indicated in comparison to WPV and Placebo groups.

with WPV presented a significant gradual increase ($P < 0.05$) of specific antibody levels during immunization when compared with baseline data (before immunization). When IgG2 was evaluated, WPV induced higher levels ($P < 0.05$) of anti-*Leishmania* IgG2 after first and third doses when compared with placebo (Fig. 3d).

4. Discussion

The natural history of CVL has been thoroughly demonstrated in several reports, focusing attention on the relationship between biochemical/haematological changes, histopathologic features, leukocytes immunophenotic patterns and the IgG isotype levels which were directly correlated with the clinical status of the disease and parasite load (Giunchetti et al., 2006, 2007a,b; Reis et al., 2006a,b,c). Nevertheless, few studies using accurate evaluations such as flow cytometric assays for canine leukocytes considering different anti-*Leishmania* vaccines have been described (Giunchetti et al., 2007c). To date, different treatment strategies have been failed to achieve consistent parasitological cure in CVL without a reliable effective therapy (Baneth and Shaw, 2002; Noli and Auxilia, 2005). In this context, a dog vaccine may be the most practical and effective method to reduce the incidence of human disease, and would permit a similar vaccine for humans (Abranches et al., 1991; Gradoni, 2001). Considering the importance of a vaccine for the control of leishmaniasis and the lack of studies on cellular and humoral events that occur during and after vaccination, the understanding of the immune response elicited by a vaccine is a prerequisite for the comprehension of the mechanisms of protection against the parasite and, therefore, the development of new and effective vaccines against this disease.

Although an increasing number of molecularly defined subunit vaccines obtained by recombinant methodologies have shown some degree of efficacy against experimental leishmaniasis (Wilson et al., 1995; Webb et al., 1996, 1998; Dole et al., 2000; Campos-Neto et al., 2001), particularly in murine model, this approach still does not reflect effective results in the dog model (Gradoni, 2001; Requena et al., 2004; Khamesipour et al., 2006). However, the semi purified FML (fucose mannose ligant) antigen associated with saponin has shown encouraging results against CVL (Silva et al., 2001; Borja-Cabrera et al., 2002). Moreover, the remarkable results obtained from the vaccination of dogs with killed parasite vaccines (Lasri et al., 1999; Mayrink et al., 1996; Panaro et al., 2001; Giunchetti et al., 2007c) showed that a successful vaccine against canine leishmaniasis is still possible.

Our results showed that immunization with WPV elicited a strong cell reactivity observed with the increase of cell proliferation after first and second doses. Thus, the ability of WPV to stimulate *in vitro* antigenic-specific T lymphocytes after immunization may play an important role during prime naive T $CD4^+$ lymphocytes, which are the central regulators of both humoral and cellular immune responses (Stockinger et al., 2006). Interestingly, a reduction in the amount of circulating T-lymphocytes $CD4^+$ was observed in comparison to the animals that received placebo alone, which might imply the migration of primed $CD4^+$ to the lymphoid organs to mediate its effectors mechanisms of immune protection. Of note, the results obtained in all experiments using the placebo group were similar to additional control animals that received saline solution (data not shown).

The expansion in T-lymphocytes Thy-1^+ in the peripheral blood after vaccination with WPV may be reflected by

the increase of subpopulation $CD8^+$ (after first and second immunizations; Fig. 2c), which would indicate a possible mechanism for the control of parasitism, since increased levels of T-lymphocytes $CD8^+$ appeared as the major phenotypic feature of asymptomatic disease and dogs bearing low parasite load (Reis et al., 2006b). As noted for T $CD4^+$ cells, circulating B-lymphocytes $CD21^+$ were also observed in reduced numbers in the peripheral blood. Again, it would imply a possible migration to lymphoid organs and a potential cooperative relationship with those activated $CD4^+$, resulting in the polyclonal B-lymphocytes activation. It is corroborated by the strong production of anti-*Leishmania* total IgG after second and third immunizations. Finally, the reduction in the expression of CD45RA by peripheral blood lymphocytes in WPV (Fig. 2e) might indicate a progressive decrease in naive canine T-lymphocytes after starting the vaccination, and therefore, likely achieving an activated status.

Immunogenicity of the WPV was also observed by the high antibody production as measured by the ELISA specifically designed to detect canine antibodies of IgG isotypes specific for the crude antigens. Since IgG1 and IgG2 responses are strictly T cell dependent, we used them as readouts to evaluate the overall immunogenicity of the recombinant antigens in dogs (Fujiwara et al., 2005b). In addition, for humans and mice, IgG1 and IgG2 subtypes have been traditionally used as surrogates of the Th2 and Th1 phenotypes of immune responses, respectively. The association between IgG subtypes and the immune response in terms of the Th1/Th2 phenotype is not so straightforward in dogs yet (Day, 2007). However, experimental evidences from published canine studies do indicate a possible association between isotype production and Th1/Th2 shifts (Chabalgoity et al., 2001; Loukas et al., 2005; Fujiwara et al., 2006). In this way, the analysis of immunoglobulin isotypes might point out to a mixed immune response in WPV group, with initial production of IgG2 (after first dose) and subsequent production of IgG1 (after second dose); after third immunization, the responses of both antibodies IgG1 and IgG2 were presented.

Furthermore, WPV group displayed significant higher anti-*Leishmania* total IgG after second and third immunizations (Fig. 3a and b). Additionally, the seric reactivity using SLcA indicated antigenic recognition against *L. chagasi*, suggesting a potential use of this vaccine against the etiological agent of visceral leishmaniasis. In fact, according to Rosario et al. (2005) the antigens of *L. amazonensis* showed cross-reactivity with serological analysis in naturally *L. chagasi*-infected dogs, demonstrating an analogous antigenic repertory between these *Leishmania* species. As expected, the vaccination with WPV also induced production of antibodies that recognized SLaA, which suggests that it might be used for vaccination against American tegumentar leishmaniasis in dogs as well.

Our findings highlight on the importance in progress of vaccines using whole parasites approach, particularly in developed countries. This vaccine design has been attractive in terms of cost, safety and stability when compared to purified subunits preparations or DNA vaccines which involved a sophisticated technology. The killed vaccines present a great diversity in antigenic repertory that would potentially activate a strong cellular response, mainly by T-lymphocytes, as a better approach when compared to purified subunits preparations or DNA vaccines. In this context, the present study showed that immunization with WPV induced both cellular and humoral immune responses, observed mainly as cellular reactivity (cell proliferation) and increase in T-lymphocytes Thy-1^+, related particularly to the subpopulation $CD8^+$ that support the hypothesis of the potential immunomodulation to control tissue parasitism. In addition, a higher production of anti-*Leishmania* total IgG, characterized by mixed isotypes profile (IgG1 and IgG2), was demonstrated. Further investigations will analyze the efficacy of the WPV in protection against the experimental challenge with *L. chagasi*.

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