Acta Tropica 127 (2013) 174–180

Contents lists available at SciVerse ScienceDirect

Acta Tropica

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

Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator P-MAPA

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ARTICLE INFO

Article history:
Received 1 April 2012
Received in revised form 16 January 2013
Accepted 5 April 2013
Available online 29 April 2013

Keywords:
CD4+ T
T lymphocytes
CD8+ T
P-MAPA
Leishmania

ABSTRACT

This study investigated the immunotherapeutic potential of the protein aggregate magnesium–ammonium phospholinoleate–palmitoleate anhydride immuno-modulator (P-MAPA) on canine visceral leishmaniasis. Twenty mongrel dogs presenting clinical symptoms compatible with leishmaniasis and diagnosis confirmed by the detection of anti-leishmania antibodies were studied. Ten dogs received 15 doses of the immunomodulator (2.0 mg/kg) intramuscularly, and 10 received saline as a placebo. Skin and peripheral blood samples were collected following administration of the immunomodulator. The groups were followed to observe for clinical signals of remission; parasite load in the skin biopsies using real-time PCR, the cytokines IL-2, IL-10 and IFN-γ in the supernatant of peripheral blood mononuclear cells stimulated *in vitro* with either total promastigote antigen or phytohemagglutinin measured by capture ELISA, and changes in $CD4^+$ and $CD8^+$ T cell subpopulations evaluated by flow cytometry. Comparison between the groups showed that treatment with the immunomodulator promoted improvement in clinical signs and a significant reduction in parasite load in the skin. In peripheral blood mononuclear cell cultures, supernatants showed a decrease in IL-10 levels and an increase in IL-2 and IFN-γ. An increase in $CD8^+$ T cells was observed in peripheral blood. In addition, the *in vitro* leishmanicidal action of P-MAPA was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and no leishmanicidal activity was detected. These findings suggest that P-MAPA has potential as an immunotherapeutic drug in canine visceral leishmaniasis, since it assists in reestablishing partial immunocompetence of infected dogs.

1. Introduction

Leishmaniases are a group of maladies that are among the most neglected diseases worldwide and the visceral form affects half a million people annually and kills 50,000 per year (WHO, 2010). In Brazil, the visceral form is endemic, notification of human cases increased between 2001 and 2010 (Brasil, 2011) and the domestic dog is considered the main reservoir of the parasite in urbanized areas (Desjeux, 2004).

In dogs, the infection caused by *Leishmania infantun* can be asymptomatic or symptomatic. When it is symptomatic, the affected animal commonly develops chronic manifestations that are characterized by one or more of nine major clinical features: appetite loss, weight loss, local or generalized enlarged lymph nodes, cutaneous and ocular lesions, epistaxis, lameness, anemia, kidney failure and diarrhea (Ferrer, 1999).

The drugs generally used to treat dogs with visceral leishmaniasis are highly toxic, costly, can be ineffective in some cases, promote clinical remission without parasitic sterilization, and once the treatment is withdrawn, relapses of the disease are the rule (Baneth and Shaw, 2002). The failure of treatment with chemotherapeutic agents available to promote sterilization of infection may lead to important epidemiological consequences, since asymptomatic infected dogs living in an endemic area could act as a reservoirs for the transmission of the parasite by sandfly (Alvar et al., 1994). Therefore, new drugs should be evaluated in an attempt

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http://dx.doi.org/10.1016/j.actatropica.2013.04.005

to find compounds capable of promoting the sterilization of infection.

The suppression of cellular immunity is the most important aspect in the pathogenesis and progression of canine disease. Symptomatic dogs present negative skin tests for antigens of the parasite (Dos-Santos et al., 2008) and a reduction in the number of T lymphocytes in peripheral blood has been observed (Bourdoiseau et al., 1997; Lima et al., 2012). In contrast, asymptomatic dogs show an increase in $CD8^+$ T in peripheral blood; a major phenotypic feature in dogs bearing a low parasite load (Reis et al., 2006).

Given that the failure of cellular immunity contributes to disease progression, immunomodulatory compounds have been studied in the treatment of visceral leishmaniasis; however, the majority of tests have been conducted on mice (Smith et al., 2000; Ghose et al., 1999; Shakya et al., 2011). In dogs, studies involving the development of vaccines against leishmaniasis have been more frequently described (Lima et al., 2010; Reis et al., 2010; Marcondes et al., 2011) than the study of immunomodulators (Borja-Cabrera et al., 2004).

The protein aggregate of magnesium–ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) is a compound obtained by fermenting the fungus *Aspergillus oryzae*. Its immunomodulatory activities include the induction of TLR2 in HEK cells (Fávaro et al., 2012), stimulation of marrow myelopoiesis (Justo et al., 2000; Melo et al., 2001), antimicrobial (Fávaro et al., 2012; Melo et al., 2001; Durán et al., 2009) and antitumoral activity (Justo et al., 2000), increase spleen cell proliferation and production of cytokines IL-2, IFN-γ and NK cell activity (Justo et al., 2003), which promotes greater stimulation of cellular immunity. Toxicological studies have determined that P-MAPA is safe in mice (Fávaro et al., 2012). In dogs, *in vivo* clinical studies on the effects of P-MAPA have not yet been performed.

Considering that dogs with visceral leishmaniasis present cellular immune suppression and that the immunomodulator P-MAPA stimulates immune cellular response in experimental models (Justo et al., 2003; Fávaro et al., 2012), this study aimed to analyze the possible effects on clinical signs, while simultaneously monitoring skin parasite load, IL-10, IL-2 and IFN-γ levels in supernatant from peripheral blood mononuclear cells cultures, $CD4^+$ and $CD8^+$ T cell percentages in symptomatic dogs treated with this compound and the leishmanicidal activity of the drug.

2. Material and methods

2.1. Animals

Twenty mixed breed adult dogs of both sexes, that presented antibodies reactive to *Leishmania* antigens, were provided by the Araçatuba Zoonosis Control Center (*Centro de Controle de Zoonoses*, CCZ), Araçatuba, SP, Brazil. The dogs were maintained in individual enclosed kennels, with access to water and balanced canine feed *ad libitum*, belonging to the Araçatuba Faculty of Veterinary Medicine (FMVA-UNESP), SP, Brazil.

The dogs were identified, weighed, received a deltamethrin-based repellent collar and, prior to the onset of the tests, were administered two doses of the dewormers pyrantel pamoate and praziquantel with an interval 15 days. They were selected based on positive serological tests for visceral leishmaniasis by ELISA and the presence of at least three clinical signs of the disease and were divided into two groups, 10 in the control group and 10 in the group treated with P-MAPA.

Skin and peripheral blood samples were collected from both groups of dogs before the onset of the experiment (day 0) and following its completion (day 45). The skin biopsies were performed with aid of the tranquilizer acepromazine (1.1 mg/kg) and local blockage with 1% xylocaine using a 4 mm "punch". The skin samples were obtained from the middle portion of the ear and stored at −80 °C until processing. Blood samples were collected by puncture of the radial vein into tubes with and without EDTA, which were used to perform the serological, hematological, biochemical, cell viability and immunophenotyping tests. The use of P-MAPA in the present study was approved by the Ministry of Agriculture, Livestock and Supply under protocol no. 322, CPV/DFIP.

2.2. Treatment with P-MAPA

The product P-MAPA is an immunomodulator developed by Farmabrasilis that is a proteinaceous aggregate of magnesium and ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) derived from *A. oryzae* (Nunes et al., 2004). This immunomodulator consists of a protein aggregate of ammonium and magnesium phospholinoleate–palmitoleate anhydride, containing 11.6 ± 4.0% of total lipids, (22.7 ± 5.0% of palmitoleic acid, 42.9 ± 2.0% of linoleic acid, and 32.0 ± 3.0% of oxidized linoleic acid), 20.1 ± 0.9% of magnesium ions, 10.0 ± 3.3% of ammonium ions, 45.2 ± 2.7% of phosphate and 0.49 ± 0.01% of protein (Asp 7.19%, Thr 3.56%, Ser 7.56%, Glu 8.53%, Pro 0.5%, Gly 9.69%, Ala 7.46%, Val 1.0%, Met 4.38%, Isoleu 2.54%, Leu 3.03%, Tyr 0.5%, Phe 1.0%, His 2.83%, Lys 3.56%, Trp 1.3%, and Arg 35.2%). The compound is produced when the *Aspergillus oryzae* fungus is cultured in a medium consisting of an aqueous solution of oat and gelatin (10:1, wt/wt) for a period of 5 days in a bioreactor maintained between 20 and 35 °C, with pH stabilized between 2 to 4, under low aeration (2 L/min) and slow agitation (5 rotations/h). The culture medium is then mechanically filtrated and the compound extracted with ethyl acetate and precipitated under pH 11 by a 20% aqueous solution of sodium carbonate. The resulting crystals are washed in ethyl acetate and ether and dried.

Dogs in the treatment group were administered P-MAPA at a dose of 2.0 mg/kg of bodyweight, intramuscularly in the dorsal region, every three days for 45 days. The control dogs were administered saline under the same inoculation schedule. On the inoculation days, all the dogs were observed for clinical signs compatible with canine leishmaniasis, including fever, skin lesions, lymphadenomegaly, muscular atrophy, pale mucous membranes and weight loss.

2.3. Clinical parameters

Based on the results of the physical examinations and levels of anti-*Leishmania* antibodies by ELISA, the dogs were classified for clinical staging according to the presence of clinical signs, as proposed by Solano-Gallego et al. (2009). Each dog received a score ranging from 0 to 4, with 0 corresponding to absence of clinical signs of CVL and negative serology by ELISA, and score values from 1 to 4, respectively corresponding to the mild, moderate, severe and extremely severe clinical stages of the disease.

2.4. Hematological analysis and biochemical analysis

Serum and blood samples were sent to the Clinical Laboratory of Araçatuba FMVA-UNESP. Complete blood counts were performed by an automatic cell counter (Celm-CC510). Determination of hemoglobin was performed by the hemiglobincyanide method (CELM E-205S spectrophotometer) and cell volume by the microhematocrit method (SIGMA 1-13 centrifuge microhematocrit). The RBC indices were calculated according to Jain (1993). For biochemical analysis of serum samples, an automated biochemical analyzer (Automatic Analyzer BTS Mod 370 plus BioSystem, Spain) was used, previously calibrated with a commercial calibrator (Calibrator serum, Cod. 18011, BioSystems, Spain), and the reactions were monitored with controls for levels I (assayed control serum level I,

Cod. 18005, BioSystems, Spain) and II (assayed control serum level II Cod. 18007, BioSystems, Spain). Using a set of commercial reagents, the concentration of total protein (Protein, Cod. 11500, BioSystems, Spain) was determined by the biuret reaction endpoint, aspartate aminotransferase (AST/GOT), Cod. 11531, BioSystems, Spain) and alanine aminotransferase (ALT/GPT), Cod. 11553, BioSystems, Spain) by continuous kinetic reaction. All biochemical reactions were processed at 37 °C, in accordance with the manufacturers' recommendations.

2.5. Quantification of parasites in skin biopsy

Parasite load was evaluated at the onset and end of treatment in both groups by real-time polymerase chain reaction (qRT-PCR). DNA extraction from skin samples was performed using DNA extraction kit (DNeasy®, USA), in accordance with the manufacturer's instructions, and 10–100 ng were used in PCR. The assessments were performed by qRT-PCR using SYBR Green (Invitrogen, USA) and primers 13 A (3′GTG GGG GAG GGG CGT TCT5′) and 13B (3′ATT TTA CAC CAA CCC CCA GTT5′) (Rodgers et al., 1990) at a concentration of 10 μM. The temperature cycles were: initial heating to 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 63 °C for 45 s and 72 °C for 30 s, plus a final extension at 72 °C for 5 min The melting curve protocol was 95 °C for 15 s, 60 °C for 15 s followed by 20 min until it reached 95 °C for 15 s. The standard curve for quantification was performed on serial dilutions at nine different concentrations of culture promastigotes of *L. chagasi*.

2.6. IFN-γ, IL-2 and IL-10 quantification

Approximately 1×10^5 of isolated PBMCs were cultivated *in vitro* with total antigen of the lysed promastigote form of *L. chagasi* MHOM/BR/00/MERO2 (10^5/well), prepared as described by Lima et al. (2003), or phytohemagglutinin in a humidified environment at 37 °C with 5% CO_2 for five days. Next, the supernatant was individually collected by removing the cells in suspension and stored at −80 °C until the measurement of cytokines by ELISA.

Cytokine quantification in culture supernatants derived from PBMCs was performed by capture ELISA using mouse monoclonal anti-canine IFN-γ, IL-2 and IL-10 antibodies (mAb) and biotinylated polyclonal goat anti-canine IFN-γ, IL-2 and IL-10 (R&D Systems, USA). Wells of 96 well-plates (Maxisorb, Nalgene Nunc International, Rochester, NY, USA) were coated with 1.0 μg/mL monoclonal antibody for cytokine capture. The detection antibodies were used at a concentration of 1.5 μg/mL. Recombinant canine IFN-γ, IL-2 and IL-10 (R&D Systems, USA) were used to generate standard curves. The test was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Promega Corporation, Madison, WI, USA), in accordance with the manufacturer's instructions, and ODs were read using a Spectra Count™ reader (Packard BioScience Company) with a 450 nm filter.

2.7. Immunophenotyping of lymphocytes by flow cytometry

To obtain mononuclear cells, 10 mL of blood with EDTA collected from treated and control dogs were used. The mononuclear cells were isolated using a Ficoll–Paque density gradient (Amersham Biosciences, USA), in accordance with the manufacturer's recommendations. After incubating with blocking buffer for 30 min at 4 °C, the cells were incubated for 20 min at 4 °C with antibodies conjugated to three different fluorochromes: anti-CD3-canine conjugated to FITC, anti-CD4-canine conjugated to RPE and anti-canine CD8-conjugated Alexa Fluor 647® (AbD Serotec, UK) or isotype control (AbD Serotec, UK). The cell suspension was then centrifuged at 2000 rpm for 5 min at room temperature. After discarding the supernatant, the cells were resuspended in 1 mL of wash buffer, recentrifuged under the same conditions, after which the supernatant was again discarded. The samples were fixed in buffered formaldehyde solution with 1% phosphate, pH 7.0. Data acquisition was performed on EasyCyte mini® equipment (Guava, Hayward, CA) and 10,000 events were acquired in each preparation. Analyses were performed using the Guava Express Plus® Software, Cytosoft 4.1.

2.8. Evaluation of direct toxic activity by P-MAPA on L. chagasi in vitro

The leishmanicidal action of P-MAPA was determined by Mosmann's test (1983) with modifications. 5×10^7 promastigotes of *L. chagasi* were grown in Schneider's medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 200 U/mL of penicillin (Sigma) and 100 μg/mL of streptomycin (Sigma) at pH 7.4, in 96-well plates (Costar®) in a volume of 100 μL/well at 26 °C for 72 h in the presence of P-MAPA concentrations of 0, 50, 100 and 200 μg/mL. Positive control was achieved by adding dead parasites that were submitted to four freezing-thawing cycles. The tests were performed in duplicate. Next, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 5 mg/mL in 0.15 M phosphate-buffered saline, pH 7.2, were added to each well, following which the plate was reincubated under the same conditions for 1 h. The reaction was then stopped by adding 100 μL of 10% sodium dodecyl sulfate in water and the plate was read in a microplate reader with a 570 nm filter.

2.9. Statistical analysis

The Wilcoxon test was used to compare clinical stage and parasitic load prior to and following treatment. The parasitic load in the saline and P-MAPA groups were compared using the Mann–Whitney test. Data concerning cytokines and lymphocyte subpopulations was assessed by the normality KS test and the unpaired *t* test was used for comparisons between the cytokine levels of the control and treated groups, and to compare the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in treated and control dogs. A paired *t* test was used to compare leishmanicidal activity. The results were considered significant when $p < 0.05$. Statistical analyses were performed with aid of GraphPad Prism version 5.00 for Windows (GraphPad Software, USA)

3. Results

3.1. Monitoring clinical signs

Prior to the onset of treatment, all the dogs presented clinical signs of canine visceral leishmaniasis and the most common symptoms were the presence of onychogryphosis (90.0%) and lymphadenopathy (78.8%), followed by alopecia and low weight (77.3%), seborrhea and dry keratoconjunctivitis (47.0%). Less frequently, skin ulcers, lameness, dull coat, vomiting, appetite loss, skin nodules, diarrhea, uveitis, skin rash and lethargy were observed.

Regarding clinical staging, each dog received a specific score, the highest corresponding to the most severe form of the disease and high positive antibodies levels. The distribution of the dogs was as follows: 55% were stage III and 45% stage II.

Following treatment, the dogs in the treated group presented improvement in their coats, reduced flaking and itching of the skin, together with an important increase in appetite and lower clinical score ($p < 0.05$, Fig. 1). In contrast, the majority of the nontreated group presented a worsening in clinical status over time and one dog died due to kidney failure.

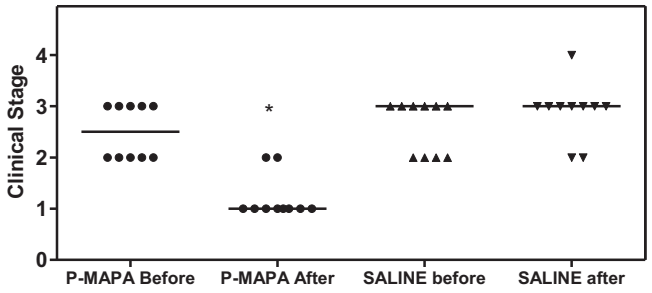

Fig. 1. Clinical staging of dogs with visceral leishmaniasis before and after treatment with P-MAPA. Each dog received a score ranging from 0 to 4, with 0 corresponding to absence of clinical signs of CVL and negative serology by ELISA, and score values from 1 to 4, respectively corresponding to increasing levels of anti-*Leishmania* antibodies by ELISA and to the mild, moderate, severe and extremely severe clinical stages of the disease. Data are shown as dot blots, and lines correspond to the median of each group. * $p < 0.05$ according to Wilcoxon test.

3.2. Potential toxicity of P-MAPA to the liver

Analysis of ALT and AST enzyme levels showed that these were not significantly affected by the treatment. Similar results were observed for the blood counts, such that the compound did not cause significant changes in the total count of erythrocytes, leukocytes and their subpopulations or total plasma proteins (results not shown).

3.3. Evaluation of parasite load in skin biopsies from the treated and control groups

The values of the linear cycle (Treshold cycle-Ct), obtained from 10 to 100 ng of DNA from each sample were analyzed with reference to the standard curve obtained for parasite load. The curve for quantifying parasite load determined $r^2 = 0.98$ and the reaction showed a slope of −3.286. Upon completion of treatment, parasite load had decreased in the P-MAPA treated group ($p < 0.05$, Fig. 2a) and showed a significant reduction compared with the placebo group ($p < 0.05$, Fig. 2a and 2b).

3.4. IFN-gamma, IL-2 and IL-10 levels

The cytokine levels were assessed in the supernatants from PBMC culture stimulated with the lysed promastigote form of *L. chagasi* or phytohemagglutinin at the end of the experimental period. PBMCs collected from dogs treated with P-MAPA secreted higher levels of IFN-γ and IL-2 when stimulated with the lysed promastigote form of *L. chagasi* or phytohemaglutinin than those of dogs in the control group ($p < 0.05$, Fig. 3a and 3b, respectively). No significant differences were observed for IFN-γ and IL-2 in the supernatant of PBMCs collected from dogs treated with P-MAPA and the control group when the culture was not stimulated.

IL-10 in the supernatant of PBMC culture stimulated with the lysed promastigote form of *Leishmania* spp., or nonstimulated culture, from treated dogs was significantly lower than in the supernatant of PBMCs collected from control group dogs ($p < 0.05$, Fig. 3c).

3.5. Immunophenotyping of lymphocytes

Comparative analysis of the percentages of $CD4^+$ T and $CD8^+$ T cells before the onset of treatment in both groups showed no statistically significant differences (results not shown). Following treatment, a slight increase in $CD4^+$ T cells was detected compared with the control group, but this was not statistically significant (Fig. 4); however, $CD8^+$ T cells showed a significant increase following treatment ($p < 0.05$) (Fig. 4).

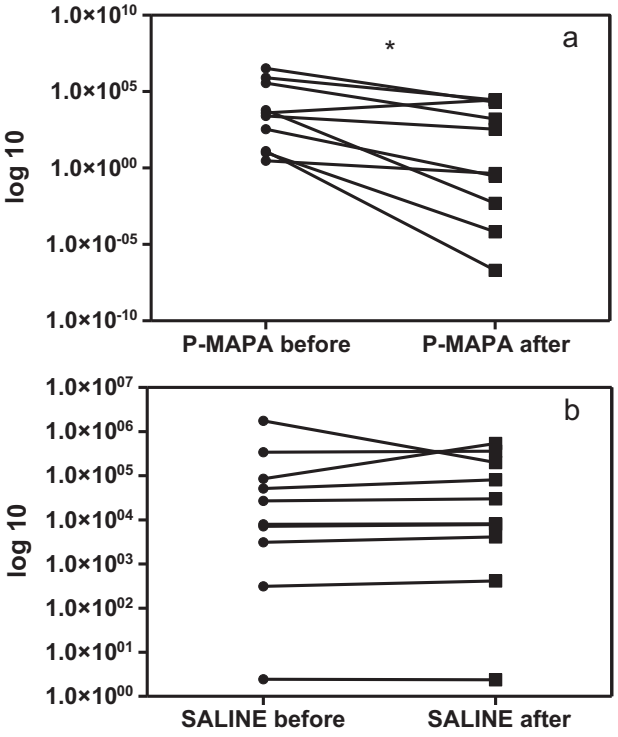

Fig. 2. Parasite burden in the skin of dogs in the P-MAPA treated (a) and saline (b) groups before and after the treatment with P-MAPA or placebo. Parasite burden was determined by qRT-PCR. Data are shown as dot blot. * $p < 0.05$ according to the Wilcoxon test.

3.6. Leishmanicidal activity

P-MAPA presented no leishmanicidal action *in vitro* (Fig. 5), no significant difference was observed between parasite viability in the presence or absence of P-MAPA ($p > 0.05$). The positive control using dead parasites showed a significant difference compared with live parasites without P-MAPA ($p < 0.05$).

4. Discussion

The current armamentarium for treatment against leishmaniases is limited and the development of alternative therapies is essential. The investigation of new drugs that can be used safely on dogs is also fundamental. P-MAPA is an immunomodulator and several studies have been conducted regarding its properties against tumors and different pathogens (Justo et al., 2000; Durán et al., 2009; Fávaro et al., 2012). Dogs with visceral leishmaniasis were treated with P-MAPA and presented a significant reduction in clinical signs and improvement in cellular immune response.

Following treatment with P-MAPA, improvements in the coat of treated dogs were observed, including reduced flaking and itching of the skin, as well as an important increase in appetite. The remission of signs resulted in a significant improvement in the disposition of the dogs. In the control group, clinical signs worsened over time and one dog died during the experimental period due to kidney failure. P-MAPA was safe in this species at the doses administered, since no changes in blood and liver enzyme levels were detected following treatment. Similar results in blood and liver enzyme levels were observed by Fávaro et al. (2012) using a murine model.

Dogs that were injected with P-MAPA showed no signs of pain at the injection site and no side effects, in contrast to the treatment of dogs with antimonials, which present important side effects, including nephrotoxicity, gastrointestinal disturbances and pain at

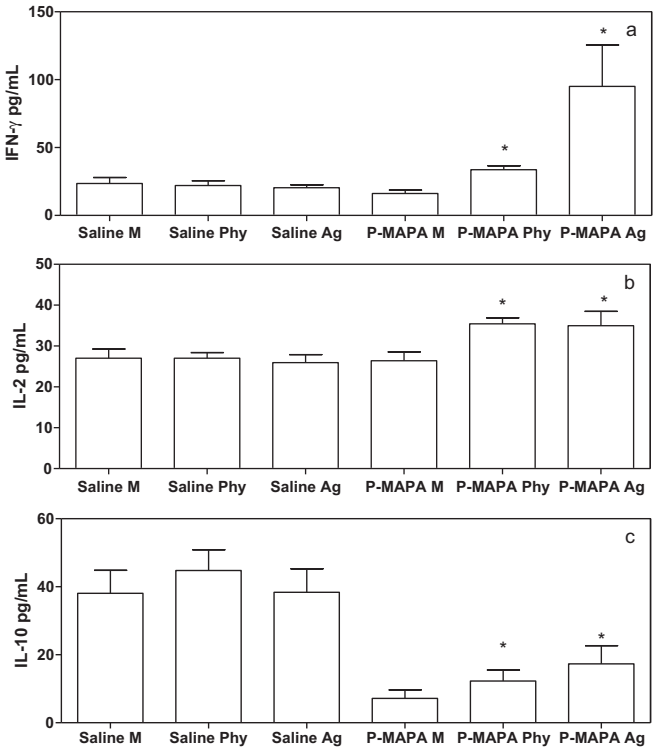

Fig. 3. Disease recovery correlates with increased IFN-γ and IL-2, and decreased IL-10 production. PBMC effector cytokines from the saline group (infected untreated) and P-MAPA treated group (infected and treated with P-MAPA) at the end of the experimental period are shown. Culture supernatants were collected from PBMC culture stimulated *in vitro* with total antigen of the lysed promastigote form of *Leishmania chagasi* MHOM/BR/00/MERO2 (10^5/well), or phytohemagglutinin for 5 days and analyzed *via* ELISA for IFN-γ (a), IL-2 (b) and IL-10 (c). Bars indicate the mean for each group (± standard deviation of the mean). *Significant differences $p < 0.05$ by unpaired *t* test.

the injection site (Baneth and Shaw, 2002; Solano-Gallego et al., 2009).

The clinical improvement observed in dogs treated with the immunomodulator P-MAPA was accompanied by a significant reduction in skin parasite burden; the evaluation of parasite burden in the skin is important because the skin is the source of parasite transmission to the vectors. In addition, the skin is considered an appropriate tissue to detect *Leishmania* spp. DNA (Maia et al., 2010; Quaresma et al., 2009) by qRT-PCR, because of the high sensitivity and specificity of the technique for the absolute quantification of parasites (Quaresma et al., 2009). Similarly, low parasite load in the spleen and lymph nodes were observed by Manna et al. (2008)

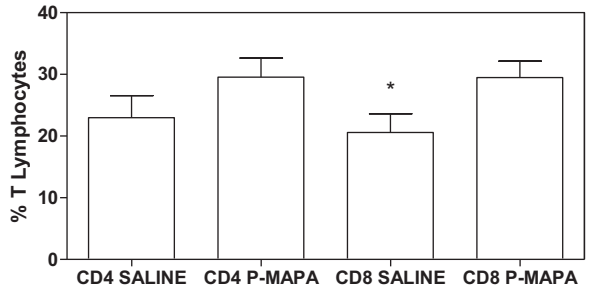

Fig. 4. Frequency of TCD4 and TCD8 cells in PBMC of dogs from the control group (infected untreated) and treated group (infected and treated with P-MAPA) at the end of the experimental period. Mononuclear cells isolated from peripheral blood were analyzed for the presence of CD3CD4 and CD3CD8 surface markers by flow cytometry. Bars indicate the mean for each group (± standard deviation of the mean). *Significant differences $p < 0.05$ by unpaired *t* test.

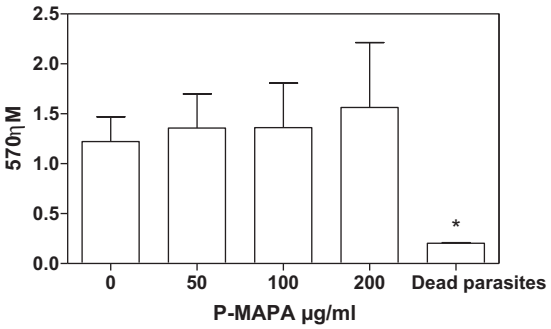

Fig. 5. Evaluation of the leishmanicidal activity of P-MAPA at different concentrations on promastigotes of *Leishmania* (L.) *chagasi*. The group dead, parasites that were submitted to four freezing–thawing cycles, was used as control. *Significant differences $p < 0.05$ by paired *t* test.

following administration of miltefosine associated with allopurinol. Reducing the parasite load occurs as a consequence of the effector action of activated macrophages that promote parasite death (Holzmuller et al., 2005). The decrease in parasites in the skin suggests that P-MAPA stimulated microbicidal mechanisms.

Regarding IL-2, our results showed increased levels in treated dogs, contrasting with the decrease in IL-10 in this group compared with the control group. IL-2 induction by P-MAPA has been previously reported. Following the administration of P-MAPA in mice for seven consecutive days, significantly higher IL-2 concentrations were secreted by immune cells (Justo et al., 2003). It is probable that IL-2 induced specific lymphocyte proliferation and the consequent IFN-γ production lead to the activation of macrophages and the killing of parasites.

Symptomatic infected dogs showed no lymphoproliferation response against parasite antigen (Fernández-Pérez et al., 2003), thus the production of IL-2 induced by P-MAPA in treated dogs suggests an improvement in cellular immune response. In fact, spleen cell proliferation has been reported following administration of P-MAPA in mice (Justo et al., 2003).

Increased levels of IFN-γ were observed following treatment with P-MAPA. IFN-γ levels appear to be inversely correlated with the parasite load and could represent a good marker in infected dogs to assist in deciding the appropriate therapeutic approach (Manna et al., 2008). The observed increase in IFN-γ production, together with the decrease in skin parasite load, are likely factors in disease recovery.

P-MAPA promoted a decrease in IL-10 in treated dogs. IL-10 has inhibitory effects on signal transduction for the enzyme iNOS (Bogdan et al., 2000), which is responsible for nitric oxide production by activated macrophages and is involved in the protection of dogs against *L. infantum* infection (Holzmuller et al., 2005; Zafra et al., 2008). P-MAPA down-regulates the immunosuppressive effects of *Leishmania* spp infection in dogs.

Treated dogs showed lower levels of IL-10 and a decrease in parasite load, indicating a rebalancing of the immune system leading to the control of parasite numbers. IL-10 is produced in infected dogs (Michelin et al., 2011) and its production is accompanied by diminished proliferative response (Boggiatto et al., 2009), as such, the decrease IL-10 levels induced by P-MAPA could be related to the recovery of specific lymphocyte proliferation, as observed by the increase in IL-2. Further studies are required to determine the relation between these cytokines and disease progression.

Low parasite load in the skin of dogs naturally infected by *Leishmania* spp could be related to an increase in TLR2. In canine visceral leishmaniasis, Amorim et al. (2011) observed that low parasite load in skin was associated with an increase in TLR2 expression. P-MAPA promoted an increase in TLR2 in HEK cells (Fávaro et al., 2012),

and in macrophages from dogs with visceral leishmaniasis (unpublished data), and although TLR2 expression was not assessed in the present work, the data shown here indicate that TLR2 could be involved in the effects of P-MAPA observed in dogs.

In this study, an increase in $CD4^+$ T and $CD8^+$ T cells was verified following treatment, though only $CD8^+$ T cells showed a significant increase. Similarly, asymptomatic dogs showed an increase in $CD8^+$ T cells with low *Leishmania* spp. burden (Reis et al., 2006), suggesting that high levels of $CD8^+$ T cells may be the key to an effective immune response against the parasite, since the destruction of parasites is associated with a high number of lymphocytes (Reis et al., 2006).

Moreover, $CD8^+$ T cells are important for controlling infections caused by protozoa, due to their ability to lyse infected cells and stimulate cytokine production (Pinelli et al., 1995; Miller et al., 2006; Martin et al., 2010). In *Leishmania* spp.-infected dogs, a negative correlation between $CD8^+$ T cells and cutaneous parasite density emphasizes the role of T cell-mediated immune response in the resistance mechanism during ongoing CVL (Guerra et al., 2009). Thus, determination of the increase in $CD8^+$ T cell population in association with the reduction in parasite load indicates that the immunomodulator stimulated the cellular immunity of the dogs.

Similar to that verified for P-MAPA, an increase in $CD8^+$ T cells has been reported following the use of other immunomodulators in the treatment of visceral leishmaniasis (Borja-Cabrera et al., 2004). In contrast, chemotherapy drugs do not seem to significantly affect this cell population, with an increase only observed in the $CD4^+$ T cell population (Moreno et al., 1999; Guarga et al., 2002).

Depending on the clinical condition of the dog, the use of chemotherapy is inefficient (Ikeda-Garcia et al., 2010), in such cases, the association of immunomodulators that potentiate the cellular response, such as P-MAPA, can provide a more satisfactory result. An effective response against *Leishmania* spp was demonstrated *in vivo* in the present study. P-MAPA showed no direct *in vitro* antimicrobial activity, so future studies are required to investigate whether P-MAPA can be combined with other leishmanicidal drugs, aimed at reducing the administration period and the dose of the drug required, while effectively reducing the side effects of these drugs.

The immunomodulator P-MAPA promoted improvement in the clinical signs of symptomatic dogs and reduced IL-10 levels and the parasite load, while increasing the levels of IL-2, IFN-γ and $CD8^+$ T cells. The results indicate a cellular immune response in the treated dogs. This study showed that P-MAPA could be a promising adjuvant in the treatment of canine visceral leishmaniasis.

Acknowledgements

The authors would like to thank the São Paulo Research Support Foundation (FAPESP) for its financial support (2009/50426-9) and Farmabrasilis, Brazil.

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