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Effects of P-MAPA immunomodulator on Toll-like receptor 2, ROS, nitric oxide, MAPKp38 and IKK in PBMC and macrophages from dogs with visceral leishmaniasis

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ABSTRACT

Leishmania (*L.*) *chagasi* is the etiologic agent of visceral leishmaniasis (VL) that can be transmitted to humans and dogs. VL in Brazil represents a serious public health problem; therefore, it is important to study new alternatives to treat infected dogs. In dogs, the therapeutic arsenal against canine VL is limited. The immunomodulator protein aggregate magnesium–ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) improves immunocompetence when the immune system is impaired, but its dependence on Toll-like receptors (TLRs) and the mechanisms involved in immune response remain unclear. The in vitro action of P-MAPA on the expression of TLR2 and TLR4, reactive oxygen species (ROS), nitric oxide (NO) and p38 mitogen-activated protein kinase (p38 MAPK) and IKK phosphorylation was studied in mononuclear cells from peripheral blood and macrophages from healthy and *Leishmania*-infected dogs. The PBMC or macrophages were isolated and cultured with different concentrations of P-MAPA (20,100 and 200 μg/ml) in a humid environment at 37 °C with 5% CO_2. Observation revealed that *Leishmania*-infected dogs showed a decrease in TLR2 in macrophages compared with healthy dogs and in induction with P-MAPA. ROS were increased in PBMCs from *Leishmania* spp.-infected dogs compared with healthy dogs and P-MAPA improved ROS production. NO production was increased in culture supernatant from macrophages stimulated by P-MAPA in both healthy and *Leishmania* spp. infected dogs. Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation, suggesting signal transduction by this pathway. These findings suggest that P-MAPA has potential as a therapeutic drug in the treatment of canine visceral leishmaniasis.

1. Introduction

Visceral leishmaniasis is an endemic disease that has spread over several continents, principally in tropical and subtropical regions. It is caused by the *Leishmania infantum*, *Leishmania chagasi and Leishmania donovani* species and affects millions of people worldwide [1]. The parasite is transmitted by sand flies to mammals, including humans and dogs, via host blood-feeding. The dog is considered the most important urban reservoir of *L. chagasi* due to its high level of infection and its proximity to humans [2].

Treatment of canine visceral leishmaniasis (CVL) has certain limitations because the therapeutic arsenal against this disease is limited and the most commonly used drugs present high toxicity (nephrotoxicity, intestinal problems, muscle pain), are costly and can be ineffective in some cases [3,4]. Treatment failures have epidemiological implications since, following treatment, the dogs become asymptomatic but remain a reservoir for transmission of the parasite to sand flies [2]. These factors indicate that new drugs should be evaluated for the treatment of CVL.

The suppression of cellular immunity is the most important aspect of the pathogenesis and progression of CVL. Dogs infected with *L. infantum* show a negative response to the cutaneous test with parasite antigens [5] and a reduction in the number of T lymphocytes in peripheral blood [6] due to increased rates of T cell apoptosis [7]. Therefore, a drug that promptly reverses immunosuppression is desirable when treating infected dogs.

Cellular immune activation involves stimulation of receptors on macrophages, which are single chain glycoproteins that recognize conserved structures on the surface of pathogens. Binding pathogens to receptors on the host cell is achieved by activating antimicrobial multiple intracellular signals, including adapter molecules, kinases like p38 mitogen-activated protein kinase (p38 MAPK), and transcription factors. This results in signal transduction, gene expression and synthesis of various molecules, including cytokines, chemokines, adhesion molecules and immunoreceptors, which regulate the innate response, while simultaneously maintaining an important link with the adaptive immune response [8].

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Since the failure of cellular immunity contributes to the progression of the disease, immunomodulatory substances have been studied to treat it [9]. Effective CVL immunotherapy requires the use of suitable antigens that stimulate cellular immunity and block negative regulatory mechanisms that prevent the immunotherapeutic effects [10]. Domperidone, a dopamine D2 receptor antagonist, has been effective at controlling and reducing clinical signs and antibody titers and increasing cellular immunity [11]; but the number of immunotherapeutic drugs available is limited.

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 Toll-like receptor (TLR) 2 in human embryonic kidney (HEK) cells [12], stimulation of marrow myelopoiesis [13,14], antimicrobial [12,14,15] and antitumoral activity [13], increased spleen cell proliferation, production of cytokines IL-2 and IFN-γ and NK cell activity [16], which all promote a greater stimulation of cellular immunity. Dogs with visceral leishmaniasis were treated with P-MAPA and presented a significant reduction in clinical signs and improvement in cellular immune response [17]. Toxicological studies have determined that P-MAPA is safe in mice [12], dogs [17] and humans [12].

P-MAPA has a stimulatory effect on the immune response; however, its mode of action remains unknown, thus this study aimed to examine, in vitro, the drug's effect on TLR2 and TLR4 expression, reactive oxygen species, nitric oxide production and p38 MAPK and IKK phosphorylation in mononuclear cells from healthy control dogs and dogs infected with *Leishmania* spp.

2. Materials and methods

2.1. Study area

The study was conducted in Araçatuba, São Paulo State, Brazil, an area endemic for both canine (CVL) and human visceral leishmaniasis.

2.2. Animals

Sixty (60) male and female dogs housed at the Araçatuba Zoonosis Control Center (CCZA) that were seropositive for *L.* (*L.*) *chagasi* by indirect ELISA [18] method were included in the study. They were symptomatic, i.e., they presented at least three clinical signs of CVL.

Sixty (60) healthy male and female dogs from private homes in Araçatuba were included in the study after the owners signed a term permitting the collection of samples.

Serology was performed on dogs using *Leishmania* spp. specific antibodies, as determined by indirect ELISA method [18], and normal blood tests were performed for those testing seronegative. Blood samples from both groups were taken from each dog, 4.5 ml of blood was collected from the cephalic vein and maintained in tubes with anticoagulant, and 1.0 ml was coagulated at room temperature and then centrifuged to extract the serum.

The local animal research ethics committee approved this study under protocol no. 322.

2.3. Treatment with P-MAPA

The product P-MAPA is an immunomodulator developed by Farmabrasilis [19] that is a proteinaceous aggregate of magnesium and ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) derived from *A. oryzae* [20]. The P-MAPA immunomodulator contains 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 *A. 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 a pH stabilized between 2 and 4, under low aeration (2 l/min) and slow agitation (5 rotations per 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.

For in vitro use, P-MAPA was prepared in RPMI-1640 (Sigma) after hydration for 12 h at 4 °C, the suspension was homogenized 3 times using a sonicator at 60 W at 4 °C. The suspension was divided into 1 ml aliquots and stored at −20 °C until use.

2.4. Purification and culture of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by a density gradient using Ficoll-Paque Plus (GE Healthcare Bio-sciences), in accordance with the manufacturer's recommendations, immediately cultured (5×10^6 cells/ml) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine (Life Technologies, Grand Island, NY) and incubated at 37 °C with 5% CO_2. Cell counts were performed in a Neubauer chamber. To obtain macrophages, the PBMCs were isolated by Histopaque® gradients 1077 and 1119 (Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer's recommendations. Mononuclear cells were immediately cultured (5×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine (Life Technologies, Grand Island, NY) and incubated at 37 °C with 5% CO_2. Macrophage acquisition was achieved as described by [21] with one modification, an incubation period of 10 days was used.

2.5. TLR2 and TLR4 quantification

To examine TLR2 and TLR4 expression in PBMCs and macrophages of *L. chagasi* infected and control dogs, the cells (5×10^6 cells/ml) were cultured with 20 μg/ml, 100 μg/ml or 200 μg/ml of P-MAPA for 3 h at 37 °C with 5% CO_2, and then double-stained with specific fluorochrome-conjugated antibodies: monoclonal fluorescein isothiocyanate (FITC) conjugated anti-human TLR2 antibody (eBioscience, San Diego, USA), and anti-human TLR4 conjugated to phycoerythrin (eBioscience, San Diego, USA) [22] or control isotypes conjugated to FITC and PE (eBioscience, San Diego, USA). Following data acquisition in EasyCyte Mini® (Guava, Hayward, CA), analysis of the data was performed using the software Guava Express® Plus.

2.6. Measurement of reactive oxygen species (ROS) levels

Intracellular ROS levels were measured in PBMCs (5×10^6 cells/ml) from infected and healthy dogs after the cells were cultured with 20 μg/ml, 100 μg/ml or 200 μg/ml of P-MAPA for 90 min at 37 °C with 5% CO_2 and incubated with 10 μM H_2DCFDA (29,79-dichlorodihydroflurescein diacetate, Invitrogen Molecular Probes—Leiden, The Netherlands) for 30 min at 37 °C, in accordance with the manufacturer's recommendations. Fluorescence was measured by flow cytometry. Following data acquisition in EasyCyte Mini® (Guava, Hayward, CA), analysis of the data was performed using the software Guava Express® Plus. Positive control was achieved by adding 10 μl of PMA (1 μM/ml) (Sigma-Aldrich, St. Louis, MO, EUA) to the cell culture, following the same protocol.

2.7. Determination of nitrite concentration (NO_2^-)

Macrophages derived from monocytes from the peripheral blood of *L. chagasi*-infected and control dogs (approx. 5×10^6 cells/ml) were

stimulated with 20 μg/ml, 100 μg/ml or 200 μg/ml of P-MAPA or LPS (0.1 μg/ml) (Sigma-Aldrich, St. Louis, MO, EUA) for 24 h at 37 °C with 5% CO_2. Nitrite ion (NO_2^-) production in the supernatants was quantified using standard Griess reagent [23]. Briefly, 100 ml of macrophage culture supernatant was mixed with an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) containing 1% sulfanilamide (Sigma-Aldrich, St. Louis MO, USA) diluted in 5% H_3PO_4 and 0.1% N-(1 naphthyl) ethylenediamine (Sigma-Aldrich Co, USA). Absorbance at 540 nm was determined using an automated ELISA plate reader (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL, USA). Conversion of the absorbance of micromolar concentrations of NO_{2-} was performed using a standard $NaNO_2$, curve with an initial concentration of 100 μM and a final concentration of 0.75 μM. All the measurements were performed in triplicate and are expressed in micromolar concentrations of NO_2^-.

2.8. Determination of p38 MAPK and IKK

Macrophages derived from monocytes from the peripheral blood of *L. chagasi*-infected and control dogs (approx. 5×10^6 cells/ml) were stimulated with 20 μg/ml, 100 μg/ml or 200 μg/ml of P-MAPA or LPS (0.1 μg/ml) (Sigma-Aldrich, St. Louis, MO, EUA) for 1 h at 37 °C with 5% CO_2. Following incubation, the cells were recovered and placed in 1.5 ml tubes and incubated with 1 ml IC fixation buffer (Invitrogen, Leiden, The Netherlands) for 10 min at 4 °C, washed with PBS (pH 7.2), then washed again with HF permeabilization buffer (eBioscience, San Diego, USA) and incubated with specific antibodies: mouse monoclonal anti-TBK1 fluorochromes conjugated to phycoerythrin (BD Biosciences) and monoclonal mouse anti-p38 MAPK conjugated to Alexa Fluor (BD Biosciences) for 30 min at 4 °C. Next, 300 μl of fixation buffer (PBS with 10% formalin) was added. Subsequently, the samples were acquired in an EasyCyte Mini® cytometer (Guava, Hayward, CA), and analysis was performed using the software Guava Express® Plus.

2.9. Statistical analysis

Statistical differences were analyzed using GraphPad PRISM 3 software (San Diego, CA, USA). Considering the nonparametric nature of all data sets, the Mann–Whitney test was used to verify significant differences between the control and infected groups and the Wilcoxon test was used to verify significant differences between the treatments. In all cases, the differences were considered significant when the probabilities of equality, P values, were $P < 0.05$.

3. Results

3.1. TLR2 and TLR4 percentage in PBMCs and macrophages from infected and control dogs

The percentage of TLR2 in PBMCs from infected and controls dogs showed no statistically significant difference ($P > 0.05$; Table 1), while the percentage of TLR4 was higher in control dogs compared with infected dogs ($P < 0.05$; Table 1). Treatment with P-MAPA in PBMC revealed no statistically significant differences in the percentages of TLR2 and TLR4 expression compared with baseline conditions (data not shown).

Table 1
TLR2/TLR4 expression the mean values in PBMCs from *Leishmania* infected dogs and healthy controls.

	CT (mean ± SD)	Inf (mean ± SD)	P value
TLR4	40.78 ± 26.86	12.09 ± 9.11	0.0229
TLR2	45.35 ± 28.65	45.55 ± 27.76	0.1094

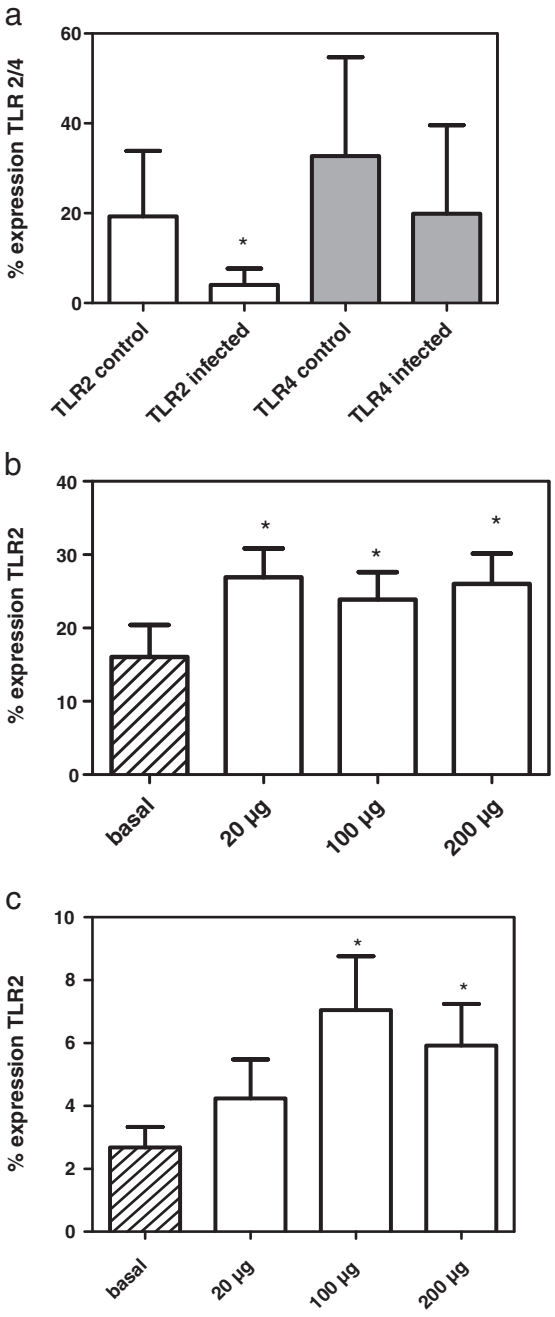

Fig. 1. TLR2/TLR4 expression in macrophages from mononuclear cells of infected dogs and controls. (a) The bars represent the mean values per group. *$P < 0.05$, significant differences between the mean baseline for dogs infected with *Leishmania* sp. and controls TLR2 and TLR4. (b) Expression of TLR2 in macrophages from control dogs was cultured in medium or with or P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml) at 37 °C, with 5% CO_2. Cells were recovered after 24 h of culture and the percentage of TLR2 expression was determined by flow cytometry. (c) Expression of TLR2 in macrophages from *Leishmania* sp. infected dogs was cultured in medium or with P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml). Cells were stained after 24 h of incubation, and the percentage of TLR2 expression was determined by flow cytometry. The bars represent the mean values of each group. The expression of TLR2 in macrophages of infected dogs, *$P < 0.05$.

Under baseline macrophage conditions, the percentage of TLR2 expression of infected dogs was decreased compared with control dogs (Fig. 1a, $P < 0.05$). P-MAPA increased TLR2 expression in control (Fig. 1b, $P < 0.05$) and infected dogs (Fig. 1c, $P < 0.05$). No statistically significant difference was observed in the percentage of TLR4 expression between control and infected dogs, and no change occurred

when macrophages were treated with the immunomodulator P-MAPA (data not shown).

3.2. Measurement of ROS levels in PBMCs

The baseline value of ROS production in mononuclear cells from infected dogs was four-fold greater than that observed in control dog cells (Fig. 2a, $P < 0.05$). Mononuclear cells from control dogs stimulated by P-MAPA at concentrations of 100 μg/ml and 200 μg/ml increased ROS production compared with that observed in cells cultured in medium alone (Fig. 2b, $P < 0.05$). In contrast, in mononuclear cells from infected dogs, all three concentrations of P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml) promoted an increase in ROS production compared with cells cultured in medium alone (Fig. 2c, $P < 0.05$). The PMA positive control showed increased ROS production compared with the baseline values of both groups (Fig. 2a, b and c, $P < 0.05$).

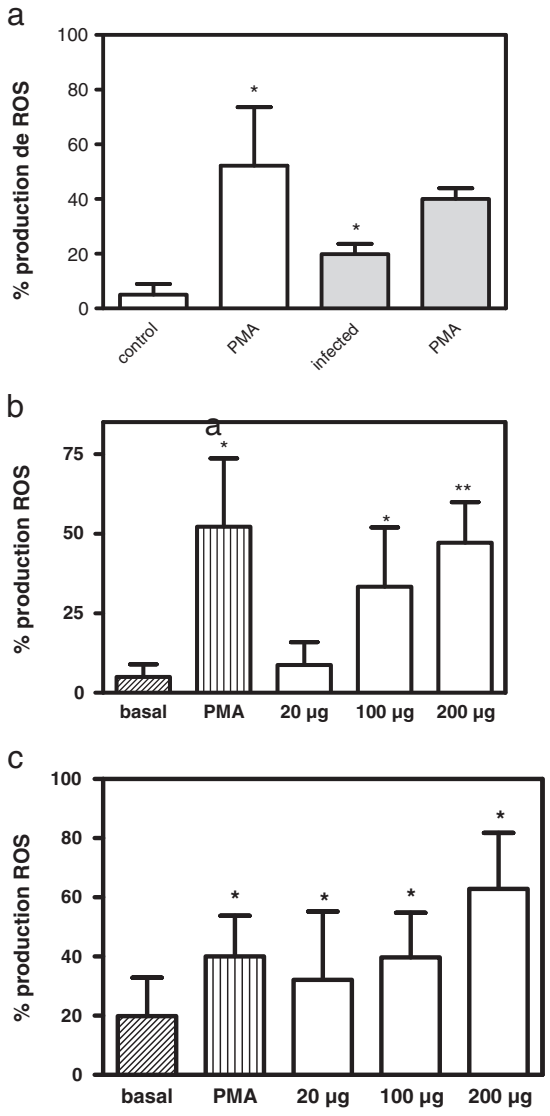

Fig. 2. (a) ROS production in mononuclear cells from significant differences between the mean baseline for the groups studied of dogs infected with *Leishmania* spp. and controls. (b) ROS production in mononuclear cells of control dogs. (c) Production of ROS in infected dogs. Mononuclear cells were cultured in media or with PMA (1 μM/ml) or P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml) at 37 °C with 5% CO_2. The cells were recovered after 24 h of culture and production of ROS was determined by flow cytometry. The bars represent the mean values of each group. *$P < 0.05$, significant differences between the averages of the values of the treatment and control.

3.3. NO production by macrophages from infected and control dogs treated with P-MAPA

In macrophages from control dogs, P-MAPA at concentrations of 100 μg/ml and 200 μg/ml induced NO production ($P < 0.05$) compared with cells cultured in medium alone (Fig. 3a). In macrophages from infected dogs, only a P-MAPA concentration of 200 μg/ml increased NO production ($P < 0.05$) compared with cells cultured in medium alone (Fig. 3b). Macrophages from control and infected dogs stimulated with LPS did not produce NO (Fig. 3a and b).

3.4. Determination of p38 MAPK and IKK

In macrophages from healthy dogs, P-MAPA increased the induction of IKK and p38 MAPK phosphorylation compared with baseline values ($P < 0.05$, Wilcoxon test), indicating that the two signal transduction pathways are stimulated by the immunomodulator. Similarly, LPS induced increased IKK and p38 MAPK phosphorylation ($P < 0.05$, Wilcoxon test; Table 2).

In infected dogs, a slight increase in p38 MAPK and IKK production was observed; however, this was not statistically significant (data not shown).

4. Discussion

The results of this study indicate that the use of P-MAPA increased TLR2 expression and induced ROS and NO production in macrophages from *L. chagasi*-infected dogs.

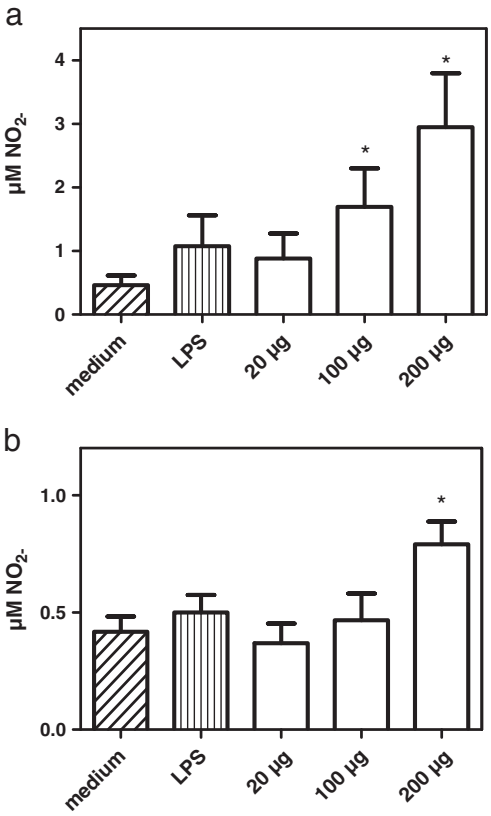

Fig. 3. Production of NO by macrophages of control dogs (a) and infected dogs (b). Macrophages were cultured in medium or with P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml) or LPS (100 μg/ml). The culture supernatant was collected after 24 h of incubation and the concentration of NO_2- was determined using a Griess reagent. The bars represent the average values of each group. *$P < 0.05$, significant difference between the values of the mean treatment and control.

Table 2
MAPKp38 protein expression (a) and IKK (b) phosphorylated in macrophages of control dogs (n = 12). The macrophages were cultured in medium or with LPS (100 μg/ml) or P-MAPA (20 μg/ml, 100 μg/ml and 200 μg/ml) at 37 °C, with 5% CO_2. After 24 h of culture the cells were recovered and detection was performed; MAPKp38 and IKK-phosphorylated by flow cytometry.

	IKK (mean ± SD)	P value	Mapk38 (mean ± SD)	P value
Basal	1.53 ± 2.52	–	0.84 ± 1.21	–
LPS	2.30 ± 2.84	0.0210	1.47 ± 1.86	0.0108
20 μg/ml	2.77 ± 2.70	0.0210	2.11 ± 2.28	0.0029
100 μg/ml	3.28 ± 3.83	0.0024	2.25 ± 2.56	0.0005
200 μg/ml	3.55 ± 4.17	0.0024	2.95 ± 3.94	0.0010

Toll-like receptor 2 is part of a family of highly conserved pattern recognition receptors (PRRs) in mammals that participate in the innate and adaptive immune responses. The role of TLRs in the pathogenesis of CVL has not been fully addressed. This study confirms that TLRs can be detected on dog white cells, as previously reported by [22]. TLR4 and TLR2 expression in canine peripheral blood leukocytes has also been previously demonstrated [24,25].

The PBMCs from symptomatic dogs naturally infected with *L. chagasi* showed diminished TLR4 expression. Similar results were observed by [26] in peripheral blood monocyte-derived macrophages (MDMs) from patients with visceral leishmaniasis. Prior studies demonstrated that TLR4 is required for efficient parasite control, probably due to the activity of inducible NO synthase. The activation of inducible NO synthase leads to NO synthase and *Leishmania* spp. death [27]. NO production by macrophages is correlated with the induction of anti-*Leishmania* activity [28,29]. In the absence of sufficient TLR4 expression, the enhanced activity of arginase increases the formation of urea and reduces NO [27]. The high parasite load observed in symptomatic dogs could be related to low TLR4 expression in PBMCs; future studies should clarify this hypothesis.

The decrease in TLR4 observed in symptomatic dogs suggests that *Leishmania* sp. uses this receptor to infect cells in dogs and the process should be regulated by cytokines. In humans, MDMs infected with *L. donovani* present suppression of TLR4 expression in late infection, when TGF-beta-1 attains high levels [26]. In symptomatic dogs, high levels of TGF-beta-1 have been observed [30,31], suggesting that this cytokine could downregulate TLR4 expression in CVL.

When treated with P-MAPA, TLR4 expression in PBMCs from infected dogs showed no increase or decrease under the experimental conditions described. The expression values obtained under basal conditions were similar to those observed in the P-MAPA treatments in vitro. The immunomodulatory effect was also assessed in mononuclear cells of healthy dogs and similar results were observed. The lack of alteration in TLR4 expression observed in the presence or absence of P-MAPA could be related to the low concentration of molecule used. Unlike the results for TLR4, macrophages stimulated by higher concentrations of P-MAPA showed an increase in TLR2 expression for both infected and control dogs. These results indicate an interaction between these molecules. Similar results were observed by [12], who showed that P-MAPA had a stimulating effect on TLR2 in HEK293 cells.

Macrophages from infected dogs showed a significant reduction in TLR2 expression compared with controls, similar to that observed in the spleen of mice chronically infected with *L. chagasi* [32]. The increase in TLR2 expression following P-MAPA treatment seems to restore the immune balance, and given that the relationship between the presence of TLR2 and the resistance to disease has been demonstrated, greater TLR2 expression may be a key point to initiating an effective immune response against this parasite. Higher levels of TLR2 mRNA were observed in mice resistant to *Leishmania* than those observed in susceptible mice [33].

Stimulation of TLRs leads to the activation of NF-kB, which can regulate the expression of cytokines [34] and the production of nitric oxide and oxygen radicals [27]. P-MAPA increased TLR2 expression, thus due to the relation observed between TLR and ROS and NO production, these molecules were investigated in PBMCs and macrophages from infected dogs.

The basal ROS production in infected dogs was higher compared with that observed in control dogs. It is known that phagocytosis of parasites by monocytes in the blood leads to ROS production, as demonstrated in human and murine macrophages [35]. However, the high level of ROS production observed in PBMCs from infected dogs suggests that the microbicidal effect generated is not sufficient to eliminate the parasite, because symptomatic dogs tend to have a high parasitic load [36]. In fact, *L. infantum* possesses a mitochondrial and cytosolic enzyme that shows peroxidase activity [37]. The overexpression of this enzyme in *Leishmania* sp. has a protective action against oxidative stress [38] and could protect the parasite from ROS.

The immunomodulator P-MAPA increased ROS levels in PBMCs from infected and control dogs. Thus, it is possible that this output could affect parasite death by eliminating their antioxidant defenses, since there is a positive correlation between the ability of ROS production and the ability of monocytes to kill canine *Leishmania* sp. in vitro [39].

The observation that P-MAPA is a potent inducer of ROS facilitates its use as an immunomodulatory drug in visceral leishmaniasis, since no toxic effect has been observed in previous experimental models [40] or dogs [17]. This contrasts from that observed with therapeutic drugs like glucantime, ketoconazole and miltefosine, which induce ROS production [41,42], but produce different side effects in dogs [4].

Apart from ROS production, the immunomodulator P-MAPA induced NO production in MDMs from infected dogs and controls. NO is generated following the activation of macrophages and plays an important role in leishmanicidal activity in canine macrophages [28].

Following stimulation with IFN-gamma associated with LPS, canine macrophages express the enzyme inducible NO synthase [21], suggesting that NO production requires two signal activators. P-MAPA induced NO production suggesting that it can trigger more than one intracellular activation signal. However, there was no nitric oxide production in macrophages of infected or healthy dogs only upon stimulation with LPS [28].

Due to its ability to induce NO, P-MAPA appears to have a similar action to other therapeutic drugs. Mononuclear cells from patients infected with visceral leishmaniasis in vitro following miltefosine stimulation produce NO [43] and murine macrophages infected with *L. donovani* and treated with sodium antimony gluconate also produce NO [41].

Treatment of macrophages from healthy dogs with immunomodulatory P-MAPA induced p38 MAPK and IKK phosphorylation at the cellular level, which stems from the recognition molecules by Toll-like receptors. The standard molecular recognition molecule MyD88 is recruited to the TIR domain of TLR, where it encounters the IRAK1/IRAK4 complex. IRAK4 phosphorylates IRAK1, creating a binding site for TRAF6, the IRAK1–TRAF6 complex dissociates and activates the protein kinase complex TKA1, and activated TAK1 phosphorylates MAP kinase and IKK, two distinct signal transductions. The phosphorylation of p38 MAPK and IKK under P-MAPA stimulation suggests that this immunomodulator binds to TLR2, since increased nitric oxide production was also observed in healthy dogs and the p38 MAPK pathway is involved in NO generation [44].

In macrophages from infected dogs treated with P-MAPA, a discrete increase in the phosphorylated proteins p38 MAPK and IKK was detected, but this was not statistically significant. It is possible that the increase in P-MAPA concentration leads to an increase in IKK and p38 MAPK, because the decrease in these molecules is a consequence of the infection, and *Leishmania* promotes failure in the MAPK signaling pathway, leading to macrophage dysfunction, due to the lack of response to IFN-gamma and the inhibition of iNOS gene expression [45]. *L. donovani* infection leads to deactivation of the signaling system, since the presence of the parasite increases the expression of phosphatases that inhibit the p38 MAPK signaling pathway [46].

Taken together, our findings indicate that P-MAPA increased TLR2 expression and induced ROS production and NO, which are related to resistance mechanisms in visceral leishmaniasis, suggesting that it has the potential to treat *Leishmania* diseases.

Acknowledgments

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