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# Down regulation of IL-10 and TGF-β1 mRNA expression associated with reduced inflammatory process correlates with control of parasitism in the liver after treating *L. infantum* infected dogs with the LBMPL vaccine therapy

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ABSTRACT

The liver plays an important role in human and canine visceral leishmaniasis, then it is considered as target to understand the mechanisms involved in the parasite control and a parameter to assess therapeutic responses. In this sense, our study focuses on evaluating the major alterations in the liver by histological (morphometric parenchyma inflammation/semi-quantitative portal inflammation), immunohistochemical assays (parasitism), and qPCR (parasitism and cytokine gene expression) in *Leishmania infantum* naturally infected dogs and treated with LBMPL vaccine. Animals were divided in four groups: NI group (n = 5): uninfected and untreated dogs; INT group (n = 7): *L. infantum*-infected dogs and not treated; MPL group (n = 6): *L. infantum*-infected dogs that received only monophosphoryl lipid A adjuvant, and LBMPL group (n = 10): *L. infantum*-infected dogs that received treatment with the vaccine composed by *L. braziliensis* disrupted promastigotes associated with MPL adjuvant. Ninety days after the end of treatments, the dogs were euthanized, and the liver was collected for the proposed evaluations. Significantly lower portal inflammatory reactions, and lower parenchyma inflammation were observed in the LBMPL group compared to INT and MPL groups. iNOS mRNA expression was higher in LBMPL group and in contrast, IL-10 and TGF-β1 mRNA expression was lower in this group when compared to INT group. Immunohistochemical and qPCR analysis showed significant parasite load reduction in LBMPL group compared to INT and MPL animals. Our data suggest that in naturally *Leishmania*-infected dogs, LBMPL vaccine reduces the damage in the hepatic tissue, being able to attenuate the type 2 immune response. It could be associated with a marked reduction in the parasitism decreasing liver inflammation in treated dogs. Along with previously obtained data, our results suggest that LBMPL vaccine can significantly contribute to the therapy strategy for *L. infantum* infected dogs.

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## 1. Introduction

Visceral Leishmaniasis (VL) caused by *Leishmania infantum*, is one of the main zoonotic diseases in the American continent and Mediterranean Basin [1,2]. VL represents a serious public health problem in Brazilian territory [3–5] where there is a distribution of autochthonous cases in 25% of the 5570 municipalities across 21 of the 27 total federal units (77.8%). Also, approximately 3.466 new human cases were reported in 2018, which corresponds to 97% of the total cases in America [5,6].

VL has a zoonotic transmission cycle in Brazil as in many American and Mediterranean countries, where dogs (*Canis familiaris*) are considered the main urban hosts of the disease [7,8]. Consequently, the management of infected dogs is regarded as an essential component by the Brazilian Visceral Leishmaniasis Control and Surveillance Program [9], since most human cases occur in regions with a high prevalence of infected animals, indicating that canine disease precedes human disease in several areas of the country [10–13].

The culling of infected animals is one of the leading disease control strategies in Brazil [14], given the risk of transmission and that the Ministry of Health does not recommend the therapeutic alternatives usually prescribed for use in humans because they have low effectiveness [15,16] addition to not preventing recurrences, the treatment of infected dogs increases the risk of drug resistance [16,17].

However, advances in the knowledge of the immune response have led to a better understanding of the pathogenesis of the disease, allowing new therapeutic strategies to be developed based on the modulation of the immune response, such as immunotherapy [18]. In this sense, the choice of an effective adjuvant is essential in this process. Adhikari et al. [19] and Dey et al. [20] observed that the MW (*Mycobacterium indicus pranii*) adjuvant has been effective against drug-sensitive and drug-resistent *L. donovani* infection, gaining a significant attention as a potential anti-leishmanial immunotherapeutic agent. Roatt et al. [21] evaluated the therapeutic response of the LBMPL vaccine composed of *L. braziliensis* antigens associated with the adjuvant Monophosphoryl lipid A (MPL) in the treatment of CVL in symptomatic dogs naturally infected with *L. infantum*. The results demonstrated restoration and normalization of biochemical and haematological parameters, control of tissue parasitism, and reduction in the intensity of clinical signs.

The findings by Roatt et al. [21] highlight the importance to evaluate compartmentalized responses in animals treated with the LBMPL vaccine to understand the complexity of biological events during treatment. Studies have demonstrated that dogs with active *L. infantum* infection present several liver alterations as hypertrophy and hyperplasia of Kupffer cells, mononuclear cell inflammatory infiltrate in the portal tract and parenchyma, ballooning degeneration of hepatocytes and fibrosis [22–24]. Considering that hepatic compartment is one of the most affected during infection, the present study evaluated the histological, immunological, and parasitological alterations in the liver of symptomatic dogs naturally infected by *L. infantum* treated with the therapeutic vaccine LBMPL.

## 2. Materials and methods

### 2.1. Ethics statement and animals

This study was approved by The Ethics Committee on Animal Experimentation of the Federal University of Ouro Preto under protocols number 2010/57, following the recommendations of CONCEA (Brazilian National Control Council of Animal Experimentation).

Twenty-three symptomatic dogs naturally infected with *Leishmania (Leishmania) infantum* were obtained from the Zoonosis Control Center of Governador Valadares, Minas Gerais (MG) State, Brazil. Screening serological tests included enzyme-linked immunosorbent assay (ELISA) and Dual Path Plataform (DPP®). Bone marrow aspirates to demonstrate parasites in NNN/LIT culture or through real-time PCR were employed to confirm the infection of the animals by *L. infantum*. Only animals showing clinical signs of CVL, and positive serological and parasitological tests were selected for the study. As negative control, five dogs uninfected and untreated were used in this study (NI - n = 5).

The animals were kept in the experimental Kennel for Drug Trial and Vaccines for Leishmaniasis of the Universidade Federal de Ouro Preto, Minas Gerais, Brazil. All dogs were distributed in collective stalls and received balanced commercial food *ad libitum* and water. The stalls were daily sanitized with 1% sodium hypochlorite. The animals were supervised by the medical veterinarian during the experimentation period to check their daily health conditions. Moreover, the dogs received daily enrichment.

Before the experimental trial, the dogs were subjected to a standard quarantine protocol, which received treatment against intestinal helminths and ectoparasite infestations. Moreover, the animals were immunized against rabies (Tecpar, Curitiba, PR, Brazil) and other infectious diseases (Vanguard HTLP 5/CV-L; Pfizer Saúde Animal, Brazil). All animals were serological tested for *Ehrlichia* spp. and *Babesia* spp. infection.

### 2.2. Experimental groups

After quarantine protocol, experimental dogs were randomly divided into three groups, as follows:

**- INT group** (n = 7): *L. infantum*-infected dogs that did not received any treatment.

**- MPL group** (n = 6): *L. infantum*-infected dogs that received treatment only with monophosphoryl lipid A adjuvant.

**- LBMPL group** (n = 10): *L. infantum*-infected dogs that received treatment with the vaccine composed by *L. braziliensis* promastigote antigens associated with MPL adjuvant.

The immunotherapeutic scheme consisted of 3 treatment series, described in detail by Roatt et al. [21].

In addition, five healthy dogs uninfected and untreated were used in this study (**NI group** - n = 5).

### 2.3. Euthanasia and necropsy

The dogs were euthanized 90 days after the end of the immunotherapeutic protocols. The animals received anesthetic protocol comprising 22 mg/Kg of ketamine (Ketamina Agener®, Agener União, Brasil) combined with 2 mg/kg of xylazine hydrochloride (Calmium®, Agener União, Brasil) by intramuscular route. Following previous anesthesia, the dogs received sodium thiopental (Thiopentax 1 g, Cristália, Brazil) at 20 mg/kg for obtaining general anaesthesia, with subsequent administration of potassium chloride produced circulatory collapse. After confirmation of death, we performed the necroscopic exam, followed by a detailed macroscopic examination of major organ systems. Subsequently, liver samples were collected for histological, gene expression and parasitological analysis.

### 2.4. Histological analysis

Liver samples collected in necropsy were maintained in 10% buffered formalin, processed, and embedded in paraffin. Sections (4 μm) were mounted on glass slides. Presence of total liver inflammation and inflammation of the hepatic portal tract were assessed histologically by examination of the HE-stained liver sections in an optical microscope. For portal inflammation, we performed a semi-quantitative analyze using optical microscopy (Olympus Optical, Japan) at 40x magnification. The portal inflammation was classified according to degree of intensity, as follow: absent (-), light ($^{+}$), moderate ($^{++}$) and intense ($^{+++}$). The total inflammation was quantified in 20 randomly selected fields (total area, 35.493 $\mu m^2$) at 40x magnification. The imagens were digitalized by AxioCam MRc microcamera associated with Leica Axio Imager.Z2 microscopy, followed by analysis using Leica Qwin V3

software. Hepatic inflammatory infiltration quantification was evaluated by counting the cell nuclei present in the distinct sections of the liver.

### 2.5. Extraction of total RNA and synthesis of first strand cDNAs

Liver fragments collected in necropsy were stored at −80 °C until the RNA analysis. For Total RNA extraction approximately 20 mg of liver tissue was homogenized with 1 mL of TRIzol reagent (Invitrogen Brasil, São Paulo, SP, Brazil) in an automatic macerator (Tissue Lyser, Qiagen, USA). The homogenate was incubated at room temperature for 10 min, mixed with 200 μL of chloroform and centrifuged at 12,000*g* for 10 min at 4∘C. The RNA extraction was continued using the SV Total RNA Isolation System (Promega, USA) following the manufacturer's recommendations. Briefly, after centrifugation, the cleared lysate was transferred to a fresh tube and 450 μL of ethanol was added. The mixture was transferred to Spin Basket and centrifuged at 12,000*g* for 1.5 min. After successive washes, the samples were incubated with DNase incubation mix for 25 min at RT. Posteriorly, 200 μL de DNAse stop solution was added, the samples were washed, and the purified RNA was transfer to elution tube. Samples were stored at −80 °C until use. cDNAs were synthesized using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA) with oligodT primers, according to the recommendations of the manufacturer. Briefly, from 1 μg of total RNA was added 10 μL of the reverse transcription mix (RT Buffer 10x, dNTP Mix − 100 Mm 25x, RT Random primers 10x, Multiscribe enzyme, RNAse inhibitor and nuclease free water). The thermal cycling conditions included a cycle for 10 min at 25 °C, followed by a cycle of 120 min at 37 °C, a cycle of 15 s at 85 °C and a cycle at 4 °C for 5 min (Veriti™ Termal Cycler, Applied Biosystems, USA). Samples were stored at −20 °C until use.

### 2.6. Primers and Real Time PCR

Primers used to amplify constitutive gene (GAPDH), cytokines (IFN-γ, TNF-α, IL-12, IL-10, TGF-β) and iNOS were designed by Gene Runner version 6.0 using specific canine sequences obtained from a genbank (http://www.ncbi.nlm.nih.gov/genbank/). The sequences of the primers employed are listed in Supplementary table 1. PCR was conducted on 7500 Real Time PCR System (Applied Biosystems, USA) using SYBR® Green PCR Master Mix (Applied Biosystems, USA), 100 mM of forward and reverse primers and cDNA diluted at 1:5. The samples were incubated at 95 °C for 10 min and then submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For the efficiency evaluation of each pair of primers a serial dilution of cDNA was performed according to the protocol developed by Applied Biosystems. To assess gene expression, three replicate analyses were realized and the amount of target RNA was normalized with respect to the control gene (GAPDH) and expressed according to the $2^{-\Delta\Delta Ct}$ method [25].

### 2.7. Evaluation of parasite load by immunohistochemistry

Representative sections with 4 μm were prepared from paraffin embedded tissue. The deparaffinization of the sections was realized with xylene, following by rehydration through a descending series of ethanol concentrations and washed in water. Posteriorly, were performed the immunohistochemical assay according by Tafuri et al. [26], with modifications. Briefly, slides were incubated with $H_2O_2$ (30, v/v) in PBS for 30 min at room temperature (RT), followed by incubation with normal goat serum (1:100 dilution). After washes, was applied primary antibody (heterologous hyperimmune serum from dog naturally infected by *L. infantum*, diluted 1:100 in PBS), followed by overnight incubation at 4 °C in humid chamber. After washing in PBS, were added biotinylated secondary antibody (DAKO, LSAB2 Kit, Agilent, USA) proceeding the incubation for 30 min at RT. Posteriorly, the slides were washed again and then incubated with the streptavidin–peroxidase complex (DAKO, LSAB2 Kit, Agilent, USA) for 30 min at RT. The reaction was revealed with 50 mg of diaminobenzidine (DAB, Sigma-Aldrich, USA), 200 mL PBS and 400 μL of hydrogen peroxide. Finally, slides were counterstained with Harris' Haematoxylin, and mounted with coverslips. The quantification of amastigotes was performed based on the count of immunomarked area through the acquisition of thirty random images. Images were captured by 40 × objective and digitized using Leica DM5000B micro camera and Leica Application Suite software (version 2.4.0 R1, Leica Ltd., Switzerland). Leica Qwin V3 software (Leica Microsystems Ltd., Switzerland) was used for image analysis.

### 2.8. Evaluation of parasite load by real time PCR

Total DNA extraction from the liver samples was carried out using Wizard SV Genomic DNA Purification System Kit (Promega Corporation, USA) following the manufacturer's recommendations. The DNA concentration and purity were determined using a spectrophotometer (NanoDrop Lite spectrophotometer; Thermo Fisher Scientific, USA), and the samples were stored at −20 °C until qPCR assay. The qPCRs were performed in 96-well plates and processed on a thermocycler (7500 Real Time PCR System - Applied Biosystems, USA). TaqMan system was used to amplify a 90-bp fragment of a single-copy gene of DNA polymerase of *L. infantum* (GenBank accession number AF009147) through specific primers (forward, 5′ TGT CGC TTG CAG ACC AGA TG 3′ and reverse, 5′ GCA TCG CAG GTG TGA GCA C 3′). The reactions were performed in a 25 μL final volume containing 1 × TaqMan Universal Master Mix (Applied Biosystems, USA), 20 pmol of the specific primers, 10 pmol of the labelled probe, and 40 ng of DNA. Thermal cycling involved an initial incubation for 2 min at 50 °C, followed by a 10-min denaturation at 95 °C, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min each. The integrity of the liver samples was evaluated by the same procedure, using GAPDH gene (AB038240) that amplifies a 115-bp fragment. Each 96-well reaction plate contained a standard curve in triplicate and samples in duplicate. The result was expressed as number of amastigotes DNA copies per milligram of liver (amastigotes/mg).

### 2.9. Statistical analysis

Statistical analyses were conducted using the GraphPad Prism 8.0 software package (Prism Software, USA). Normality of the quantitative data was evaluated by Kolmogorov–Smirnov test. One-way analysis of variance and Tukey post-tests were used to determine the differences between groups. Analysis of portal inflammation was carried out using the Chi-square test. Pearson rank correlation was computed to investigate associations between spleen parasite burden versus cytokine expression. Results were considered significant if $p < 0.05$.

## 3. Results

### 3.1. LBMPL treated dogs demonstrate reduction of the portal and total hepatic inflammation

The semi-quantitative analyses of portal inflammation showed a higher ($p < 0.05$) intensity in INT and MPL groups than NI group (Fig. 1A). Moreover, the LBMPL group presented lower ($p < 0.05$) intensity of inflammation in the portal tract in comparison to INT and MPL groups (Fig. 1A). Fig. 1A (bottom panel) shows representative images of portal inflammation concerning the animals of different experimental groups. It is possible to observe a normal histological pattern across the animals of NI group (Fig. 1-I). On the other hand, a moderate to intense portal inflammation is seen in dogs of the INT and MPL groups (Fig. 1 -II and III). The Figure I-IV demonstrates mild portal inflammation, present in an animal from the LBMPL group, similar to the NI group. The portal inflammatory process in the different groups was composed mainly of lymphocytes and macrophages.

The analysis of total inflammation demonstrated a higher ($p < 0.05$)

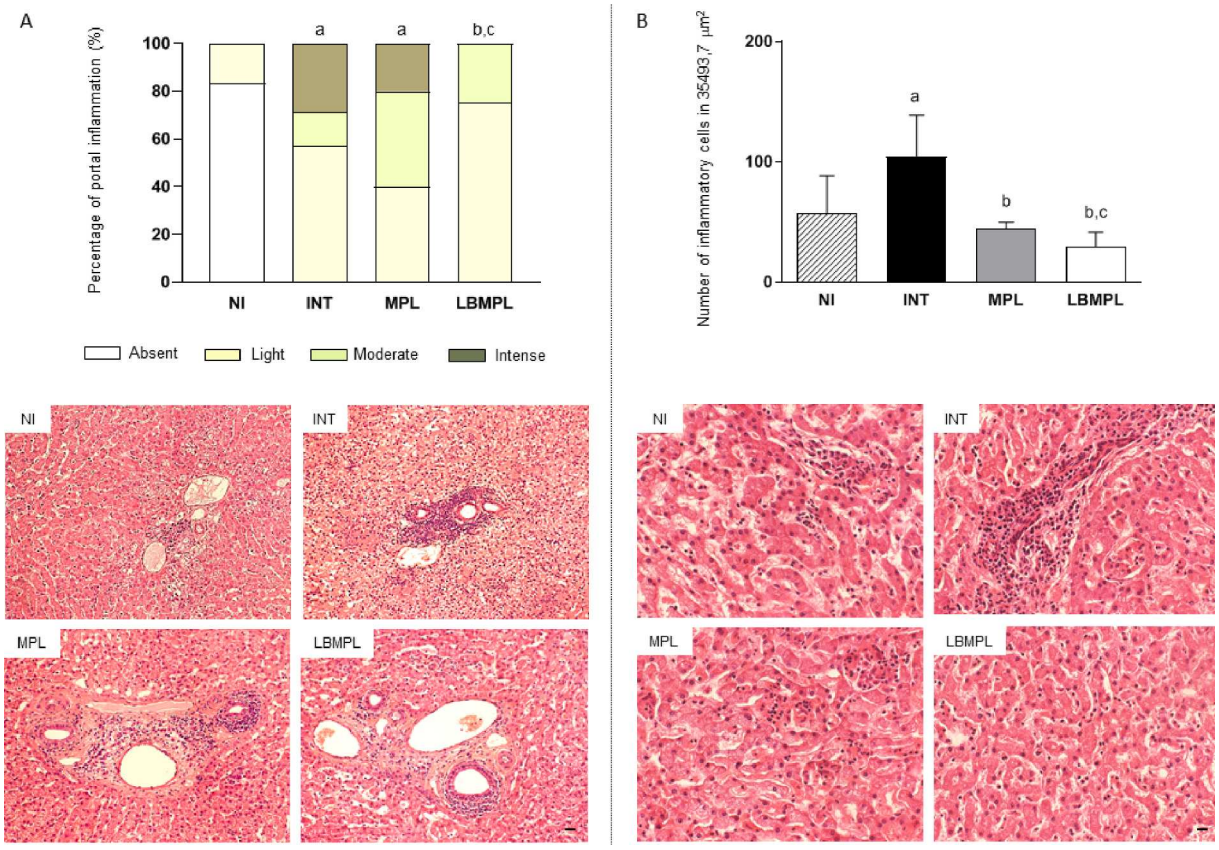


**Fig. 1. Hepatic histopathological evaluation of dogs naturally infected by *Leishmania infantum* and treated with the LBMPL vaccine. (A)** Semi-quantitative analysis of portal inflammation and **(B)** quantitative total liver inflammation in dogs uninfected and untreated (NI), dogs infected by *L. infantum* and untreated (INT), dogs infected by *L. infantum* and treated with the Monophosphoryl lipid A (MPL) adjuvant alone and dogs infected by *L. infantum* and treated with the vaccine composed of *L. braziliensis* antigens associated with the MPL adjuvant (LBMPL). **(A)** The results are expressed as a percentage. The bars are represented as follows: white (-): absent portal inflammation; light yellow (+): light portal inflammation; yellow (++): moderate portal inflammation; gray (+++): intense portal inflammation. Below are shown representative photomicrographs of the portal inflammatory process in the liver in all groups. Hematoxylin and eosin staining. Images shown at 20x magnification. Bar = 100 µm. **(B)** The results are expressed as mean ± standard deviation of number of inflammatory cells. The significant differences ($p < 0.05$) are represented by "a", "b" and "c", referring to NI, INT and MPL groups, respectively. Below are shown representative photomicrographs of the total liver inflammation in all groups. Hematoxylin and eosin staining. Images shown at 40x magnification. Bar = 50 µm.

inflammation process in INT group compared to NI group (Fig. 1B). Moreover, we observe a reduction ($p < 0.05$) of inflammation in MPL and LBMPL groups in relation to INT group, and a reduction ($p < 0.05$) in LBMPL group compared to MPL group. In general, histopathological findings showed different magnitude degree in groups of *L. infantum* infected dogs, with a lower mononuclear inflammation in LBMPL group, similar to NI group (Fig. 1B – bottom panel). The chronic inflammatory reaction was composed by a diffuse mononuclear infiltrate in all hepatic parenchyma, with few granuloma formations. Rare neutrophils and eosinophils were observed (Figue 1B – bottom panel).

### 3.2. LBMPL treated dogs show high expression of iNOS and lower expression of IL-10 and TGF-β1 in the liver

The mRNA expression of iNOS was increased ($p < 0.05$) in LBMPL group when compared to INT group (Fig. 2). On the other hand, there was less mRNA expression ($p < 0.05$) of IL-10 and TGF-β1 in the LBMPL group in relation to INT group (Fig. 2).

### 3.3. LBMPL vaccine leads to an important parasite burden reduction in the liver

As shown in Fig. 3, a low number ($p < 0.05$) of amastigotes was observed in the liver of the LBMPL group when compared with the INT group by immunohistochemistry assay. Fig. 3B (bottom panel) shows representative images of amastigotes (immunostained area) in hepatic tissue of *L. infantum* infected dogs. It is possible to observe an intense stainning of amastigotes in the INT group (Fig. 3-I). On the other hand, a reduction in the parasitism was demonstrated in the LBMPL group (Fig. 3-III).

In the same way, it was observed a decrease ($p < 0.05$) of parasitism in the LBMPL group compared to the INT and MPL groups by qPCR analysis (Fig. 3).

### 3.4. Enhanced IL-10 and TGF-β1 cytokines may represent an essential condition for parasite replication in the liver of INT and MPL groups

Correlation analyses between hepatic parasitism and cytokine expression were performed (Table 1). Our results indicated a positive correlation between parasite load and IL-10 expression in INT ($r = 0.78$; $p = 0.02$) and MPL ($r = 0.88$; $p = 0.02$) groups, besides a positive correlation between parasite load and TGF-β expression in INT ($r = 0.84$; $p = 0.02$) and MPL ($r = 0.97$; $p = 0.005$) groups.

## 4. Discussion

The development of compartmentalized lesions is directly associated to the host immune response and the clinical evolution in canine VL. To better understand the mechanisms underlying upon immunotherapy in *L. infantum* infected dogs, this study aimed to investigate the main

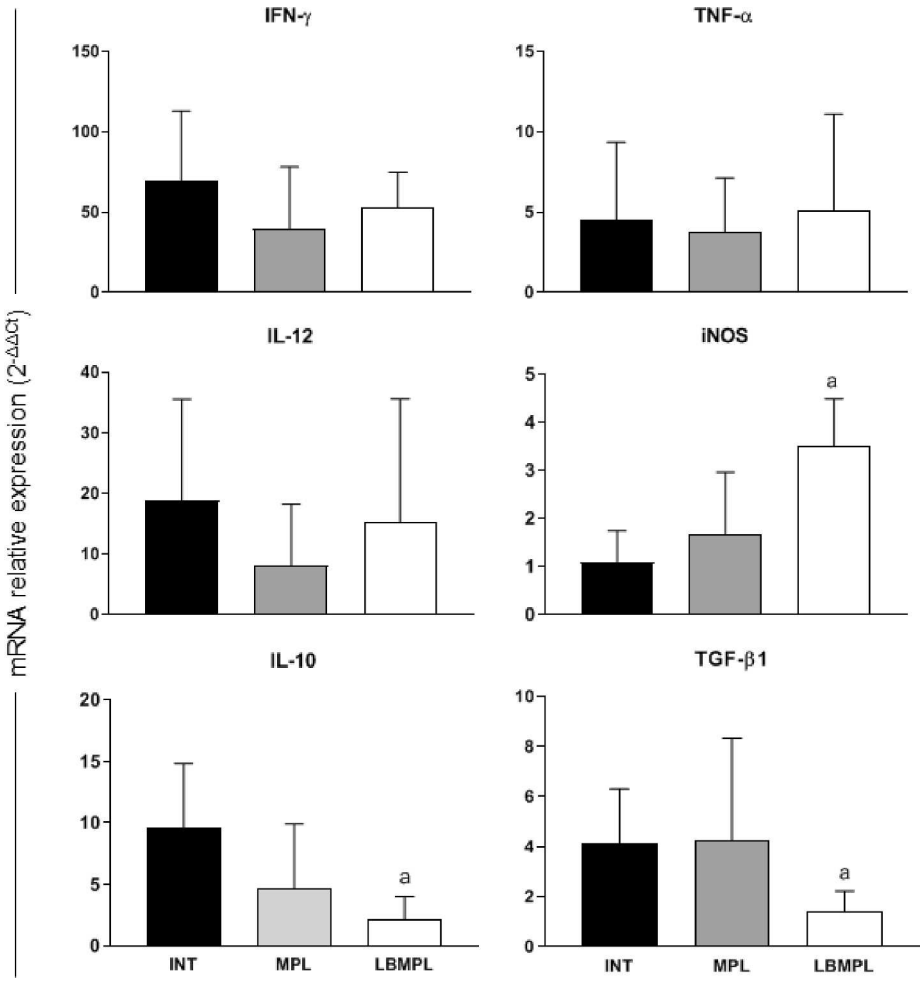


**Fig. 2. Profile of cytokines and iNOS in the liver of dogs naturally infected by *Leishmania infantum* and treated with the LBMPL vaccine.** Analyses of the expression of mRNAs for hepatic cytokines and iNOS in dogs infected by *L. infantum* and untreated (INT), dogs infected by *L. infantum* and treated with the Monophosphoryl lipid A (MPL) adjuvant alone and dogs infected by *L. infantum* and treated with the vaccine composed of *L. braziliensis* antigens associated with the MPL adjuvant (LBMPL). The results are expressed as mean ± standard deviation of mRNA relative expression ($2^{-\Delta\Delta Ct}$) of IFN-γ, TNF-α, IL-12, iNOS, IL-10 and TGF-β1 in the liver. The significant differences ($p < 0.05$) are represented by "a" referring to INT group.

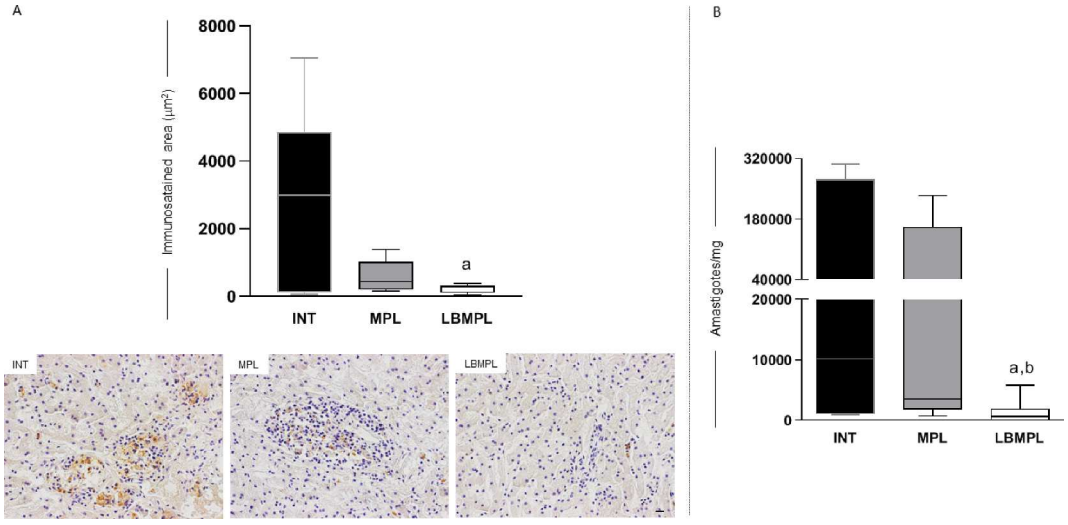


**Fig. 3. Quantification of parasite burden in liver of dogs naturally infected by *Leishmania infantum* after immunotherapy with LBMPL vaccine.** Evaluation of hepatic parasite burden by **(A)** immunohistochemistry and **(B)** qPCR in dogs infected by *L. infantum* and untreated (INT), dogs infected by *L. infantum* and treated with the Monophosphoryl lipid A (MPL) adjuvant alone and dogs infected by *L. infantum* and treated with the vaccine composed of *L. braziliensis* antigens associated with the MPL adjuvant (LBMPL). **(A)** The results are expressed as mean ± standard deviation of immunostained area. The significant difference ($p < 0.05$) is represented by "a", referring to INT group. Below are shown representative photomicrographs of immunostaining for *Leishmania infantum* in hepatic tissue, revealed with DAB and against stained with Hematoxylin in all groups. Images shown at 40x magnification. Bar = 50 μm. **(B)** The results are expressed as median and minimum and maximum values of number of amastigotes per milligram of tissue. The significant differences ($p < 0.05$) are represented by "a" and "b" referring INT and MPL groups, respectively.

**Table 1**
Correlation indexes between liver parasitism by qPCR and expression of cytokines.

| Groups | Hepatic parasite burden versus expression of cytokines | | | | | | |
|---|---|---|---|---|---|---|---|
| | *IFN-γ* | | *TNF-α* | *IL-12* | *iNOS* | *IL-10* | *TGF-β1* |
| **INT** | *r* | −0.62 | 0.22 | 0.71 | 0.24 | **0.78** | **0.84** |
| | *p* | 0.14 | 0.64 | 0.07 | 0.60 | **0.02** | **0.02** |
| **MPL** | *r* | 0.82 | 0.78 | **0.89** | 0.44 | **0.88** | **0.97** |
| | *p* | 0.09 | 0.12 | **0.04** | 0.45 | **0.02** | **0.005** |
| **LBMPL** | *r* | −0.36 | −0.41 | −0.34 | −0.44 | −0.29 | 0.33 |
| | *p* | 0.33 | 0.27 | 0.37 | 0.23 | 0.22 | 0.39 |

Numbers in bold represent correlation values presenting significant differences ($p < 0.05$).
(INT) dogs infected by *L. infantum* and untreated.
(MPL) dogs infected by *L. infantum* and treated with Monophosphoryl lipid A (MPL) adjuvant alone.
(LBMPL) dogs infected by *L. infantum* and treated with the vaccine composed of *L. braziliensis* antigens associated with the MPL adjuvant (LBMPL).

histopathological, immunological, and parasitological alterations in the liver tissue after treating with the LBMPL vaccine. Overall, our results demonstrated a reduction of inflammatory infiltrate, an increase of iNOS expression and a decreased of IL-10 and TGF-β1 expression, and an intense reduction in parasite load in dogs treated with LBMPL vaccine.

Hepatic disease and several associated histopathological alterations have been described in the course of *L. infantum* infection in dogs. Inflammation is considered the main histopathological liver alteration and has been associated with the persistence of the parasite in this organ [23,27]. In our study, a striking difference is that a relatively smaller portal and total inflammation were observed in the LBMPL group, similar to the uninfected control group. These results are in line with previous studies that described a lower hepatic inflammation in symptomatic dogs compared to asymptomatic or non-infected dogs [22,24]. Decreasing inflammatory process was associated with lower hepatic parasite load, which is consistent with the results of Verçosa et al. [28], Giunchetti et al. [22], Guerra et al. [29], and Cardoso et al. [30]. These authors demonstrated a positive correlation between parasitism and inflammation in different tissues of *L. infantum* infected dogs. Some studies have described granuloma formation associated with resistance profile to *Leishmania* in experimental models, as well as in human disease [31]. In contrast, this formation was few highlighted in our study, even in the LBMPL group. Supporting this view, no association between the well-organized granulomas and clinical presentation was observed in *L. infantum* infected dogs [24]. Furthermore, Reis et al. [32] did not observe granuloma formation even in mice successfully treated with liposomal meglumine antimoniate, indicating that granuloma formation may not be associated with a resistance profile in experimental VL.

The resolution of *Leishmania* infection is mediated by oxygen reactive species-producing macrophages activated by T cell derived cytokines [33,34]. In this sense, we evaluated the expression of iNOS and cytokines in the liver of treated dogs. Our results demonstrated a higher mRNA expression of iNOS and lower mRNA expression of IL-10 and TGF-β1 in the LBMPL group. Similarly, several reports have been demonstrated that the high expression of iNOS in macrophages is related to lower parasite load in the skin, lymph nodes, and liver of dogs naturally infected by *L. infantum* [34,35]. In the same way, previous reports have been associated the IL-10 and TGF-β1 cytokines to susceptibility profile in CVL [36,37]. Interestingly, Roatt et al. [21] observed a reduction in IL-10 in PBMC after specific stimuli in dogs treated with the LBMPL vaccine. Some reports have been demonstrated the role of TGF-β1 in the development of hepatic fibrosis following HSC activation [38]. In CVL, the liver fibropoiesis inducing by TGF-β1 is related to parasite load and inflammation in comparison to uninfected dogs [39,40]. Together, these data reinforce the possible role of the LBMPL vaccine in preventing further liver damage.

After treatment, it was possible to observe a lower liver parasite load in dogs treated with the LBMPL vaccine, either by a less sensitive methodology such as immunohistochemistry or more sensitive like qPCR. This reduction was associated with an attenuation of the anti-inflammatory immune profile and reduction of histopathological alterations in the liver. Consist with our results, Roatt et al. [21] observed a decrease of parasite load in bone marrow and skin after in dogs treated with LBMPL vaccine. In the same way, Reis et al. [32] observed a reduction of hepatic parasite load, associated with a pro-inflammatory profile and attenuation of histopathological alterations in the experimental VL after treatment with liposomal meglumine antimoniate.

In conclusion, our results demonstrated that the LBMPL vaccine was able to reduce the damage to hepatic tissue, being able to deactivate the type 2 immune response, with decreased inflammation, associated with a reduction in the parasite burden in the liver of treated dogs. The data in addition with previous results highlight the potential of LBMPL vaccine as a possible therapeutic alternative in *L. infantum* dogs.

### *CRediT authorship contribution statement*

**Bruno Mendes Roatt:** Conceptualization, Methodology, Formal analysis, Writing – original draft. **Jamille Mirelle de Oliveira Cardoso:** Methodology, Formal analysis, Writing – original draft. **Rory Cristiane Fortes de Brito:** Methodology, Formal analysis. **Levi Eduardo Soares Reis:** Methodology. **Gabriel José Lucas Moreira:** Methodology, Writing – original draft. **Paula Melo de Abreu Vieira:** Methodology, Formal analysis. **Flávia Marques de Souza:** Methodology. **Wanderson Geraldo de Lima:** Methodology. **Rodrigo Dian de Oliveira Aguiar-Soares:** Methodology, Formal analysis. **Rodolfo Cordeiro Giunchetti:** Conceptualization. **Alexandre Barbosa Reis:** Supervision, Project administration, Funding acquisition, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2022.155838.

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