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Heterologous vaccine therapy associated with half course of Miltefosine promote activation of the proinflammatory response with control of splenic parasitism in a hamster model of visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a serious and neglected disease present worldwide. Chemotherapy using pentavalent antimony (Sb^V) is the most practical and inexpensive strategy available for the VL treatment today, however, it has high toxicity. Alternatively, other drugs are used as viable leishmanicidal therapeutic options. Miltefosine is the only anti-leishmanial agent administered orally, however, it has been reducing its effectiveness. In this sense, there is no ideal therapy for VL since the drugs currently used trigger severe side effects causing discontinuation of treatment, which carries an imminent risk for the emergence of parasite resistance. With that, other therapeutic strategies are gaining prominence. Among them, immunotherapy and/or immunochemotherapy, which the activation/modulation of the immune system can redirect the host's immune response to an effective therapeutic result. Therefore, this work was designed to assess an immunochemotherapy protocol composed of half course of Miltefosine associated with LBSap vaccine (Milt+LBSap) using the hamster *Mesocricetus auratus* as an experimental model for VL treatment. When evaluating the main hematobiochemical, immunological and therapeutic efficacy parameters, it was demonstrated that the treatment with Milt+LBSap showed restoration of hematobiochemical condition and reduced serum levels of IgG-anti-*Leishmania* compared to animals infected non treated (INT). Beyond that, an increase in the number of $CD4^+$ lymphocytes producers of IFN-γ in relation to INT or to animals treated with miltefosine during 28 days, and TNF-α increased compared to INT were observed. Also, it was found a reduction of IL-10-production in relation to INT, or animals that received LBSap vaccine only, or miltefosine, following by a reduction in the splenic parasitic burden. These results demonstrate that the immunochemotherapy protocol used can stimulate the immune response, inducing an expressive cellular response sufficient to control spleen parasitism, standing out as a promising proposal for the VL treatment.

1. Introduction

Visceral leishmaniasis (VL) is a chronic and severe disease that estimated 50,000 to 90,000 new cases occur worldwide annually (WHO, 2017). Anthroponotic transmission of VL is caused by *Leishmania donovani* and prevails in the Indian subcontinent and East Africa, while the zoonotic transmission is caused by *L. infantum* in the Mediterranean region, South America, and South-west and Central Asia (Alvar et al., 2012). These protozoan parasites cause systemic disease, infecting macrophages in visceral organs, mainly the spleen, bone marrow and lymph nodes. Despite most infections being asymptomatic, individuals that develop VL can die, if they do not receive adequate treatment

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(Wilson et al., 2005; van Griensven and Diro, 2012).

Chemotherapy is the most practical and effective treatment strategy applied to VL, despite the few available therapeutic agents (Chakravarty and Sundar, 2019; Roatt et al., 2020). The effectiveness of anti-leishmanial drugs varies with the host immune status and the geographic location of *Leishmania* infection (WHO, 2017). The pentavalent antimonials (Sb^v) compounds had been the main treatment of VL for many decades until it becomes obsolete in the Indian subcontinent due to the development of resistance, being replaced by intravenous amphotericin B deoxycholate (AmB-D) (Sundar, 2001). Despite the discontinued use of this drug in India, its use is still common in some regions of the world, such as in Brazil (Aronson et al., 2017). Nevertheless, Sb^v, as well AmB-D, require a long treatment period, they have elevated toxicity, and require intensive clinical and laboratory monitoring. In order to reduce adverse events, the liposomal amphotericin B formulation (Ambisome®) has been used in some regions of the world (Sundar et al., 2010). However, this treatment has a high cost, preventing its use in the global population (Meheus et al., 2010). Another important antileishmanial agent is miltefosine (hexadecylphosphocholine), the only available oral drug in use approved for leishmaniasis. Although initially developed as an anticancer drug, miltefosine is relatively safe and became the first-line therapy in India, after increased resistance to pentavalent antimonials (Croft and Engel, 2006). Several clinical trials performed showed high cure rates (approximately 94–97% at 6 months follow-up) with miltefosine. However, there was a reduction in the effectiveness after extensive use in the Indian subcontinent over the ensuing decade (Rahman et al., 2011; Sundar et al., 2012).

Whereas the therapeutic arsenal for VL has limitations related to either toxicity, cost, parenteral administration, and long duration, which reduces adherence to treatment and increases drug resistance, one solution is to combine these drugs to allow shorter, less toxic and more affordable treatment. Other alternatives are immunotherapy and/or immunochemotherapy, which activation/modulation of the immune system can redirect the host immune response for an effective therapeutic outcome (Musa et al., 2010). Recently, Roatt et al. (2017) treated symptomatic dogs naturally infected by *L. infantum* with a therapeutic vaccine composed by *L. braziliensis* antigens associated with the adjuvant Monophosphoryl lipid (MPL) (LBMPL vaccine). This vaccine elicited a strong cellular immune response, besides to improving clinical parameters and drastic reducing the parasitic load on bone marrow and skin (Roatt et al., 2017).

In the last years, our research group has been working on the development of prophylactic vaccines for VL (Giunchetti et al., 2007; Roatt et al., 2012; Aguiar-Soares et al., 2014). A study has been demonstrated that the administration of a vaccine consisting of *L. braziliensis* antigens with saponin as an adjuvant (LBSap vaccine), conferred protection to dogs challenged with *L. infantum*. This protection was associated with a significant decrease in spleen parasite burden and increases of $CD4^+$ and $CD8^+$ T cells producing IFN-γ and total anti-*Leishmania* IgG1 and IgG2 antibody levels (Roatt et al., 2012). However, to date, this vaccine has not been evaluated as a therapeutic tool.

Considering that the immune status of VL patients may have impact in the efficacy of certain drugs and that the immune modulation can act synergistically with drugs to control the parasitic load. Therefore, immunotherapy in conjunction with chemotherapy (immunochemotherapy) can quickly induce a protective immune response, suggesting that this strategy provides a promising tool for VL treatment. In this way, the present study was designed to assess an immunochemotherapy protocol composed by Miltefosine associated with LBSap vaccine using the hamster *Mesocricetus auratus* as an experimental model for VL treatment.

2. Materials and methods

2.1. Animals and infection

One-month-old male and female Syrian golden hamsters (*Mesocricetus auratus*), weight range 60g–80g, were acquired from Centro de Ciência Animal, Universidade Federal de Ouro Preto (UFOP), Brazil. The hamsters were housed in plastic cages and fed with rodent food and water *ad libitum*.

L. infantum (strain MCAN/BR/2008/OP46) was used for the experimental infections of hamsters (Moreira et al., 2012; Carvalho et al., 2021). The animals were infected by intraperitoneal route with 2×10^7 promastigotes of *L. infantum* in the stationary growth phase. One hundred days after infection, they were euthanized, and blood and spleen were collected. All the presented experiments in this work were performed in duplicate. The L. *infantum* OP46 strain used in this study has approval from the National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SISGen (A55DE5A).

2.2. Experimental groups and treatment schedules

In this study, the experiments refer to the treatment schedule using an immunotherapy protocol with a vaccine or an immunochemotherapy protocol which intercalates immunotherapy during chemotherapy with miltefosine 2% (Milteforan™ – Virbac, Carros, France). The vaccine used in these treatment protocols was the LBSap, which is composed of total crude extract of *Leishmania braziliensis* antigens (LB) associated with the adjuvant saponin (Sigma Chemical Co., St. Louis, EUA). The hamsters were infected with 2×10^7 promastigotes of *L. infantum* and sixty days after infection were distributed in different treatment protocols groups, described below:

(i) **Infected and untreated group (INT)**: the animals received orally sterile 0.85% saline for twenty-eight consecutive uninterrupted days;
(ii) ***L. braziliensis* group (LB)**: the animals were treated by subcutaneous route with *L. braziliensis* antigen (60 μg) in 3 series of five days each, with an interval of five days between the series;
(iii) **Saponin group (Sap)**: the animals were treated by subcutaneous route with adjuvant saponin (100 μg) in 3 series of five days each, with an interval of five days between the series;
(iv) **LBSap group (LBSap)**: the animals were treated by subcutaneous route with *L. braziliensis* antigen (60 μg) associated with the adjuvant saponin (100 μg) in 3 series of five days each, with an interval of five days between the series;
(v) **Miltefosine group (Miltefosine)**: the animals received oral treatment with miltefosine (2 mg/kg) for twenty-eight consecutive uninterrupted days (full course of treatment);
(vi) **Miltefosine + LB group (Milt. + LB)**: the animals received oral treatment with miltefosine (2 mg/kg) for fourteen consecutive uninterrupted days (half course of treatment). After seven days of treatment with miltefosine, the animals received the *L. braziliensis* antigen (60 μg) by subcutaneous route in 2 series of five days each, with an interval of five days between the series;
(vii) **Miltefosine + LBSap group (Milt. + LBSap)**: the animals received oral treatment with miltefosine (2 mg/kg) for fourteen consecutive uninterrupted days (half course of treatment). After seven days of treatment with miltefosine, the animals received the *L. braziliensis* antigen (60 μg) associated with the adjuvant saponin (100 μg) by subcutaneous route in 2 series of five days each, with an interval of five days between the series.

An uninfected control group composed of seventeen animals was also used, whose analyzes were used to generate normal hematological, biochemical, and immunological parameters.

2.3. Hematological and biochemical analysis

In this study, hematological (leukogram and eritrogram) and biochemical (renal and hepatic function) parameters were determined. After anesthesia with sodium thiopental 2.5% at a dose of 10 mg/kg intraperitoneally, blood was collected by cardiac puncture and was transferred to tubes containing or not EDTA anticoagulant (Sigma Chemical Co). As hematological assessments were performed the total count of leukocytes, erythrocytes, hemoglobin, hematocrit, and platelets using the Auto Hematology Analyzer equipment (Mindray BC-2800 Vet, Hamburg, Germany). Complementary, we also made the differential leukocyte count by blood smears on slides stained with the InstantProv Fast Panotic kit (Newprov®) and evaluated by optical microscopy in an immersion objective. The biochemical parameters were used Labtest commercial reagent kits (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil) being performed following the manufacturers' recommendations. For this, urea and creatinine levels in serum samples were determined to assess kidney function. Also, the analysis of serum protein, alanine aminotransferase (ALT/STGP) and aspartate aminotransferase (AST/STGO) was performed to liver function analysis. For the evaluation of all these biochemical parameters was used the Semi-Automatic Biochemical System (CELM SBA-200, Barueri, SP, Brazil).

2.4. Humoral immune response

To measure the anti-*Leishmania* IgG titers it was used serum from hamsters distributed in the different experimental groups. For this, the conventional enzyme-linked immunosorbent assay (ELISA) was performed according to Reis et al. (2006). ELISA microplates (MaxiSorp, Nalge Nunc) were coated, with soluble *Leishmania* (MHOM/BR/1972/BH46) promastigotes antigen – SliAg (Reis et al., 2006) diluted in carbonate buffer at a concentration of 1μg/well. Then, free binding sites were blocked. Following a washing step, the plates were incubated with the sera sample diluted 80 times. The plates were washed and incubated with goat anti-hamster IgG (1:1000, Goat Anti-Hamster - Caltag Laboratories). After a washing step, the plates were incubated with the substrate and chromogen (O-phenylenediamine, Sigma–Aldrich) and sulfuric acid 2N was added to stop the reactions. Optical density (OD) values were read in ELISA reader (ELX800 Biotek Instruments VT, USA) at 490 nm. In all plates, were used positive and negative hamsters control sera, conjugate control, and reaction control - "blank". The *cut-off* point was established as mean absorbance value + 2 SD from seventeen sera from uninfected hamsters.

2.5. Cellular immune response

2.5.1. In vitro culture CD4 and intracellular cytokine phenotyping

The procedure was performed in accordance with Carvalho et al. (2021). Briefly, in a solution of RPMI (Sigma, St. Louis, MO, USA) plus heparin (Cristália, Itapira, SP, Brazil), splenocytes were obtained by maceration of spleens harvested from the animals under sterile conditions. After centrifugation at 450*g*, 4 °C for 10 min, the splenocytes were plated (5×10^5 cells/well) in 96-well round-bottom (Costar, Cambridge, MA, USA) culture plates and were incubated for 48 h at 37 °C with 5% CO_2 in RPMI, in the presence or absence of *Leishmania* Antigen - SLiAg (50 μg/mL) or with phorbol 12-myristate 13-acetate (PMA, 5 ng/mL, Sigma, St. Louis, MO, USA) and ionomycin (ION, 0.2 μg/mL, Sigma, St Louis, MO, USA) as our positive control of cytokine production. After, Brefeldin A (BFA, 10 μg/mL, Sigma, St Louis, MO, USA) and 2 mM ethylenediamine tetra acetic acid (EDTA) (Sigma, St Louis, MO, USA) were added. Following centrifugation at 400*g* at 4 °C for 7min, cells were labeled with Fixable Viability Stain 450 (FVS450, BD Biosciences) (12.5 μg/mL). Then, the splenocytes were stained with 6 μg/mL of the anti-mouse CD4 FITC (clone Gk1.5 - BD Biosciences Bioscience, USA) antibody and were fixed with FACS fixing solution. After washing and permeabilization steps, the cells were stained with anti-mouse TNF-α PerCP-Cy5.5 (clone MP6-XT22), anti-mouse IFN-γ AF-647 (clone XMG1.2) and anti-mouse IL-10 PE (clone JES5-2A5) (BD Biosciences Bioscience, USA) in a concentration of 1.5 μg/mL of all the cytokines. It is important to notice that internal controls such as unstained cells and isotypic control of the monoclonal antibodies were used in all the assays.

2.5.2. Flow cytometry acquisition and analysis

Flow cytometer LSR Fortessa cytometer (BD Biosciences, USA) using FACSDiva software was used to acquire the cells (100,000 events/second) and FlowJo software for data analysis. After singlet selection based on their size (forward scatter area—FSC-A and forward scatter height—FSC-H), dead cells were excluded from subsequent analyzes by gating FVS450 cells (SSC-A versus FVS450 stain dot plots). Then, lymphocytes were quantified using their FSC-A and granularity (side scatter area—SSC-A) and the cytokines expressed by them in FSC-A versus PerCP-Cy5.5/AF647/PE-Fluorescence dot plots graphs. We also gated $CD4^+$ lymphocytes (SSC-A versus FITC-Fluorescence dot plots) and the cytokines-$CD4^+$ cells were selected by gating FITC-Fluorescence versus PerCP-Cy5.5/AF647/PE-Fluorescence dot plots graphs. The Supplementary Fig. 1 illustrates the analysis strategy used.

2.6. Evaluation of splenic parasite load

Splenic parasite load was determined 100 days after infection through quantitative real-time PCR (qPCR) as described by Moreira et al. (2012). Briefly, at the endpoint, hamsters were euthanized (using the anesthetic dose of 30 mg/kg of 2.5% sodium thiopental; after confirming that the anesthetic plan had been reached, the animals received a lethal dose of 120 mg/kg of 2.5% sodium thiopental), and approximately 20 mg of spleen were used for genomic DNA isolation with the CTAB reagent, as follow: 250 μL of lysis buffer (5 mM Tris pH 7.5, 1 mM EDTA, n-lauryl sarcosine 1%) was added to the spleen samples. The mixture was incubated in a dry bath at 37 °C for 1 h. The samples underwent mechanical digestion for 30 s at a frequency of 30/second. After, 100 μL of 5 M NaCl was added, (incubation for 10 min at 65 °C) and 50 μL of 10% CTAB solution (v/v) was added incubation for 20 min at 65 °C). Thus, 400 μL of chloroform (Sigma, St. Louis, MO, USA) was added under and the samples were centrifuged (12,000×*g* for 10 min). The aqueous phase was transferred to a new tube and 400 μL of isopropanol (Merck®, Darmstadt, Germany) was added. Following precipitation and centrifugation step (10 min at 12,000×*g*), the supernatant was discarded, and the pellet was washed with 70% cold ethanol. After centrifugation (5 min at 12,000×*g*), the supernatant was discarded, and the DNA precipitate was homogenized in 50 μL of nuclease-free water. The quantification of *L. infantum* parasites was assessed using a conserved region of *Leishmania* kDNA (kinetoplast DNA) (forward primer: 5′- GGG(G/T) A GGG GCG TTC T(C/G) CGA A-3'; reverse primer: 5′-C/G) (C/G) (C/G) (A/T) CTA T(A/T) TTA CAC CAA CCC C-3';) with Bryt Fluorescent Signal detection system (GoTaq® qPCR Master Mix - Promega Corporation, EUA). GAPDH primers (5′-TGG AGT CTA CTG GCG TCT TC-3′ e reverse: 5′ GGA GAT GAT GAC CCT CTT G-3′) were used as constitutive for the amounts of hamster chromosomal DNA. The PCR reactions were conducted as follows: one step to activate Taq enzyme (95 °C for 2 min) followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min. All samples were run on MicroAmp optical 96-well reaction plates (Applied Biosystems) sealed with MicroAmp optical adhesive film (Applied Biosystems). Each 96-well reaction plate contained a standard curve in triplicate (efficiency, 96.0%; r2 - 0.99) in duplicate samples. The result was expressed as the number of amastigotes DNA copies per mg of the hamster's spleen.

2.7. Statistical analysis

Statistical tests were performed using the GraphPad Prism 8.0 software (Prism Software, Irvine, CA, USA). To analyze the normality, was

assessed the Shapiro-Wilk test. The analysis of variance (one-way ANOVA) was performed for parametric or non-parametric tests. For parametric data, Tukey test was used to determine the specific differences between the means (longitudinal analysis within the same group). For non-parametric data, the Kruskal-Wallis test was performed to determine the specific differences between the median. Pearson's r test was assessed to perform the correlation analyses. Differences with p-values less than 0.05 ($p < 0.05$) were considered significant.

3. Results

3.1. Evaluation of the hematological and biochemical profile

Based on the hemogram evaluation, white blood cells (WBC) did not show many modifications in all groups of hamsters. It was observed an increase ($p < 0.05$) of circulating monocytes in Milt.+LBSap group (Miltefosine plus LBSap vaccine) compared to INT (Infected non treated), LB (*L. braziliensis* antigens), SAP (Saponin), LBSap (*L. braziliensis* antigens plus Saponin), Miltefosine and Milt.+LB (Miltefosine + *L. braziliensis* antigens) groups (Table 1). In red blood cells (RBC) parameters, was observed an increase ($p < 0.05$) in erythrocytes and hemoglobin in LB, SAP, LBSap, Miltefosine, Milt.+LB, Milt.+LBSap groups concerning to INT group (Table 1). The platelet number was outside the reference values in the INT group (Table 1).

The biochemical evaluation showed a reduction ($p < 0.05$) in urea levels in LB, SAP, LBSap, Miltefosine, Milt.+LB, Milt.+LBSap groups compared to the INT group (Table 1). Moreover, we observed an increase ($p < 0.05$) in this parameter in the Milt.+LB and Milt.+LBSap groups in relation to the LB, SAP, LBSap groups (Table 1). Serum creatinine levels were reduced ($p < 0.05$) in the LBSap group compared to LB and SAP groups. Furthermore, we observed a decrease ($p < 0.05$) in this parameter in Miltefosine, Milt.+LB and Milt.+LBSap groups concerning the INT, LB, SAP and LBSap groups (Table 1). Regarding hepatic function, decreased ($p < 0.05$) levels of serum protein were observed in LB, SAP, Milt.+LB Milt.+LBSap groups compared to INT group. Moreover, it was observed an increase ($p < 0.05$) in Miltefosine group in relation to LB group and a decrease ($p < 0.05$) in the Milt.+LBSap group in relation to Miltefosine group (Table 1). In relation to serum AST/SGOT levels, all groups demonstrated reducing ($p < 0.05$) compared to the INT group (Table 1). Furthermore, we observed a reduction ($p < 0.05$) in the LBSap group compared to LB group and a reduced in the Milt.+LBSap group compared to the Milt.+LB group (Table 1). We also observed a decrease ($p < 0.05$) of ALT/SGPT levels in Miltefosine group in relation to the INT, LB, SAP, Milt+LB groups (Table 1). Besides, were observed a decrease ($p < 0.05$) in the Milt.+LBSap group compared to INT, SAP and LBSap groups (Table 1). The serum ALT/SGPT levels were above the reference values in SAP, LBSap and Milt+LB groups (Table 1).

3.2. Production of anti-L. infantum total IgG

Significant decreases ($p < 0.05$) in the serum levels of anti-*Leishmania* total IgG were observed in all treated groups when compared to the INT control group (Fig. 1). Only Miltefosine, Milt.+LB and Milt.+LBSap groups were capable of reducing ($p < 0.05$) circulating IgG levels compared to LB and SAP groups (Fig. 1). Miltefosine, Milt.+LB and Milt.+LBSap groups showed lower levels of total IgG ($p < 0.05$) concerning the LBSap group (Fig. 1). Moreover, the Milt.+LB group elicited higher levels ($p < 0.05$) of total IgG compared to the Miltefosine group (Fig. 1).

3.3. TNF-α, IFN-γ and IL-10 profile by splenic lymphocytes

To evaluate the cytokine profile produced by splenic total lymphocytes and CD4 lymphocyte subtype, the intracytoplasmic synthesis of TNF-α, IFN-γ and IL-10 was assessed after *Leishmania* antigen-specific stimulus (Fig. 2). Regarding total lymphocytes, a significant increase was observed in the percentage of these cells producing TNF-α ($p < 0.05$) in all treated groups compared to the INT group (Fig. 2). Furthermore, we observed an increase ($p < 0.05$) of total lymphocytes producing IFN-γ in LBSap, Miltefosine, Milt.+LB and Milt.+LBSap groups in relation to the INT group and an increase ($p < 0.05$) in Milt.+LBSap group when compared to the Sap group (Fig. 2). All treated groups were capable to

Table 1
Hematology and Biochemical results of infected hamsters after treatment.

WBC (/mm^3)	Normal range	INT	LB	SAP	LBSap	Miltefosine	Milt.+LB	Milt.+LBSap
Leukocytes	1403 – 4855	3450±1217	3200±1316	3944±1030	3400±1263	3629±1806	3260±1544	4957±1621
Neutrophils	175 – 1034	901±588	840±356	927±404	672±220	1056±672	1181±771	1345±906
Eosinophils	10 – 48	45±16	35±17	42±13	41±22	34±9	39±30	48±14
Lymphocytes	989 – 3282	2283±792	2402±1070	2611±1005	2653±1041	2013±716	3063±1430	2187±821
Monocytes	17 – 45	35±11	40±27	35±15	54±24	53±38	40±20	89±40^{a,b,c,f}
Erythrogram								
Erythrocytes (10^6/mm^3)	2,4 - 7,6	5,0±1,4	7,6±0,6^a	6,9±0,6^a	7,8±0,7^a	7,4±1,3^a	6,9±1,6^a	7,2±1,2^a
Hemoglobin (g/dL)	6,8 – 14,8	11,4±2,6	14,7±1,3^a	14,6±1,6^a	14,8±1,7^a	15,8±2,1^a	14,5±3,6^a	16,3±1,7^a
Hematocrit (%)	15,2 – 44,4	35,0±9,8	41,8±2,8	39,2±6,0	41,2±5,5	42,7±7,3	40,7±3,5	43,4±6,3
Platelets (10^3/mm^3)	172,0 – 458,4	**155±82**	233±50	247±60	250,0±54	227±121	198±65	338±71
Renal Function								
Urea (mg/dL)	12,4 – 85,6	63,4±3,9	45,8±1,7^a	44,7±6,2^a	43,9±8,8^a	50,2±10,6^a	57,5±3,3^{a,b,c,d}	56,5±1,9^{a,b,c,d}
Creatinin (mg/dL)	0,2 – 0,5	0,42±0,12	0,49±0,03	0,48±0,05	0,36±0,14^{b,c}	0,11±0,08^{a,b,c,d}	0,22±0,07^{a,b,c,d}	0,26±0,07^{a,b,c,d}
Hepatic Function								
Serum Protein (g/dL)	6,0 - 9,7	8,5±1,2	6,9±0,6^a	7,0±1,2^a	7,7±0,9^b	7,7±0,5^b	7,1±0,6^a	7,1±0,5^{a,e}
AST/SGOT (U/L)	58,6 - 150	**177±58**	98±37^a	94±39^a	108±39^{a,b}	96±27^a	118±30^a	88±21^{a,f}
ALT/SGPT (U/L)	57,8 – 86,5	**96±28**	83±22	**99±37**	**94±21**	62±11^{a,b,c,d,f}	**92±28**	53±42^{a,c,d}

Significant differences ($P < 0.05$) are represented by the letters "a", "b", "c", "d", "e" and "f" related to INT, LB, Sap, LBSap, Miltefosine and Milt.+LB groups respectively. Data are shown as mean +/- standard deviation.

N=8/per group. For statistical analysis, the samples were processed and evaluated individually.

Red numbers represent values outside the reference values.

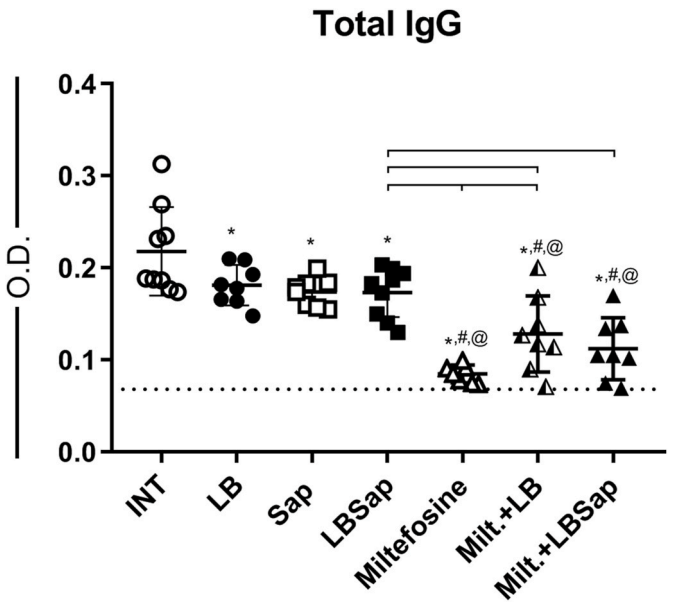

Fig. 1. Humoral response of hamsters infected by *Leishmania infantum* and submitted to immunotherapy, chemotherapy and immunochemotherapy. Analysis of IgG response in sera of hamsters intraperitoneal infected with 2×10^7 *Leishmania infantum* promastigotes in untreated (infected and non-treated - INT) or treated with immunotherapy with *Leishmania braziliensis* antigen (LB), saponin (Sap) and LBSap vaccine (LBSap); chemotherapy with Miltefosine (Miltefosine); or immunochemotherapy with Miltefosine with LB (Milt.+LB) and Miltefosine with LBSap vaccine (Milt.+LBSap) groups. n = 8/group. For statistical analysis, the samples were processed and evaluated individually. The *cut-off* value (dashed line) was calculated using the mean ± three times the standard deviation of all negative samples (uninfected and untreated animals). The results are expressed as mean ± standard deviation. Significant differences ($p < 0.05$) are shown by the "*", "#" and "@" representing differences related to INT, LB and SAP, respectively. Connecting lines—represent differences ($p < 0.05$) between the LBSap, Miltefosine, Milt.+LB and Milt.+LBSap groups.

increase ($p < 0.05$) the production of TNF-α by $CD4^+$ T cells when compared to INT group and only in LBSap and Milt.+LBSap groups there was an increase ($p < 0.05$) in the production of this cytokine when compared to the LB group (Fig. 2). Moreover, we observed an increase ($p < 0.05$) of $CD4^+TNF\text{-}\alpha^+$ lymphocytes in the LBSap group concerning to the Sap group (Fig. 2). Based on intracellular IFN-γ production by $CD4^+$ lymphocytes, we observed a significant increase ($p < 0.05$) in the Miltefosine, Milt.+LB and Milt.+LBSap groups when compared to INT group and an increase ($p < 0.05$) in Milt.+LB and Milt+LBSap groups concerning to the LB and Sap groups (Fig. 2). Further, we observed that the Miltefosine and Milt.+LB groups were capable of decreasing ($p < 0.05$) the production of IFN-γ by $CD4^+$ T cells when compared to LBSap and Milt.+LBSap groups (Fig. 2).

Regarding immunomodulatory cytokine IL-10, we observed a decreased production ($p < 0.05$) by total lymphocytes in all treated groups when compared to INT group (Fig. 2). Moreover, we observed a reduction of IL-10 ($p < 0.05$) in Milt.+LBSap groups in relation to Miltefosine and Milt.+LB groups and ($p < 0.05$) in Milt.+LB group when compared to Miltefosine group (Fig. 2). According to $CD4^+$ lymphocytes analysis, we observed lower percentage of $CD4^+IL\text{-}10^+$ cells ($p < 0.05$) in LB, LBSap and Milt.+LBSap groups when compared to INT group and a decrease ($p < 0.05$) of $CD4^+IL\text{-}10^+$ lymphocytes in Milt+LBSap group compared to LB and Sap groups (Fig. 2). Further, we observed a reduction ($p < 0.05$) of IL-10 production by $CD4^+$ T cells in LBSap and Milt.+LBSap groups in relation to Miltefosine and Milt.+LB groups and a decrease ($p < 0.05$) in Milt.+LBSap group when compared to LBSap group (Fig. 2).

3.4. Splenic parasite burden quantification

Total spleen DNA was used for quantification of amastigotes copies using qPCR. Our results demonstrated a low number ($p < 0.05$) of amastigotes in all treated groups when compared with the INT group (Fig. 3). A significant reduction ($p < 0.05$) of parasite load in the Miltefosine and Milt.+LBSap groups was observed compared to the LB group (Fig. 3). Moreover, we observed a decrease ($p < 0.05$) of parasite load in LBSap, Miltefosine and Milt.+LBSap groups in relation to Sap group (Fig. 3).

We also performed correlation analyses between the spleen parasite load and the intracytoplasmic cytokine production by total lymphocytes and $CD4^+$ cells presented in Table 2 and in Supplementary Fig. 2. Our data showed a positive correlation between spleen parasitism and production of IL-10 by total lymphocytes in INT ($p = 0.037$; Pearson's $r = 0.606$), LBSap ($p = 0.002$; Pearson's $r = 0.935$) and Milt.+LBSap ($p = 0.038$; Pearson's $r = 0.837$) groups (Table 2). Based on spleen parasite load and $CD4^+IL\text{-}10^+$, a positive correlation was observed in INT ($p = 0.026$; Pearson's $r = 0.730$), LBSap ($p = 0.001$; Pearson's $r = 0.987$) and Milt.+LBSap ($p = 0.024$; Pearson's $r = 0.870$) groups (Table 2).

4. Discussion

The restricted conventional therapeutic options available for the treatment of human VL (HVL) and canine VL (CVL) have several limitations such as serious side effects, high cost, and emergence of drug-resistant strains leading to treatment failure (WHO/Fairlamb et al., 2003). Besides, there is still no vaccine available for HVL and a limited number of vaccines for CVL. Moreover, only Milteforan™ is available for treatment of dogs in Brazil, which considerably makes it difficult to control the disease in the country. Therefore, it is essential to search for new therapeutic strategies. In this scenario, immunotherapy, and combined therapies (immunochemotherapy) are gaining prominence as a rational strategy for safe maintenance of the drugs already available across the resistance of parasites, restoration of immunity in non-responsive animals and therapeutic success faster and secure with reduced treatment time and fewer side effects (Joshi et al., 2014; Roatt et al., 2017; Viana et al., 2018). Based on this, we performed the evaluation of an immunochemotherapy protocol employing the association between miltefosine and LBSap vaccine which has been shown to promote a cellular immune response by splenic $CD4^+$ lymphocytes producing Th1 cytokines leading to a reduction of the parasitic load in the spleen of hamsters infected with *Leishmania infantum*.

It is known that hamsters *M. auratus* is highly susceptible to infection by viscerotropic *Leishmania* species (*L. donovani* and *L. infantum*) (Moreira et al., 2012; Loria-Cervera and Andrade-Narvaez, 2014). When experimentally infected with any of these parasite species, they present intense tissue parasitism in organs such as spleen, liver, and bone marrow. Also, the clinical signs, such as hepatosplenomegaly, pancytopenia and hypergammaglobulinemia are similar to HVL and CVL (Corbett and Laurenti, 1998; Eberhardt et al., 2016). In this work, it was demonstrated that untreated infected hamsters showed blood hemato-biochemical changes characteristic of human or canine disease. Moreira et al. (2016) showed that these changes first appear in blood and serum samples from infected hamsters with *Leishmania infantum* (OP46 strain). The altered hemato-biochemical parameters are also noticed in infected dogs, being restored after immunotherapy with the LBMPL vaccine (Roatt et al., 2017). Added to this, we found that treatment with Miltefosine (chemotherapy) or its association with LBSap vaccine (immunochemotherapy) showed a restoration of serum levels of liver enzymes ALT/SGPT and AST/SGOT, suggesting the safety of the treatment. Therefore, our data demonstrate that these hemato-biochemical alterations are only related to the infection, which was able to generate an immunopathological process, and not to the treatment, suggesting that the therapeutic regimens proposed here have low toxicity.

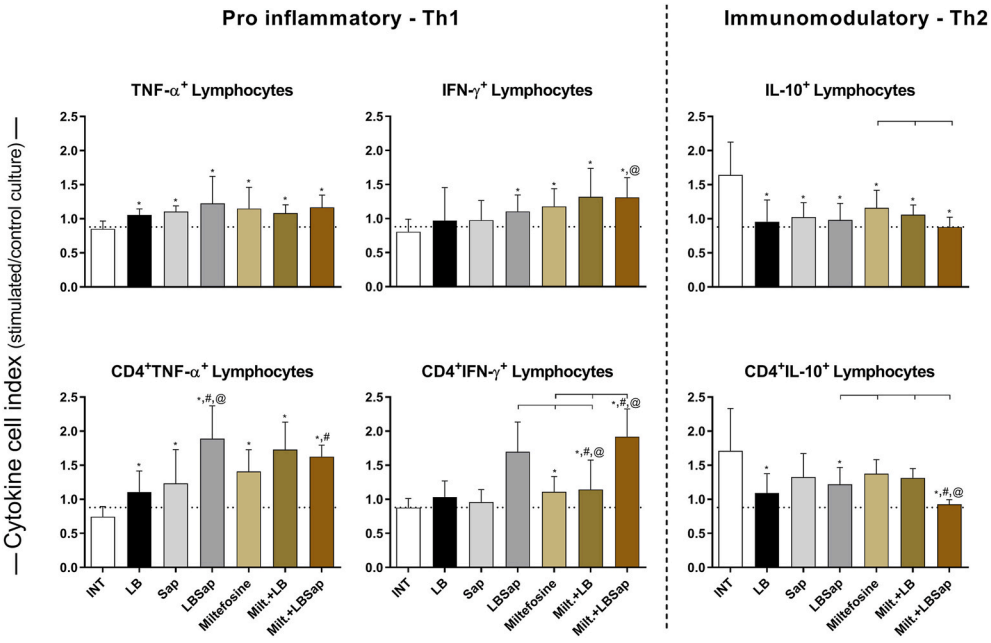

Fig. 2. Profile of intracytoplasmatic cytokines in splenocytes of hamsters infected by *Leishmania infantum* and submitted to immunotherapy, chemotherapy and immunochemotherapy. Analysis of intracellular pro inflammatory - Th1 (TNF-α, IFN-γ) and immunomodulatory - Th2 cytokines^{+} (IL-10) in total and CD4^{+} lymphocytes in spleen lymphocytes of hamsters intraperitoneal infected with 2×10^{7} *Leishmania infantum* promastigotes after *in vitro* stimulation with soluble *Leishmania infantum* antigens (SLiAg) in untreated (infected and non-treated - INT) or treated with immunotherapy with *L. braziliensis* antigen (LB), saponin (Sap) and LBSap vaccine (LBSap); chemotherapy with Miltefosine (Miltefosine); or immunochemotherapy with Miltefosine with LB (Milt.+LB) and Miltefosine with LBSap vaccine (Milt.+LBSap) groups. n = 8/group. For statistical analysis, the samples were processed and evaluated individually. The total and CD4^{+} lymphocytes cytokine indexes were calculated as the proportion of cytokine^{+} cells observed in SLiAg-stimulated cultures divided by the control culture (CC) (SLiAg/CC ratio). The dashed lines indicate the percentage of lymphocytes producing cytokines from uninfected and untreated animals. The results are expressed as mean ± standard deviation. Significant differences ($P < 0.05$) are shown by the "*", "#" and "@" representing differences related to INT, LB and SAP, respectively. Connecting lines—represent differences ($p < 0.05$) between the LBSap, Miltefosine, Milt.+LB and Milt.+LBSap groups.

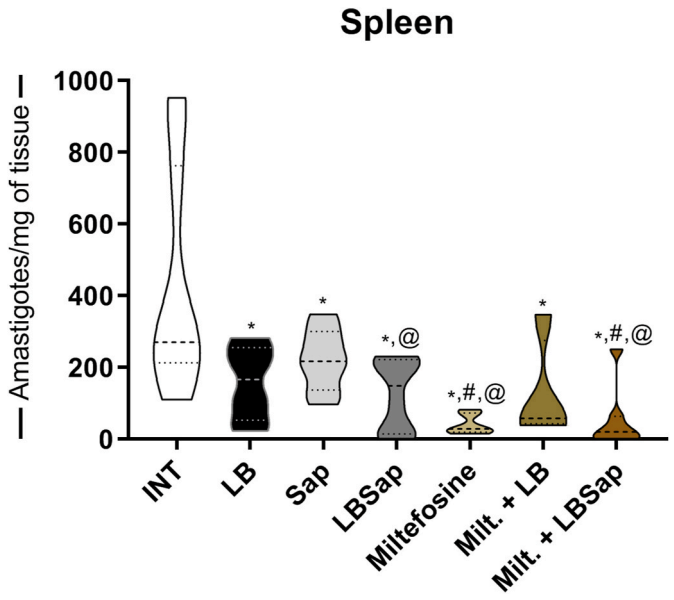

Fig. 3. Quantification of parasitism in the spleen of hamsters infected by *Leishmania infantum* and submitted to immunotherapy, chemotherapy and immunochemotherapy. Analysis of parasite burden in spleen of hamsters intraperitoneal infected with 2×10^{7} *Leishmania infantum* promastigotes in untreated (infected and non-treated - INT) or treated with immunotherapy with *Leishmania braziliensis* antigen (LB), saponin (Sap) and LBSap vaccine (LBSap); chemotherapy wit Miltefosine (Miltefosine); or immunochemotherapy with Miltefosine with LB (Milt.+LB) and Miltefosine with LBSap vaccine (Milt.+LBSap) groups. n = 8/group. For statistical analysis, the samples were processed and evaluated individually. The results are expressed as median ± standard deviation. Significant differences ($p < 0.05$) are shown by the "*", "#" and "@" representing differences related to INT, LB and SAP, respectively. Connecting lines—represent differences ($p < 0.05$) between the LBSap, Miltefosine, Milt.+LB and Milt.+LBSap groups.

Regarding to IgG immunoglobulin in serum samples from hamsters, it was observed that in the INT group 100% of the animals showed levels of specific *Leishmania*-IgG above the *cut-off*, thus confirming the establishment of infection. High serum IgG levels are considered biomarkers of active disease in dogs and hamsters (Reis et al., 2006; Moreira et al., 2012; Lopes et al., 2018). A recent study revealed that there is a prevalence of highly avidity antibodies without evolutionary maturation in serum from hamsters infected by *L. infantum*, affecting the progression of the disease (de Carvalho et al., 2020). It is known that the humoral response with antibody production is dependent on parasite persistence (Gonçalves et al., 2020). The reduction of levels of immunoglobulin in the serum of treated animals is probably related to the decrease of the parasitic load in the spleen of these animals observed in this work. Corroborating these data, a strong positive correlation was demonstrated between the spleen parasite load and IgG levels in the serum of hamsters infected with *L. infantum* (Carvalho et al., 2021), that is, the persistence of the parasite appears to be directly related to the presence of anti-*Leishmania* antibodies. In this sense, the induction of an immunogenic reaction, but with controlled production of anti-*Leishmania* IgG, is interesting for the establishment of a good post-treatment prognosis.

The cellular immune response with production of Th1 or Th2-type cytokines is intricately linked to the outcome of *Leishmania*-infection. In VL, the immune response compartmentalized directly influences the resistance or susceptibility to disease (Reis et al., 2009). Therefore, knowledge and understanding of the components of the cellular response after evaluation of treatment protocols is critical to determine the success of the therapy. However, in relation to the hamster model, there is a shortage of immunological reagents available, which makes it a challenge for the research community. Based on this, our research group has been working on standardizing an antibody panel to assess the immune response in hamsters (Carvalho et al., 2021). Thus, we evaluated by flow cytometry the production of intracytoplasmic cytokines IFN-γ, TNF-α and IL-10 by splenic lymphocytes after the treatment protocols. The immunochemotherapy (MILT+LBSap) proposed here was able to cause an increase mainly in CD4^{+}IFN-γ^{+}, with a stronger

Table 2
Correlation indexes between spleen parasitism and cytokine cell production.

Groups	Spleen Parasitism vs	*IFN-γ⁺ Lymphocytes*	*CD4⁺IFN-γ⁺*	*TNF-α⁺ Lymphocytes*	*CD4⁺TNF-α⁺*	*IL-10⁺ Lymphocytes*	*CD4⁺IL-10⁺*
INT	*r*	−0.145	−0.103	−0.460	0.058	***0.606***	***0.730***
	P	0.671	0.762	0.212	0.882	***0.037***	***0.026***
LBSap	*r*	−0.498	−0.586	−0.266	−0.487	***0.935***	***0.987***
	P	0.209	0.127	0.565	0.221	***0.002***	***0.001***
Miltefosine	*r*	0.563	0.076	0.480	0.496	0.102	0.678
	P	0.187	0.903	0.275	0.258	0.827	0.138
Milt.+LBSap	*r*	−0.410	−0.601	0.737	−0.589	***0.837***	***0.870***
	P	0.420	0.115	0.058	0.124	***0.038***	***0.024***

Numbers in bold represent correlation values presenting significant differences ($P < 0.05$).
For statistical analysis, the samples were processed and evaluated individually.

reduction in $CD4^+IL\text{-}10^+$ also regarding all other treated groups. Corroborating, it has been shown that hamsters treated with Miltefosine or its derivative showed increased Th1 response and reduced Th2 when quantifying cytokine expression by RT-PCR in the spleen of animals (Gupta et al., 2012; da Silva et al., 2020). Hence, the present work presents in an unprecedented way the evaluation of a more specific compartmentalized immune response. The cytokine production profile presented by treated animals probably contributed to the reduction of the parasitic load in the spleen compared to the INT or SAP group. These results indicate that the response profile of saponin-treated animals is similar to that observed by untreated animals demonstrating that the saponin adjuvant was not able to generate a specific immune response against the parasites. On the other hand, there was an even more marked reduction in the splenic parasitic load of the animals that received immunochemotherapy. The pro-inflammatory cytokines IFN-γ and TNF-α work synergistically acting to induce the production of nitric oxide, helping to eliminate the pathogen (Kaye et al., 2004). On the other hand, IL-10 is usually related to susceptibility to the disease (Reis et al., 2010). We found a high splenic parasitic burden in the INT group accompanied by higher frequencies of IL-10. When observing the animals treated with the LBSap vaccine or with Milt+LBSap, we have noticed that the spleen parasitism decreased while IL-10-lymphocytes and $CD4^+IL\text{-}10^+$ reduced. In fact, we have elucidated a strong positive correlation between interleukin-10 production and parasitic load, that is, there was no control of tissue parasitism in those animals whose IL-10 levels were high. Similarly, studies elucidated that spleen from hamsters infected presented a gradual increase in interleukin-10 (Melby et al., 1998; Moulik et al., 2021).

Collectively, our data demonstrated that the immunochemotherapy proposed (miltefosine plus LBSap vaccine) promotes enhancement of Th1-immune response with spleen parasitic burden control, even reducing by half the time of treatment with Miltefosine.

5. Conclusion

This study strengthens the fact that the hamster is an excellent model for conducting initial pre-clinical trials in research for new anti-*Leishmania* drugs, new therapeutic protocols (including immunotherapy and immunochemotherapy) or vaccine development. This occurs since these animals make easier pharmacokinetic and pharmacodynamic studies in addition to requiring a lower amount of drug and, especially, because they are a low-cost and easy-to-handle model (Balaña-Fouce et al., 2019). It is important to note that in this study we proposed an immunochemotherapeutic protocol with reduction of the time of the treatment with Miltefosine, which can contribute to reducing the cost of the treatment. Although, there is a need for additional studies to guarantee therapeutic efficacy in canine models. Therefore, it can be concluded that immunochemotherapy using the association between Miltefosine and LBSap vaccine is a promising proposal for the VL treatment. Especially when it deals with infectious diseases, immunochemotherapy has an increased synergistic effect, with activation of the immune response, thus strengthening the immune system concomitantly with direct action of the drug against the infectious agent.

Ethics statement

All animals used in this study were approved by the Ethical Committee for the use of Experimental Animals from UFOP (protocol CEUA ID number — 2016/57) and all procedures were performed according to recommendations of the Guide for Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA).

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CRediT authorship contribution statement

Lívia Mendes Carvalho: Writing – original draft, Methodology, Validation, Visualization, Data curation. **Francielle Carvalho Ferreira:** Investigation, Methodology. **Miriã Rodrigues Gusmão:** Methodology, Validation. **Ana Flávia Pereira Costa:** Methodology, Validation. **Rory Cristiane Fortes de Brito:** Methodology, Validation. **Rodrigo Dian de Oliveira Aguiar-Soares:** Visualization, Validation, Writing – review & editing. **Alexandre Barbosa Reis:** Writing – review & editing, Visualization, Funding acquisition. **Jamille Mirelle de Oliveira Cardoso:** Visualization, Validation, Writing – review & editing. **Cláudia Martins Carneiro:** Writing – review & editing, Visualization, Funding acquisition. **Bruno Mendes Roatt:** Writing – review & editing, Visualization, Funding acquisition, Supervision, Project administration.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2021.10.003.

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