

## Immunotherapy against murine experimental visceral leishmaniasis with the FML-vaccine

Wania Renata Santos<sup>a</sup>, Ivan Augusto Aguiar<sup>a</sup>, Edilma Paraguai de Souza<sup>a</sup>,  
Valéria M.F. de Lima<sup>b</sup>, Marcos Palatnik<sup>c</sup>, Clarisa Beatriz Palatnik-de-Sousa<sup>a,\*</sup>

<sup>a</sup> Instituto de Microbiologia, “Professor Paulo de Góes”, Universidade Federal do Rio de Janeiro (UFRJ), CCS, Cidade Universitária, Ilha do Fundão, CP 68040, CEP 21941-590, Rio de Janeiro, Brazil

<sup>b</sup> Curso de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, CEP 16050-680, Brazil

<sup>c</sup> Hospital Universitário Clementino Fraga Filho-Faculdade de Medicina UFRJ, Rio de Janeiro, Brazil

Received 25 March 2003; accepted 3 July 2003

### Abstract

The fucose mannose ligand (*Leishmania donovani* FML)-saponin vaccine has earlier shown its immunoprophylactic potential against visceral leishmaniasis in the CB hamster (87.7% of parasite load reduction), Balb/c (84.4%) and Swiss albino mouse (85–93%) models. In this investigation its specific immunotherapeutic efficacy against *L. donovani* infection in Balb/c mice was studied. The effects of vaccine treatment on the humoral response, delayed type of hypersensitivity to promastigote lysate (DTH), cytokine levels in sera and reduction of the liver parasitic load of *L. donovani* infected mice, were examined. The types and subtypes of anti-FML antibodies increased significantly in the vaccinees over the saline and saponin controls. As expected for a saponin vaccine, the highest ratios were found in relation to IgG1, IgG2a and IgG2b (4.4, 5 and 2.5, respectively). The DTH response and the in vitro ganglion cell proliferative response against FML antigen were also significantly higher than controls ( $P < 0.005$ ). Concomitantly, an impressive and specific decrease of liver parasitic burden was detected only in vaccine-treated animals (94.7%). Our results indicate that the therapeutic FML-vaccine has a potent effect on modulation of the murine infection leading to the reduction of parasitic load and signs of disease, being a new potential tool in the therapy and control of visceral leishmaniasis.

© 2003 Elsevier Ltd. All rights reserved.

**Keywords:** Visceral leishmaniasis; Kala-azar; FML-vaccine; Immunotherapy; Immunoprophylaxis; Saponin; Vaccination

### 1. Introduction

Human visceral leishmaniasis or kala-azar is a severe and frequently lethal infectious disease against which chemotherapy treatment is very toxic, show failing in 5–10% of cases [1]. In addition, chemotherapy demands hospitalization for 30 days. Alternatively, immunotherapy or immunochemotherapy were assayed against tegumentar human leishmaniasis, a less severe form of disease [2–4]. Effective immunotherapy or immunochemotherapy against canine visceral leishmaniasis would also be a good tool for control of dissemination of human disease. Indeed, zoonotic visceral leishmaniasis is a re-emerging disease that causes 500,000 new human cases all over the world. Dogs, foxes and wild canids are the reservoir for visceral leishmaniasis in the Mediterranean, Asia, North Africa and South Amer-

ica. The etiological agent (*Leishmania chagasi* or *Leishmania infantum*) is introduced into the domestic cycle when infected foxes visit houses to scavenge poultry. Peridomestic sand flies acquire the parasite by feeding on the foxes' skin and transmit it to dogs. The subsequent transmission to humans by sand flies causes human visceral leishmaniasis (reviewed in [5]). One of the tools used in epidemiological control of the disease is the sacrifice of seropositive infected dogs, since they expose the parasite to sandflies [5]. Despite the great number of investigations [6–9] no effective chemotherapy treatment against the canine disease is available [10–13]. Therapeutic failure has epidemiological implications since treated animals became infectious after treatment although they might remain asymptomatic [12]. Since infectivity to sandflies is highly correlated to the loss of an effective cellular immune response [14], the immunotherapy or combined immunochemotherapy treatment of infected dogs is an alternative that has begun to be explored [15].

In previous reports, we described the isolation of a *Leishmania donovani* promastigote glycoproteic complex

\* Corresponding author. Tel.: +55-21-256-26742; fax: +55-21-25608344/25608028.

E-mail address: [clarisaps@infolink.com.br](mailto:clarisaps@infolink.com.br) (C.B. Palatnik-de-Sousa).

named fucose mannose ligand (FML) since it contains the neutral sugars fucose and mannose and behaved as a ligand that strongly inhibits the *in vitro* infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [16,17]. This inhibition was species-specific for the genus *Leishmania* [18]. The FML antigen is present on the surface of the parasite throughout the life cycle [17] being a potent immunogen in rabbits and mice [17,19–21] and a sensitive, predictive and specific antigen in serodiagnosis of human [22] and canine kala-azar [23]. FML-electrophoretic (SDS-PAGE) analysis disclosed the presence of several proteic bands [16]. Two of them: 36 and 55 kDa, were also stained for carbohydrates [16]. Rabbit anti-FML hyperimmune serum, 22 IgG secreting hybridomas [17] and human kala-azar patients sera specifically reacted with the 36 kDa glycoprotein [24] that strongly protected Balb/c mice from *L. donovani* infection [25]. Its proteic moiety was cloned and expressed in *Escherichia coli* system as the nucleoside hydrolase of *L. donovani*, being strongly antigenic for *L. chagasi* infected dogs's sera [26]. A majority of acid and non-polar amino acid residues were detected in FML (16% glutamic acid, 12% glycine, 12% alanine) [16]. The analysis of FML sugar molar composition disclosed the presence of: ribose (16.8%), fucose (5.3%), xylose (12.6%), mannose (38.5%), galactose (3.3%) glucose (13.7%), *N*-acetyl glucosamine (7.4%) and NANA (0.2%) [27]. Two major FML *N*-linked oligosaccharides were identified. The major fraction corresponded to linear oligosaccharides of 4-*O* (42%), 3-*O* (22%) linked Man<sub>p</sub>, 2-*O* (3%) linked Fuc<sub>p</sub>, 2-*O* (tr) linked Man<sub>p</sub> in trace amounts and Gal<sub>p</sub> as terminal units. The second major fraction is a branched oligosaccharide composed of 4-*O* and 3-*O* Man<sub>p</sub> and 4-*O* linked GlcNac alternating units with GlcNac as a branching point and Gal<sub>p</sub>, Man<sub>p</sub> and Fuc<sub>p</sub> as terminal residues [27].

The FML antigen, although isolated from the *L. donovani* species, has an impressive antigenic and diagnostic potential on American human [22] and canine [23] visceral leishmaniasis caused by *L. chagasi* infection and on sera of humans or dogs infected with *L. infantum* in Spain (unpublished results). Coincidentally canine vaccination with FML induced a highly immunoprophylactic potential in dogs infected with either *L. donovani* [28] or *L. chagasi* [29,30].

The FML-vaccine was assayed in the mice and hamster models of visceral leishmaniasis, corresponding to Phase I–IIa trials [19–21,31]. Significant and specific protection was achieved in the Balb/c (87.7%;  $P < 0.01$ ) [19], Swiss Albino (85%,  $P < 0.025$ ) [20] and CB hamster 84% ( $P < 0.001$ ) [31] models, respectively. Vaccination with FML along with saponin was superior to aluminum hydroxide and Freund's incomplete adjuvant and had no toxic effect [20]. The reduction of parasitic load in the liver in response to each FML-vaccine formulation was: 52% ( $P < 0.025$ ) for BCG (*Mycobacterium bovis* Bacille Calmette Guérin-Moreau)-FML, 73% ( $P < 0.005$ ) for saponin R (Riedel De Haën)-FML, 93% ( $P < 0.005$ ) for QuilA-FML and 79.2% ( $P < 0.025$ ) for QS21-FML treated animals,

respectively [21]. Protection was specific for R-FML and QS21-FML while the QuilA saponin treatment itself induced 69% of leishman donovan units (LDU) reduction [21]. The FML-saponin vaccines (SAPF) promote significant, specific and strong protective effects against murine visceral leishmaniasis [19–21]. BCG-FML induced minor and non-specific protection while IL-12-FML, although enhancing the specific antibody and delayed type of hypersensitivity to promastigote lysate (DTH) response, failed to reduce the parasitic load of infected animals [21]. Recently, Phase III trials of efficacy of the FML-vaccine in dogs were performed in Brazil, an endemic area both for human and canine visceral leishmaniasis [29,30]. In the first trial, 2 years after vaccination, 92% of protection was achieved where only 8% of vaccinees showed mild signs of visceral leishmaniasis with no deaths, while 33% of controls developed clinical or fatal disease (76% of vaccine efficacy) [29]. In a second Phase III trial, using the FML antigen in formulation with QuilA saponin, 25% of the control animals and 5% of the vaccinees developed clinical and fatal disease until the end of the experiment (95% protection; 80% of vaccine efficacy) [30]. After 3.5 years of vaccination, no leishmanial DNA was detected in vaccinees while *Leishmania* infection was confirmed in saline controls. The FML-vaccine induced a significant, long-lasting and strong protective effect against canine visceral leishmaniasis in the field [29,30].

In the development and improvement of a vaccine formulation several parameters must be analyzed in a suitable animal model before the trials in the target individuals occur. For the analysis of the prophylactic potential of the FML-vaccine we used the mice [19] and the hamster [31] models to define the appropriate dosage, administration route [20,31] and best adjuvant formulation [20,21] being able to obtain robust data on safety and immunogenicity (Phase I) and on the protective potential against experimental infection (Phase IIa). The mice model has the advantage of giving clear results in 45 day's experiments while comparative experiments in dogs demand at least 1 year of follow-up. We passed from inbred models [19,31] to outbreed model (Swiss Albino mice) [20,21] in order to obtain results from a more variable heterogenic population, more closely related to the heterogenic target population of vaccine candidates (dogs). In the dog model, we first defined the saponin dosage (best adjuvant) [28]. According to the literature [32], and to the saponin manufacturer advise, we administered a 10-fold increased formulation for outbred dogs experimentally infected with *L. donovani* in our kennel. This 2 year assay represented a Phase I–IIa analysis in the target animal [28]. Further Phase III trials of the vaccine in the endemic area (400 dogs) confirmed the strong protective potential of the FML-vaccine on canine visceral leishmaniasis [29,30]. The experiments in mice, therefore, allowed us to make rapid and helpful inferences for the development of the canine formulation. Experiments in outbreed dogs take much longer periods and are more limiting, due mainly to economical and ethical reasons. All these

stages were needed in order to obtain enough information of the immunoprophylactic potential of the FML-vaccine.

In this investigation, we aimed to initiate the characterization of the possible immunotherapeutic effect of the FML-vaccine on experimental visceral leishmaniasis. For this preliminary approach we used the murine Balb/c model.

## 2. Material and methods

### 2.1. Immunotherapy with the FML-saponin vaccine in *L. donovani* infected mice

Female Balb/c mice (3-month old;  $n = 42$ ) obtained from the central animal care facilities, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, RJ, Brazil, were infected through the intravenous (i.v.) route with  $2 \times 10^8$  *L. donovani* (LD-1S/MHOM/SD/00-strain 1S) amastigotes obtained from hamster's spleens. Animals were further treated with three subcutaneous (s.c.) doses of 0.2 ml sterile saline solution containing 150  $\mu\text{g}$  of FML antigen combined and 100  $\mu\text{g}$  of Riedel De Haen saponin, Saponin pure (R) (8047-15-2) EINECE (West Germany), in the hind footpads, on day 15, 22 and 29 after infection. Each footpad received 0.1 ml of vaccine. Control animals received only saline or 100  $\mu\text{g}$  saponin. Isolation and chemical characterization of the fucose mannose ligand obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (LD-1S/MHOM/SD/00-strain 1S) was performed as previously described [16]. Briefly, promastigotes were submitted to an aqueous extraction followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on a Bio-Gel P-10 column yielding the FML glycoproteic complex in void volume [16]. After chemical characterization, the lyophilized FML was quantitated by dry weight and solubilized together with saponin in 0.9% NaCl saline solution. The protective response was evaluated by the analysis of: sera by the FML-enzyme-linked immunosorbent assay (ELISA) assay, the delayed type of hypersensitivity assay against leishmanial antigen, the specific lymphoproliferative in vitro ganglion cell response against the FML antigen and the serum interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-10 (IL-10) levels. The mice were sacrificed by etherization and their liver parasite loads were monitored in leishman donovan units of Stauber on Giemsa-stained imprints (LDU = number of amastigotes/1000 cell nuclei  $\times$  organ weight (mg)). The FML-vaccine is registered as a Patent: INPI number: P11100173-9 (18.3.97), Federal University of Rio de Janeiro, Brazil.

### 2.2. FML-ELISA assay

Mice's blood was collected from the caudal vein. The serum fraction was obtained after centrifugation, diluted in glycerol (1:1 (v/v)) and conserved at  $-20^\circ\text{C}$  until use. The

anti-FML antibody levels were assayed in animals' sera, at days 015 and 45 post infection using the FML-ELISA as previously described [22], with 2  $\mu\text{g}$  antigen per well and: goat anti-mouse IgG or anti-IgM peroxidase conjugate (Sigma) or goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 horseradish peroxidase conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) in a 1:4000 dilution in blocking buffer. The reactions were developed with *O*-phenyldiamine (Sigma), interrupted with 1N sulfuric acid, and monitored at 492 nm. Sera were collected as pools, titrated by double-blind tests, in triplicate. Positive and negative control sera were included in each test. Results are expressed the mean value of pools of two different experiments (seven mice per group in each experiment). Results are expressed as log<sub>2</sub> titers and according to conventional serology, titration differing in two or more dilutions is significant [20].

### 2.3. Delayed type hypersensitivity (intradermal reaction to promastigote lysate)

This was determined, 42 days after infection, by injecting mice with  $10^7$  freeze-thawed stationary phase promastigotes of *L. donovani* in 0.1 ml sterile saline solution, intradermally, in the right front footpad, measuring the footpad thickness with a Mitutoyo apparatus, both before and 24, 48 and 72 h after injection. Each animal was also injected with 0.1 ml sterile saline in the left front footpad for control. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. Previous experiments carried out in Balb/c mice and CB hamsters demonstrated that 24 h after inoculation saline treated footpads returned to base levels [31].

### 2.4. In vitro mouse draining lymph node cell proliferation against FML antigen

Forty days after infection, mice were sacrificed by etherization. To assess the specific proliferative in vitro response, popliteal draining lymph nodes were aseptically removed and disrupted in Hank's saline solution (Sigma) using a Petri dish and stainless steel mesh. The mononuclear cells were separated by centrifugation at  $400 \times g$  for 5 min at  $4^\circ\text{C}$ . The pellet was washed by centrifugation in order to remove erythrocytes and cellular debris and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 0.2 g/ml L-glutamine, 0.04 mM 2-mercaptoethanol and antibiotics (200 U/ml of penicillin and 200  $\mu\text{g}/\text{ml}$  of streptomycin). The cell suspension was distributed in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark), with each well containing  $10^6$  cells in a final volume of 100  $\mu\text{l}$ . RPMI supplemented medium was added as negative control. Concanavalin A (0.4  $\mu\text{g}$  per well) or  $10^7$  freeze-thawed *L. donovani* promastigotes per well or 4.4  $\mu\text{g}$  per well FML obtained by T cell blot, with its respective nitrocellulose control [25,33] were

added in triplicates as a stimulus for lymphocyte proliferative responses. Briefly, FML proteins were separated by SDS-PAGE gel, electrophoretically transferred to a nitrocellulose membrane, identified using Rosso Ponceau S (Carlo Erba, Italy) in acetic acid and excised. The nitrocellulose strips (NCP) were dried at room temperature for 12 h, fractionated in small pieces, solubilized with 2 ml dimethylsulfoxide (DMSO) (Sigma) for 2 h at room temperature and precipitated with 5 ml carbonate buffer (pH 9.6). The precipitated NCP were then washed five times in sterile PBS and stored frozen at  $-20^{\circ}\text{C}$  [25,33]. The protein content of FML was monitored using a calibration curve that correlates known concentrations of BSA to their SDS-PAGE densitometric profile [25]. Images were acquired using Adobe Photoshop and the NIH Image 1.58 programs for densitometric analysis. Nitrocellulose strips containing no antigen were eluted by the same protocol and used for control experiments. The mononuclear cells with the antigens were further incubated for 2 days at  $37^{\circ}\text{C}$  under a 5%  $\text{CO}_2$  atmosphere. Cell proliferation was monitored as described by Mossman [34]. Briefly,  $10\ \mu\text{l}$  of a 5 mg/ml solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) (Sigma) were added to each well and the plates further incubated for 4 h at  $37^{\circ}\text{C}$  under a  $\text{CO}_2$  atmosphere. Reaction was interrupted by the addition of  $100\ \mu\text{l}$  10% SDS in 0.04N HCl. Plates were further incubated at  $37^{\circ}\text{C}$  for 17 h in the dark and absorbency reading was performed in an Elisa BioRad Microplate Reader Model 550 at 570 nm.

### 2.5. *IFN- $\gamma$* and interleukin-10 measurement in mouse sera

The *interferon- $\gamma$*  and interleukin-10 levels in serum were measured by enzyme-linked immunosorbent assay (ELISA). Capture and biotinylated monoclonal antibodies for *interferon- $\gamma$*  (R4-6A2, XMG1.2) and for IL-10 (JES5-2<sup>A</sup>5, JES5-16E3) and the recombinant cytokines were purchased from Pharmingen (San Diego, CA, USA). Levels of cytokines were determined following manufacturer's instructions (Pharmingen). Briefly, anti-cytokine capture antibody was diluted (1  $\mu\text{g}/\text{ml}$ ) in binding solution, incubated overnight at  $4^{\circ}\text{C}$  on Nunc maxisorb immunoplates, washed, blocked and further incubated with sera samples diluted in glycerol (1:1) for 2 h, at room temperature. The biotinylated anti-cytokine detection antibody (1  $\mu\text{g}/\text{ml}$ ) diluted in blocking buffer, was added and incubated for 1 h at room temperature. Development of the reaction was performed with streptavidin-horseradish peroxidase (Dako) and *O*-phenyldiamine (Sigma) and stopped with sulfuric acid 1N. Reading of the optical density was done in a microplate reader set to 492 nm. A standard curve using preparations with known concentration of mouse recombinant r-*IFN- $\gamma$*  and IL-10 was performed for each assay. The detection limit was 15 pg per ml for both cytokines.

### 2.6. Statistical analysis

Means were compared by a standard *t*-test, ANOVA analysis, simple factorial test and by one way ANOVA, Student–Newman–Keuls method (SPSS for windows). Correlation coefficient analysis was determined on a Pearson bivariate, two tailed test of significance (SPSS for windows).

## 3. Results

### 3.1. Immunotherapy with the FML-saponin vaccine in *L. donovani* infected mice

In order to examine the possibility of controlling visceral leishmaniasis in the already infected animal we used the mouse model in which we have already demonstrated the induction of a prophylactic response after FML-saponin vaccination. Mice were infected with  $2 \times 10^8$  *L. donovani* amastigotes and treated with three weekly doses of the FML antigen of *L. donovani* and Riedel De Haen saponin, starting by day 15 after infection. Before vaccination (day 15), the anti-FML antibody levels in sera of infected mice were not significantly increased, in any type or subtype of immunoglobulins (not shown). On the other hand, pronounced increase in anti-FML IgG, IgG1, IgG2a, IgG2b and IgG3 antibody was achieved in vaccine treated Balb/c mice, 45 days after infection (Fig. 1A). A discrete IgM response was detected, as expected for a secondary antibody response in the chronic visceral leishmaniasis. The anti-FML antibody titers were significantly and specifically enhanced in animals treated with the FML-vaccine, over their saline and saponin controls, in all types and subtypes of immunoglobulines. As expected for FML-saponin treated group, the highest responses were observed for the IgG1, IgG2a and IgG2b subtypes (4.4, 5 and 2.5 ratio to saline, respectively) (Fig. 1A). The intradermal response to the promastigote antigen of *L. donovani* was also significantly higher in FML treated animals than in saline or saponin controls ( $P < 0.005$ ) at all tested times (24–72 h) (Fig. 1B). Maximal swelling was achieved 72 h after antigen injection characterizing a delayed type of hypersensitivity. The increase in IgG, IgM, IgG1, IgG2a and IgG2b anti-FML was highly correlated to the increase of intradermal reaction ( $P = 0.039$ , at 24 h and 48 h) and revealed the high specificity of the FML-saponin vaccine formulation.

We tested the hypothesis that the therapeutic FML-vaccine altered the T cell responses induced by infection leading to the reduction of parasitic load and signs of disease. We examined the in vitro ganglion cell proliferative response against the FML antigen, obtained by T cell blot (Fig. 2A) and the levels of the TH1 cytokine *IFN- $\gamma$*  and the TH2 cytokine IL-10. A higher proliferative response was detected in FML treated animals, over their saline controls ( $P < 0.005$ ) on day 45 post infection (p.i.). Control animals treated with saponin only showed however, a non-specific increase

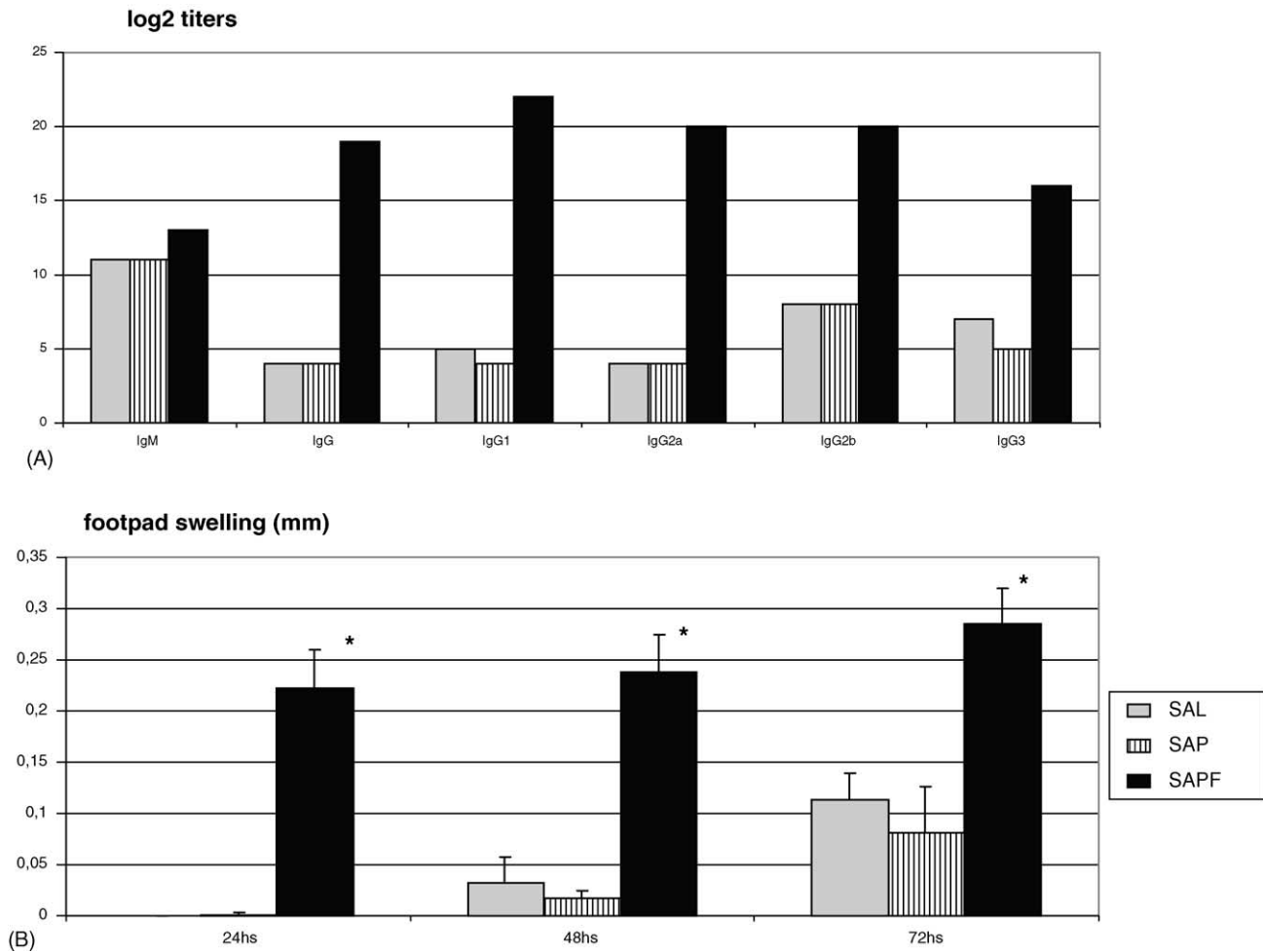


Fig. 1. (A) Anti-FML IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies in animals infected with *L. donovani* and treated with the FML-saponin vaccine (SAPF). Control animals received only saline (SAL) or saponin (SAP), as indicated. The y-axis represents the mean of the FML-ELISA log<sub>2</sub> titers of two pools of sera obtained from each treatment ( $n = 7$ , two experiments), 45 days after infection with  $10^8$  amastigotes of *L. donovani*, by the intravenous route. Titration differing in two or more dilutions is significant. (B) Delayed type hypersensitivity of infected and vaccine-treated animals, on day 42 after infection. The y-axis represents the thickness of skin reaction in mm. From left to right: the intradermal reaction at 24, 48 and 72 h after injection with  $10^7$  freeze-thawed stationary phase promastigotes of *L. donovani*, ( $n = 7$ , two experiments). At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. T-bars represent the standard deviation. The symbol asterisks (\*) represent statistically significant differences compared to saline controls ( $P < 0.005$ ).

over saline controls as well (Fig. 2A). Although, the IFN- $\gamma$  level in serum was increased after 15 days of infection, no significant differences were observed between saline, saponin or FML-saponin treatments on day 45 p.i. (Fig. 2B). On the other hand, the examination of the IL-10 cytokine profile provided a different picture. Mice vaccinated with FML-saponin showed a significant reduction ( $P < 0.005$ ) in IL-10 levels present in infected untreated controls, both at 15 or 45 days after infection (Fig. 2B).

Finally, a noteworthy reduction of liver parasitic load was observed in animals treated with the FML-vaccine (94.7%) when compared to saline ( $P < 0.05$ ) and saponin ( $P < 0.025$ ) controls (Fig. 3). Our results showed that immunotherapy treatment was highly specific, since saponin or saline treated controls showed no significant difference in LDU levels. The LDU decrease is positively correlated

with the decrease in IL-10 ( $P = 0.0001$ ) and negatively correlated to the enhancement of in vitro ganglion proliferative response ( $P = 0.0001$ ). Taken together, the effect on humoral and cellular immune responses, and the reduction in parasitological signs of disease, the FML-saponin vaccine induced a significant, specific and strong immunotherapy effect against murine visceral leishmaniasis in the experimental model of infection with *L. donovani*.

#### 4. Discussion

Chemotherapy is the first line of treatment against visceral leishmaniasis. It includes glucamine antimoniate and Amphotericin B or Pentamidine, which are used in case of resistance or recurrent infections [35,36]. In Brazil,

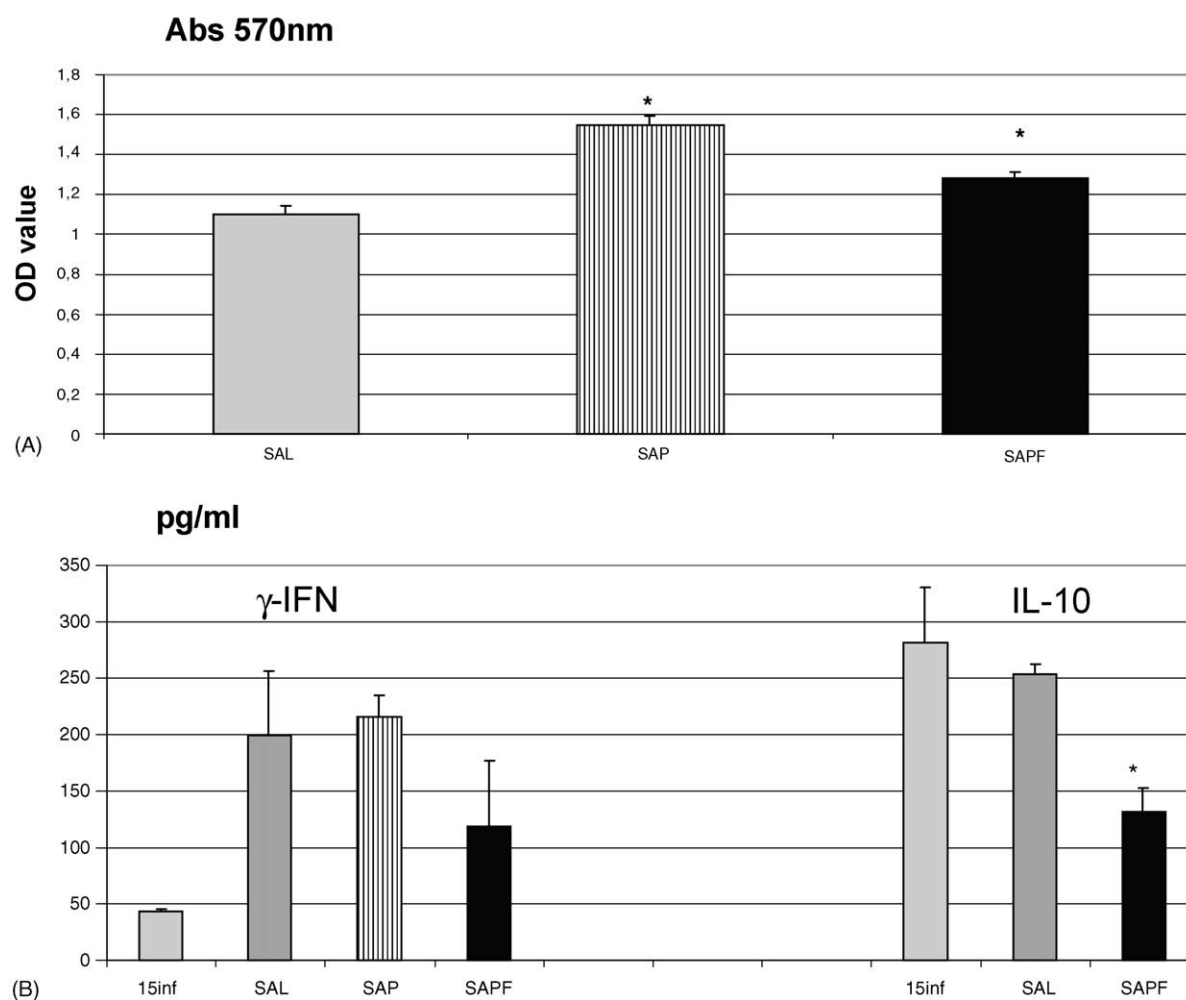


Fig. 2. (A) Proliferative response of ganglion lymphocytes against FML antigen of mice infected and later treated with the FML-vaccine. This evaluation was performed in saponin-FML treated animals and controls, on day 45 after infection. Triplicates of  $10^6$  cells, in RPMI supplemented medium, were treated with  $4.4 \mu\text{g}$  of FML antigen and incubated for 48h at  $37^\circ\text{C}$  under a 5%  $\text{CO}_2$  atmosphere. Cell proliferation was monitored as described by the MTT method. T-bars represent the standard deviation. The y-axis represents the absorbancy readings at 570 nm. The symbol asterisks (\*) represent statistically significant differences compared to saline controls ( $P < 0.005$ ). (B) Analysis of interferon- $\gamma$  and IL-10 levels in sera of infected (15inf) and further vaccine treated and control animals. The y-axis represents the levels of cytokines, detected by a specific ELISA assay, expressed in pg/ml ( $n = 7$ ).

20 mg/kg per day glucamine antimoniate are administered during 20–40 days through the i.v. route. Therefore, human kala-azar treatment demands hospitalization and shows many undesired effects such as: myalgia, arthralgia, nausea, fever, headache and acute renal failure. Eventually, leukopenia, hematuria, cardiac arrhythmias, pancreatitis and kidney lesions might occur. Besides that, parasites resistant to the usual chemotherapy have already been described [36–38]. A large number of drugs have been tested for their therapeutic potential against leishmaniasis: Amphotericin B-containing liposomes, allopurinol, IFN- $\gamma$ , paromomycin [39,40] and recently, miltefosine. Additionally, several medicinal natural plant products are being assayed: terpenes, alkaloids, acetogenins, chalcones, lactones, coumarines, tetralones and saponins (reviewed in [41]).

Another approach investigated exclusively in the treatment of tegumentar leishmaniasis, is the use of thera-

peutic vaccines. In Venezuela, Convit et al. [2] compared immunotherapy with *L. (L.) mexicana* promastigote lysate + BCG versus chemotherapy with glucantime, on patients with localized cutaneous leishmaniasis. Similar cure ratios were detected in both treated groups (94%) [2]. In Brazil, Mayrink et al. [3], using a prophylactic vaccine composed of five stocks of *Leishmania* (Leishvacin) developed an immunotherapy treatment on 62 patients. Among them, 47 (76%) were considered clinically cured: 41 required 2–10 treatment courses and another six 11–19 courses. None of the patients treated with immunotherapy developed any side effect [3]. Immunotherapy proved then to be effective in the treatment of single cutaneous lesions, multiple cutaneous lesions and in cases of mucocutaneous leishmaniasis. In comparison with glucantime, immunotherapy was less efficient and more prolonged but can be safely used when antimonials are contra-indicated

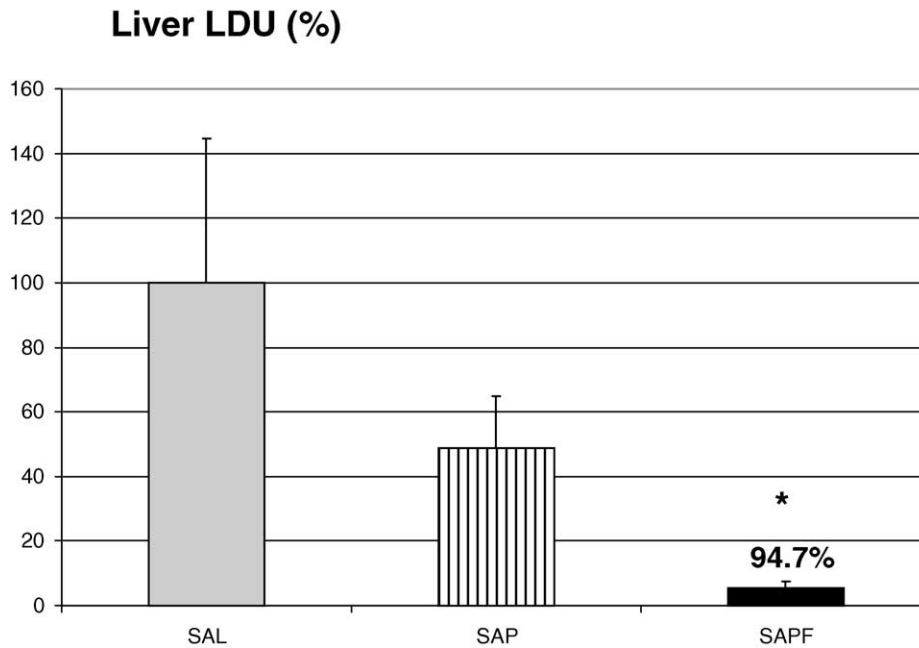


Fig. 3. Liver parasitic burden in mice infected with *L. donovani* and further treated with the FML-saponin vaccine (SAPF). Control animals received only saline (SAL) or adjuvant (SAP) as indicated. The y-axis represents average liver parasitic load in percent of leishman donovan units (LDU= number of amastigotes/1000 cell nuclei  $\times$  organ weight (mg)) of seven animals for each treatment (two experiments), 45 days after infection with  $10^8$  amastigotes of *L. donovani* and 15 days after complete vaccination. T-bars represent the standard deviation. The symbol asterisks (\*) represents the statistical significance compared to the saline control ( $P < 0.05$ ).

or found ineffective [3]. Recently, chemotherapy was compared to immunochemotherapy using the same vaccine plus pentavalent ammonium salts [4]. Although no differences in time of cure were detected between the two treatments, patients submitted to immunochemotherapy ( $n = 50$ ) showed higher lymphoproliferative responses, decreased IL-10 and IFN- $\gamma$  secretion associated with healing with no increase in CD8+ cells, indicating lower toxicity [4]. In a larger experiment performed on 350 patients, immunotherapy with Leishvacin was a good alternative for tegumentar leishmaniasis treatment with high cure ratio (98.2%). Antimony combined with the vaccine (immunochemotherapy) showed the same cure ratio as the standard antimony treatment (100%) and significantly reduced the salt volume used for treatment (17.9%) and the time for cure, from 87 to 62 days; reducing consequently the undesirable side effects (Dr Mayrink's personnel communication). Furthermore, using the Balb/c murine model for tegumentar leishmaniasis, Handman et al. [42] showed the therapeutic potential of the PSDA-2 DNA vaccine, against the Balb/c experimental infection by *L. major*, in resistant and susceptible mice. The use of the vaccine promoted reduction in lesion size at week 5 after infection and a shift in the existing disease-promoting Th2 cytokine profile towards a host-protective Th1, even in genetically susceptible mice [42].

All the above mentioned vaccine formulations showed, however, their potential against the tegumentar form of leishmaniasis which is considered, within the broad spectrum of the *Leishmania* infections, the mildest form of disease.

Visceral leishmaniasis on the other hand, involves severe immunosuppression making more difficult the control of the disease after the onset of infection. In the hamster model, Mukhopadhyay et al. [43] demonstrated that the UR6-*L. donovani* strain, a mutant which lacks LPG, expresses KMP-11 and is unable to infect hamsters when injected by the intracardiac route, develops an immunotherapeutic and immunoprophylactic effect against experimental visceral leishmaniasis. Recently, the efficacy of immunochemotherapy against *L. infantum* natural dog infection was examined [15]. Animals received 21 subcutaneous doses of glucan-time (100 mg/kg) and three applications of the soluble fraction released from *L. infantum* promastigotes after freeze and thaw-sonication treatment. Protection was evident in: the reduction of infectivity for *Phlebotomus perniciosus*, and the maintenance of CD4/TcR $\alpha\beta$  and CD4/CD45RA $^+$  lymphocyte normal levels which were expected to be reduced during canine kala-azar [14,15,44,45]. The lymph node aspirates however, remained positive in 3/5 dogs and relapse of clinical symptoms were also detected [15].

The question addressed in this investigation was whether FML-saponin vaccination, which induces an exclusively protective response when administered prophylactically in murine [19–21] and canine [29,30,46] models, can switch the immunosuppressive response in the infected Balb/c mice into a protective response, avoiding or reducing the disease manifestations. Although the Th1/Th2 dichotomy is not so clearly defined in the murine model of visceral leishmaniasis [33], the parasite-specific proliferation of

CD4+ lymphocytes with secretion of IFN- $\gamma$  and low levels of IL-10 was demonstrated in *L. chagasi* resistant mice, while CD4+ cells produce IFN- $\gamma$ , IL-4 and high levels of IL-10, in mouse strains susceptible to infection [47] such as Balb/c [48]. In addition, a mixed Th1/Th2 response with their typical cytokines is expected in animals treated with saponin adjuvants [49]. In this investigation then, high levels of IL-10 were expected, both because maximal parasitic burden in liver is expected at day 15 after infection, and also because a saponin treatment was performed later. In spite of that, the therapeutic effect of the FML-vaccine led to a strong reduction in IL-10 levels in sera as well as an impressive specific and protective antibody response (IgG2a and IgG2b) and an intradermal reaction to promastigote lysate which correlated with the pronounced reduction in parasitic load. Indeed, the levels of protection achieved in this variable are in agreement with those developed by the prophylactic vaccination in the CB hamster [31], Balb/c [19] and Swiss Albino models [20,21] using different saponins. Preliminary results also indicate that the immunotherapy treatment with FML-QuilA saponin vaccine on dogs experimentally infected with *L. donovani* amastigotes induces protection against visceral leishmaniasis, if the treatment is performed when infection is subclinical in asymptomatic animals [50].

This study represents the first Phase I–IIa trial of the immunotherapeutic potential of the FML-vaccine in a susceptible murine model of infection. We were able to describe the promising potential of the FML-saponin formulation towards the development of a host-protective alternative treatment of visceral leishmaniasis.

## Acknowledgements

This study received financial support from: Brazilian National Council for Scientific and Technological Development (CNPQ); Coordination for the Improvement of Higher Education Personnel (CAPES); MCT/PRONEX (Brazilian Ministry of Science and Technology); Rio de Janeiro State Research Foundation (FAPERJ) and Council for Graduate Studies-UFRJ (CEPG-UFRJ), Brazil. English language editing was done by Dr Jack Woodall, Federal University of Rio de Janeiro.

## References

- [1] Schmunis GA, López Antuñano FJ. World-wide importance of parasites. In: Colier L, Balows A, Sussman M, editors. *Microbial Infections*. Topley & Wilson; 1997. p. 19–38.
- [2] Convit J, Castellano PL, Rondon A, Penardi ME, Ulrich M, Castes M, et al. Immunotherapy versus chemotherapy in localized cutaneous leishmaniasis. *Lancet* 1987;1:401–14.
- [3] Mayrink W, Magalhães PA, Michalick MSM, Costa CA, Lima AO, Melo MN, et al. Immunotherapy as treatment of American cutaneous leishmaniasis: preliminary studies in Brazil. *Parasitologia* 1992;34:159–65.
- [4] Toledo VP, Mayrink W, Gollob KJ, Oliveira MA, Costa CA, Genaro O, et al. Immunotherapy of American cutaneous leishmaniasis: immunological aspects before and after treatment. *Mem Inst Oswaldo Cruz* 2002;96:89–98.
- [5] Tesh R. Control of zoonotic visceral leishmaniasis. Is it time to change strategies. *Am J Trop Med Hyg* 1995;52:287–92.
- [6] Lanotte G, Rioux JÁ, Pereires J, Völlhardt Y. Ecologie des leishmanioses dans le sud de la France. 10. Les formes évolutives de la leishmaniose viscérale canine. Elaboration d'une typologie biologique à finalité épidémiologique. *Ann Parasitologie* 1979;54:277–95.
- [7] Reiter IV, Kretzschmar A, Boch J, Krampitz H. Zur leishmaniose des hundes. Infektionsverlauf, diagnose un therapieversuche nach exp. Infection vom Beagles mit *Leishmania donovani* (st. Kalkutta). *Berl Münch Tierärztl Wschr* 1985;98:40–4.
- [8] Gradoni L, Maroli M, Gramiccia M, Mancianti F. Leishmania infantum infection rates in *Phlebotomus perniciosus* fed on naturally infected dogs under antimonial treatment. *Med Vet Entomol* 1987;1:339–42.
- [9] Mancianti M, Gramiccia M, Gradoni L, Pieri S. Studies on canine leishmaniasis control. I Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. *Trans R Soc Soc Trop Med Hyg* 1988;82:566–7.
- [10] Ranque J, Ranque M, Cabassu J, Cabassu H. Le diagnostic précoce de la leishmaniose canine par la ponction ganglionnaire. Reflexions à propos de soixante examens positifs obtenus en dix mois dans la région Marseillaise. *Bull Acad Nat Med* 1948;132:339–40.
- [11] Marzochi MCA, Coutinho SG, Souza WJS, Toledo LM, Grimaldi Jr G, Momen H, et al. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977–1983). *Mem Inst Osw Cruz* 1985;80:349–57.
- [12] Alvar J, Molina R, San Andrés M, Tesouro M, Nieto J, Vitutia M, et al. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. *Ann Trop Med Parasitol* 1994;88:371–8.
- [13] Oliva G, Gradoni L, Cortese L, Orsini S, Ciaramella P, Scalone A, et al. Comparative efficacy of meglumine antimoniate and aminosidine sulphate, alone or in combination, in canine leishmaniasis. *Ann Trop Med Parasitol* 1998;92:165–71.
- [14] Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribanez MA, Alvar J, et al. Canine leishmaniasis transmission: higher infectivity among naturally infected dogs to sandflies is associated with lower proportions of T helper cells. *Res Vet Sci* 2000;69:249–53.
- [15] Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribañez MA, Castillo JÁ. Evaluation of a specific immunochemistry for the treatment of canine visceral leishmaniasis. *Vet Immunol Immunopathol* 2002;88:13–20.
- [16] Palatnik CB, Borojevic R, Previato JO, Mendonça-Previato L. Inhibition of *Leishmania donovani* promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate. *Infect Immun* 1989;57:754–63.
- [17] Palatnik de Sousa CB, Dutra HS, Borojevic R. *Leishmania donovani* surface glycoconjugate GP36 is the major immunogen component of the fucose-mannose ligand (FML). *Acta Trop* 1993;53:59–72.
- [18] Palatnik CB, Previato JO, Mendonça-Previato L, Borojevic R. A new approach to phylogeny of *Leishmania*: species-specificity of glycoconjugate ligands for promastigote internalization into murine macrophages. *Parasitol Res* 1990;76:289–93.
- [19] Palatnik de Sousa CB, Paraguai de Souza E, Gomes EM, Borojevic R. Experimental murine *Leishmania donovani* infection: immunoprotection by the fucose mannose ligand (FML). *Braz J Med Biol Res* 1994;27:547–51.
- [20] Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination with the FML antigen (fucose mannose ligand) of *Leishmania donovani* in the Swiss Albino model. *Vaccine* 1999;17:2554–61.

- [21] Santos WR, de Lima VMF, Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Saponins, IL12 and BCG adjuvant in the FML-vaccine formulation against murine visceral leishmaniasis. *Vaccine* 2002;21:30–43.
- [22] Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Luz K, Palatnik M, Borojevic R. *Leishmania donovani*: titration of antibodies to the fucose mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. *Trans Roy Soc Trop Med Hyg* 1995;89:390–3.
- [23] Borja Cabrera GP, da Silva VO, da Costa RT, Barbosa Reis A, Mayrink W, Genaro O, et al. The FML-ELISA assay in diagnosis and prognosis of canine visceral leishmaniasis. *Am J Trop Med Hyg* 1999;61:296–301.
- [24] Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Santos WR, Macedo SR, Medeiros LV, et al. The FML (fucose mannose ligand) of *Leishmania donovani*. A new tool in diagnosis, prognosis, transfusional control and vaccination against human Kala-azar. *Rev Soc Bras Med Trop* 1996;29:153–63.
- [25] Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*. *Vaccine* 2001;19(23–24):3104–15.
- [26] Santana DM, Paraguai de Souza E, Borja-Cabrera GP, Sturm NR, Palatnik de Sousa CB, Campbell DA. Nucleoside hydrolase from *Leishmania (L.) donovani* is an antigen diagnostic for visceral leishmaniasis. *Mol Biochem Parasitol* 2002;120:315–9.
- [27] Bernardo RR, Palatnik de Sousa CB, Parente JP. N-linked oligosaccharide structures of the FML antigen of *Leishmania (L.) donovani*. *Mem Inst Oswaldo Cruz* 1998;93(SII):179.
- [28] Borja-Cabrera GP. Análise do potencial diagnóstico, prognóstico e imunoprotetor do antígeno FML (Ligante de Fucose Manose) de *Leishmania (L.) donovani*, no calazar canino experimental e de área endêmica. PhD Thesis. Department of Experimental Pathology. Federal Fluminense University; 2000. p. 81–98.
- [29] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, Paraguai de Souza E, Luz KG, Palatnik M, et al. A Phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amarante, RN). *Vaccine* 2001;19:1068–81.
- [30] Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante, RN). *Vaccine* 2002;20(27–28):3277–84.
- [31] Palatnik de Sousa CB, Moreno MB, Paraguai de Souza E, Borojevic R. The FML vaccine (fucose-mannose ligand) protects hamsters from experimental Kala-Azar. *Ciência e Cultura (J Braz Assoc Adv Sci)* 1994;46:290–6.
- [32] Schettters TPM, Kleuskens J, Scholtes N, Bos HJ. Vaccination of dogs against *Babesia canis* infection using parasite antigens from in vitro culture. *Parasite Immunol* 1992;14:295–305.
- [33] Kaye PM, Curry AJ, Blackwell JM. Differential production of Th1 and Th2 derived cytokines does not determine the genetically controlled or vaccinated induced rate of cure in murine visceral leishmaniasis. *J Immunol* 1991;146:2763–70.
- [34] Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
- [35] WHO. Control of the leishmaniases. World Health Organization—Technical Report Series 793; 1990.
- [36] Olliaro PL, Bryceson ADM. Practical progress and new drugs for changing patterns of Leishmaniasis. *Parasitol Today* 1993;9:323–8.
- [37] Pal S, Mandal A, Duttgupta S. Studies on stibionate resistant *Leishmania donovani* isolate of Indian origin. *Indian J Exp Biol* 2001;39:249–54.
- [38] Sundar S. Drug resistance in Indian visceral leishmaniasis. *Trop Med Int Health* 2001;6:849–54.
- [39] Koff AB, Rosen T. Treatment of cutaneous leishmaniasis. *J Am Acad Dermatol* 1994;31:693–708.
- [40] Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Inf Dis* 1997;24:684–703.
- [41] Akendengue B, Ngou-Milama E, Laurens A, Hocquemiller R. Recent advances in the fight against leishmaniasis with natural products. *Parasite* 1999;6:3–8.
- [42] Handman E, Noormohammadi AH, Curtis JM, Baldwin T, Sjölander A. Therapy of murine cutaneous leishmaniasis by DNA vaccination. *Vaccine* 2000;18:3011–7.
- [43] Mukhopadhyay S, Sem P, Bhattacharyya S, Majumdar S, Roy S. Immunoprophylaxis and immunotherapy against experimental visceral leishmaniasis. *Vaccine* 1999;17:291–300.
- [44] Bourdoiseau G, Bonnefont C, Hoareau E, Boehringer C, Stolle T, Chabanne L. Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. *Vet Immunopathol* 1997;59:21–30.
- [45] Moreno J, Nieto J, Chamizo C, Gonzalez F, Blanco F, Barker F, et al. The immune response and PBMC subsets in canine visceral leishmaniasis before and after chemotherapy. *Vet Immunopathol* 1999;30:181–95.
- [46] Mendes CO, Paraguai de Souza E, Borja-Cabrera GP, Melo Batista LM, Santos MA, Parra LE, et al. IgG1/IgG2 antibody dichotomy in sera of vaccinated or naturally infected dogs with visceral leishmaniasis. *Vaccine* 2003;21:2589–97.
- [47] Gomes NA, Barreto-de-Souza V, Dos Reis GA. Early in vitro priming of distinct Th cell subsets determines polarized growth of visceralizing leishmania in macrophages. *Int Immunol* 2000;12:1227–33.
- [48] Bradley DJ. Genetics of susceptibility and host resistance in the vertebrate host. In: *The Leishmaniasis*, vol. 2. London: Academic Press; 1987. p. 551–81.
- [49] Marciani DJ, Pathak AK, Reynolds RC, Seitz L, May RD. Altered immunomodulating and toxicological properties of degraded Quillaja saponaria Molina saponins. *Int Immunopharmacol* 2001;1:813–8.
- [50] Cruz Mendes A, Borja Cabrera GP, Paraguai de Souza E, Santos WR, Gomes EM, Palatnik de Sousa CB. Immunotherapy against canine kala-azar with the FML antigen of *L. (L.) donovani* and the QuilA saponin. In: *Proceedings of Fifthe Meeting on Applied Research on Leishmaniasis (Abs.)*. Uberaba, MG, Brasil; 2001. p. 80–81.