



## Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis

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### ABSTRACT

Four new antigenic proteins located in *Leishmania* ribosomes have been characterized: S4, S6, L3 and L5. Recombinant versions of the four ribosomal proteins from *Leishmania major* were recognized by sera from human and canine patients suffering different clinical forms of leishmaniasis. The prophylactic properties of these proteins were first studied in the experimental model of cutaneous leishmaniasis caused by *L. major* inoculation into BALB/c mice. The administration of two of them, LmL3 or LmL5 combined with CpG-oligodeoxynucleotides (CpG-ODN) was able to protect BALB/c mice against *L. major* infection. Vaccinated mice showed smaller lesions and parasite burden compared to mice inoculated with vaccine diluent or vaccine adjuvant. Protection was correlated with an antigen-specific increased production of IFN- $\gamma$  paralleled by a decrease of the antigen-specific IL-10 mediated response in protected mice relative to non-protected controls. Further, it was demonstrated that BALB/c mice vaccinated with recombinant LmL3 or LmL5 plus CpG-ODN were also protected against the development of cutaneous lesions following inoculation of *L. braziliensis*. Together, data presented here indicate that LmL3 or LmL5 ribosomal proteins combined with Th1 inducing adjuvants, may be relevant components of a vaccine against cutaneous leishmaniasis caused by distinct species.

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### 1. Introduction

Infection with *Leishmania* protozoan parasites can result in the development of leishmaniasis. Several species cause cutaneous leishmaniasis (CL) including *Leishmania major* and *L. braziliensis* (in the Old or New World, respectively); the latter is also related with development of mucocutaneous leishmaniasis (MCL). Visceral leishmaniasis (VL) is caused by the infection of *L. chagasi* in the New World or *L. infantum* and *L. donovani* in the Old World [1]. In natural and experimental leishmaniasis, effective primary immunity requires the production of IFN- $\gamma$  by CD4<sup>+</sup> T cells and to a minor

extend by CD8<sup>+</sup> T cells, which mediates nitric-oxide-depending parasite killing by the activation of infected macrophages [2]. Disease progression is related with the induction of humoral and IL-10 mediated responses [3].

A preparation of biochemically purified *Leishmania* ribosomal proteins (LRP) administered with CpG-oligodeoxynucleotides (CpG-ODN) conferred protection against challenge with *L. major* parasites in susceptible BALB/c and resistant C57BL/6 mice. Protection was correlated with a LRP-specific IL-12 dependent production of IFN- $\gamma$  (in both mouse strains) and a diminished production of both IL-4 and IL-10 in BALB/c mice [4]. Also, BALB/c mice vaccinated with LRP plus CpG-ODN and subsequently infected were able to resist a secondary challenge [5]. A protective response against *L. chagasi* and *L. amazonensis* was observed in BALB/c mice when a LRP-specific IFN- $\gamma$  mediated response was induced by administration of LRP combined with saponin [6].

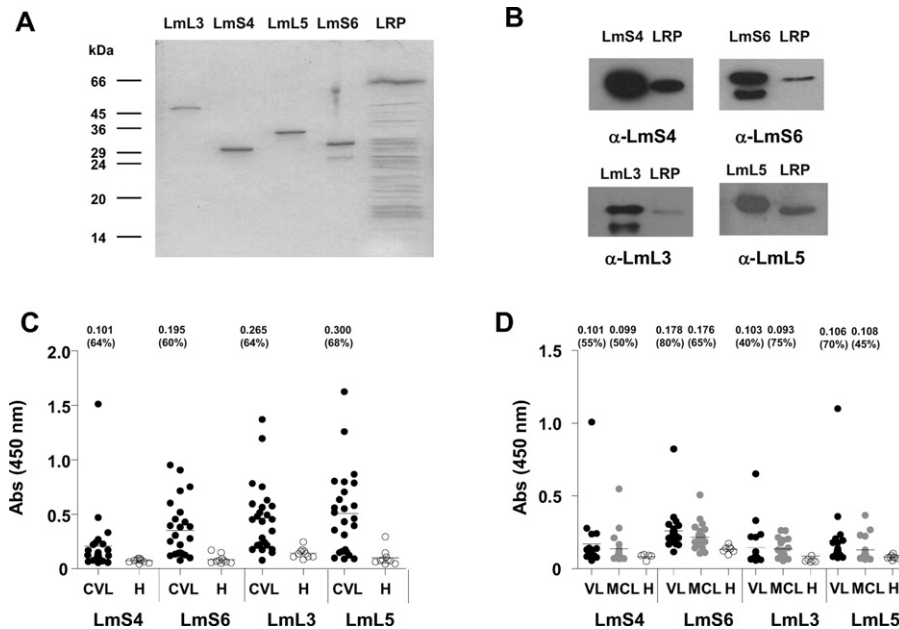
In this work, four *L. major* ribosomal antigens have been characterized: Lms4, Lms6, LmL3 and LmL5. Their prophylactic properties were assayed in the *L. major*-BALB/c mouse model of CL. Since immunization of LmL3 or LmL5 combined with CpG-ODN elicited

**Abbreviations:** CpG-ODN, CpG-oligodeoxynucleotides; VL, visceral leishmaniasis; MCL, mucocutaneous leishmaniasis; CL, cutaneous leishmaniasis; LRP, *Leishmania* ribosomal proteins; SLA, soluble *Leishmania* antigens; MRP, mouse ribosomal proteins; CR, coding region; DLN, draining lymph nodes; OPD, orthophenyldiaminedihydrochloride.

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**Fig. 1.** Characterization of the LmS4, LmS6, LmL3 and LmL5 as antigenic components of the *Leishmania* ribosome. (A) One  $\mu\text{g}$  of each recombinant protein and 10  $\mu\text{g}$  of *Leishmania major* LRP extracts were electrophoresed on a linear 10–13% gradient SDS-PAGE gel stained with Coomassie blue (B). Gels loaded with each one of the recombinant proteins and the LRP extracts were blotted and individually probed with the sera from mice immunized with LmS4, LmS6 and LmL3 (1/100) or with anti-LmL5 antibody fraction affinity purified from five canine VL sera. Horseradish peroxidase-conjugated anti-mouse (1/2000) or anti-dog IgG (1/2000) antibodies were used as the secondary reagents. (C) Antibody responses of canine VL (CVL,  $n=25$ ) and healthy animals' sera (H,  $n=8$ ) against the four recombinant proteins. All sera were tested for IgG reactivity by ELISA (1/200). Horseradish peroxidase-conjugated anti-dog (1/2000) or anti-human IgG (1/2000) antibodies were used as the secondary reagents. The OD value of each individual serum is shown. Bars represent means of each group. The cut-off value for negative and positive samples, calculated by comparison of the reactivity values from each group with the healthy sera using a Receiver-Operating Characteristic (ROC) analysis is indicated. It was defined as the lower O.D. value with a 100% of specificity. In brackets the percentage of positive sera is also indicated.

protective responses against *L. major* infection, their capacity to modulate *L. braziliensis* infection in the same mice strain was tested. Mice vaccinated with both proteins were able to control parasite growth in the site of infection in this New World species experimental model.

## 2. Materials and methods

### 2.1. Mice strains and parasites

Female BALB/c mice (6–8 week old) were purchased from Harlan (BCN, Spain) or were obtained from the Centro de Pesquisa Gonçalo Moniz, FIOCRUZ. Promastigotes of *L. major* strains (WHOM/IR/-/173) or clone V1 (MHOM/IL/80/Friedlin) and from *L. braziliensis* (MHOM/BR/01/BA788), were cultured at 26 °C in Schneider medium (Gibco, NY) supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin.

### 2.2. CpG-ODN

Phosphorothioate-modified CpG-ODN (5'-TCAACGTTGA-3' and 5'-GCTAGACGTTAGCGT-3') were synthesized by Isogen (The Netherlands) and employed for their capacity to induce Th1 responses in mice when immunized with various leishmanial antigenic preparations [7,8].

### 2.3. Cloning of DNA sequences coding for *L. major* ribosomal proteins LmS4, LmS6, LmL3 and LmL5

The *L. major* LmS4, LmS6, LmL3 and LmL5 coding regions were obtained from the *L. major* genome database ([www.genedb.org/genedb/leish](http://www.genedb.org/genedb/leish)) using the *Saccharomyces cerevisiae* orthologous protein sequences as probes [9]. Coding regions

were PCR amplified using specific primers (Supplementary Fig. 1) and the DNA from *L. major* (MHOM/IL/80/Friedlin). Amplified DNAs were cloned into the pQE30 prokaryotic expression vector (Qiagen, Germany). The four clones were double-stranded sequenced in the same plasmid.

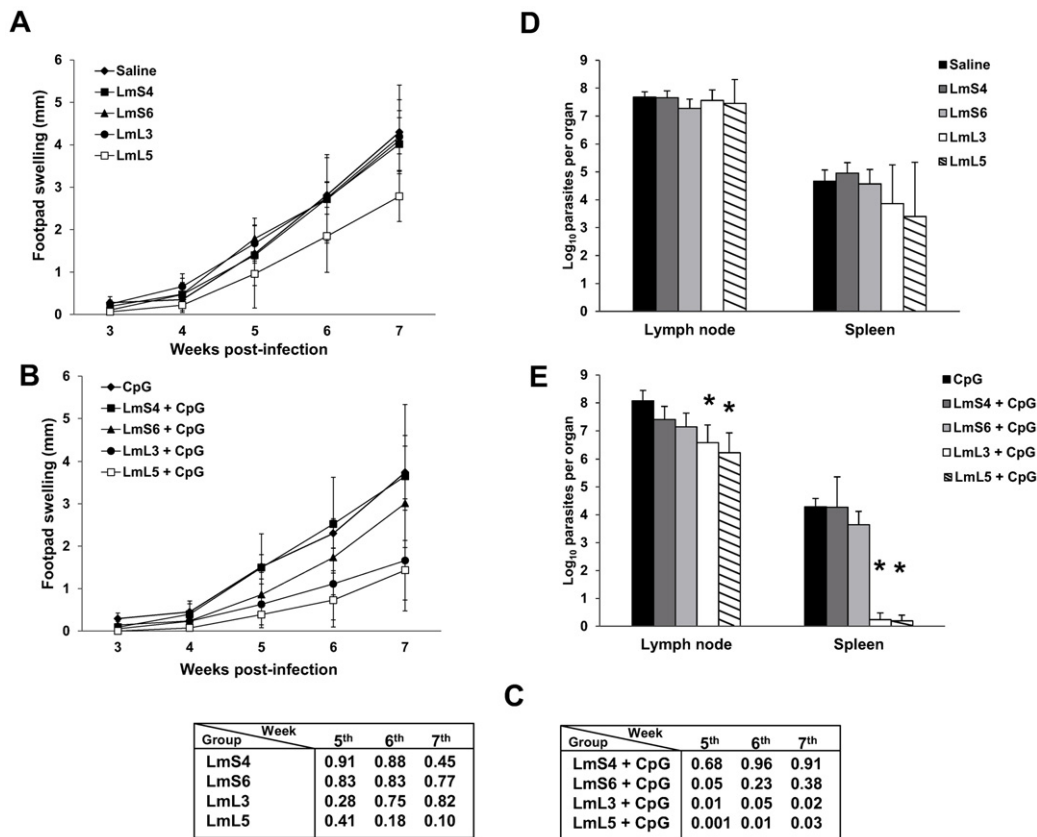
### 2.4. Protein purification

Recombinant proteins were over-expressed in *Escherichia coli*, purified under denaturing conditions onto Ni-nitrilotriacetic-acid-agarose columns (Qiagen) and refolded on the affinity column as described [10]. Polymyxin-agarose columns (Sigma, MO) were employed to remove residual endotoxin content (<10  $\mu\text{g}$  of LPS per 1  $\mu\text{g}$  of recombinant protein, measured by the Quantitative Chromogenic *Limulus* Amebocyte Assay QCL-1000 (BioWhittaker, MD)).

### 2.5. Sera, immunoblotting and ELISA assays

Mice sera were collected at the beginning of the experiment, before challenge with parasites and at the 7th week after challenge with *L. major*. Human VL and MCL sera were obtained from clinical and parasitologically diagnosed Brazilian patients. Canine symptomatic VL sera were collected in the Extremadura region of Spain [11]. Control sera were obtained from healthy individuals.

Soluble *Leishmania* antigens (SLA), mouse ribosomal proteins (MRP) and LRP were prepared as described in [4]. For immunoblotting, recombinant proteins and LRP extracts were electrophoresed and blotted as described in [11]. Anti-LmL3, anti-LmS4 or anti-LmS6 polyclonal sera were obtained from the immunized mice described below. Anti-LmL5 antibodies were obtained by passing canine VL sera through a recombinant LmL5 affinity chromatography column prepared as in [12]. For ELISA, recombinant proteins were used at 1.0  $\mu\text{g}$  per well. Murine, canine and human sera were employed



**Fig. 2.** Course of *L. major* infection in BALB/c mice vaccinated with ribosomal proteins. Mice ( $n=6$  per group) were vaccinated with the indicated formulations and challenged in the footpad with  $5 \times 10^4$  *L. major* promastigotes. Footpad swelling in groups vaccinated with the corresponding proteins without (A) or with (B) adjuvant is shown. Data correspond to the mean  $\pm$  SD of the difference of thickness between the infected and the uninfected contra-lateral footpads. (C) *P* values (saline versus antigen or CpG-ODN versus antigen plus CpG-ODN) from data shown in panels A and B. The number of viable parasites (mean  $\pm$  SD) in the draining lymph node on the infected leg (popliteous) and in spleen were determined by limiting dilution at week seven post-challenge in mice vaccinated with the recombinant proteins without (D) or with (E) adjuvant. Serial dilution of each mouse sample was individually performed in triplicates. Comparison were established between each one of the vaccinated groups and their respective control (saline [D] and CpG-ODN [E]) (\* $P < 0.05$  significant differences between vaccinated and control mice). Results in each panel are representative of  $\geq 2$  independent experiments.

as described in [4,12,13], respectively. Secondary antibodies were purchased from Nordic (Tilburg, The Netherlands). The reciprocal endpoint titre, defined as the inverse of the highest serum dilution factor giving an absorbance  $>0.15$  was determined by serial dilution of the sera.

## 2.6. Immunizations, parasite challenge and parasite quantification

For the *L. major*-BALB/c model, mice ( $n=6$  per group) were independently inoculated in the right hind footpad with  $10 \mu\text{g}$  of each recombinant protein (LmS4, LmS6, LmL3 or LmL5), alone or combined with  $25 \mu\text{g}$  of each CpG-ODN. As control groups, mice were inoculated with  $25 \mu\text{g}$  of each CpG-ODN or with saline. Each group was boosted two and four weeks later with the same dose. Parasite challenge was carried out by subcutaneous inoculation with  $5 \times 10^4$  stationary-phase promastigotes of *L. major* (WHOM/IR/173) into the left footpad, four weeks after the last immunization. Footpad swelling was measured with a metric caliper (thickness of the left footpad minus thickness of the right footpad). For the *L. braziliensis* model BALB/c mice ( $n=5$  per group) were intradermally (i.d.) inoculated with  $10 \mu\text{g}$  of each recombinant protein (LmL3 or LmL5) combined with  $25 \mu\text{g}$  of each CpG-ODN or with a mixture of the proteins ( $5 \mu\text{g}$  each) plus  $25 \mu\text{g}$  of each CpG-ODN in the left ear. Inoculation schedule was the same indicated above. Mice were challenged one month after the last inoculation in the dermis of the right ear with  $1 \times 10^5$  stationary-phase promastigotes

of *L. braziliensis* in the presence of sand fly saliva, as described [14]. Ear thickness was monitored weekly using a caliper. For parasite load determination the ears (in the case of *L. braziliensis* infection), draining lymph nodes (DLN) and spleen from each mouse were independently processed as described in Ref. [7] and assayed in triplicates by limiting dilution [15].

## 2.7. Measurement of cytokines

The release of IFN- $\gamma$ , IL-10 and IL-4 was measured in culture supernatants of splenocytes or DLN cells obtained from the different mice groups, following stimulation with the corresponding recombinant proteins, using commercial ELISA kits (eBioscience, CA) as described [4]. Briefly, spleen or lymph node cells obtained from each mouse were seeded and independently cultured (at  $5 \times 10^6$  cells per ml) during 48 h at  $37^\circ\text{C}$  alone or with the next stimuli: recombinant LmL3 ( $10 \mu\text{g}/\text{ml}$ ), recombinant LmL5 ( $10 \mu\text{g}/\text{ml}$ ), SLA ( $12 \mu\text{g}/\text{ml}$ ) or MRP ( $12 \mu\text{g}/\text{ml}$ ). When indicated cells were stimulated with a mixture of the two recombinant proteins ( $5 \mu\text{g}/\text{ml}$  each one).

## 2.8. Statistical analysis

The Receiver Operating Characteristic (ROC) curves were used to analyze the data obtained with sera samples from patients. Statistical analysis with the vaccinated and infected mice was performed

by a two-tailed Student's *t*-test. Differences were considered significant when  $P < 0.05$ .

### 3. Results

#### 3.1. *Leishmania* ribosomal proteins S4, S6, L3 and L5 are antigenic in canine and human leishmaniasis

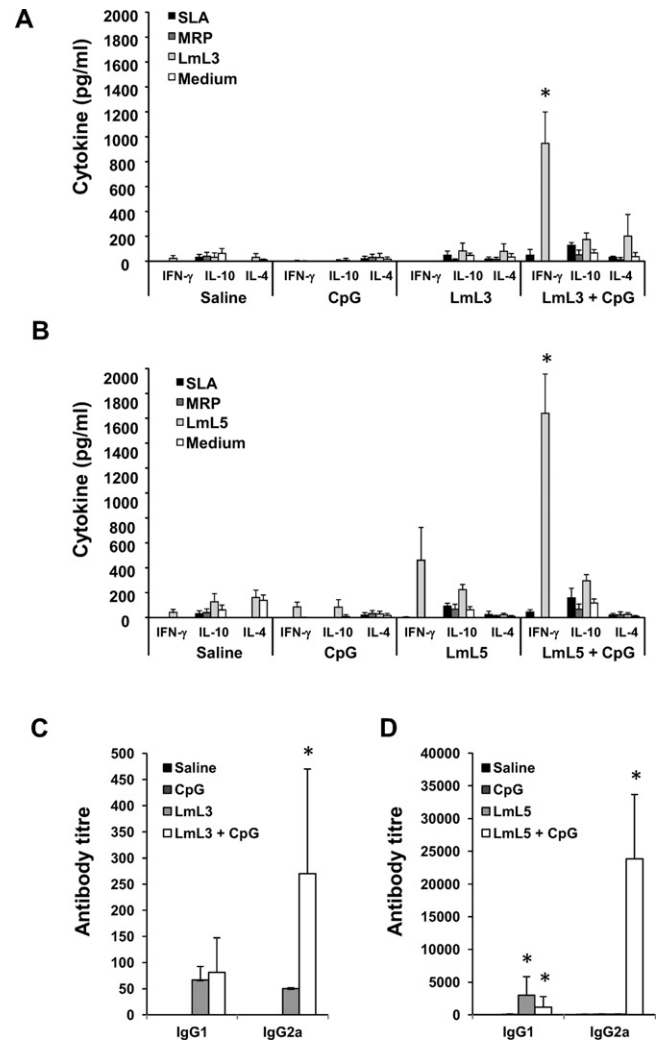
The putative *L. major* S4, S6, L3 and L5 ribosomal proteins were identified using as probes the *S. cerevisiae* homologous amino acid sequences [9] in a BLASTP search (Supplementary Fig. 2A). The degree of sequence identity with yeast and human ribosomal proteins (Supplementary Fig. 2A–B) supports the statement that they are components of ribosomes. In addition, antibodies specific for each protein revealed single bands with the expected molecular weights in a LRP preparation by Western blot (Fig. 1A and B). A high degree of sequence identity was observed for the proteins in different *Leishmania* species (Supplementary Fig. 2B).

The four recombinant proteins were recognized by the sera from dogs affected by VL (Fig. 1C). The percentages of positive sera ranged from 60 to 68% (Fig. 1C). They were also recognized by sera samples from Brazilian patients with VL and MCL, infected by *L. chagasi* and *L. braziliensis*, respectively (Fig. 1D).

#### 3.2. Outcome of CL due to *L. major* following vaccination of BALB/c mice with recombinant ribosomal proteins

Next, we analyzed whether the immunization with the respective recombinant proteins was able to induce protection against *L. major* infection. Different groups of BALB/c mice were independently vaccinated with each recombinant protein in the absence or in the presence of CpG-ODN. Mice groups inoculated with the vaccine diluent (saline) or with the adjuvant alone were established as controls. After the challenge with *L. major* the course of infection was followed-up for 7 weeks (Fig. 2A in the absence and Fig. 2B in the presence of adjuvant). In the absence of adjuvant no significant differences in the footpad swelling between control and vaccinated groups were observed (Fig. 2C). When mice were immunized with the recombinant proteins combined with the adjuvant, the LmL3 plus CpG-ODN or LmL5 plus CpG-ODN groups showed a decrease in their lesion size compared to the control (CpG-ODN vaccinated mice) (Fig. 2B). Differences were significant from week 5 to week 7 (Fig. 2C). LmS6 plus CpG-ODN vaccinated mice showed a delay in the evolution of CL until week 5, but differences were not maintained at the end of the assay (Fig. 2B and C). Mice vaccinated with the proteins without adjuvant had a number of parasites similar to that of the saline control group mice in their DLN and their spleens (Fig. 2D). An approximately 2-log reduction in parasite burden was observed in the DLN cells from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN, relative to the CpG-ODN control group (Fig. 2E). Moreover, spleens from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN were almost free of parasites, contrary to the other vaccinated groups and controls (Fig. 2D and E).

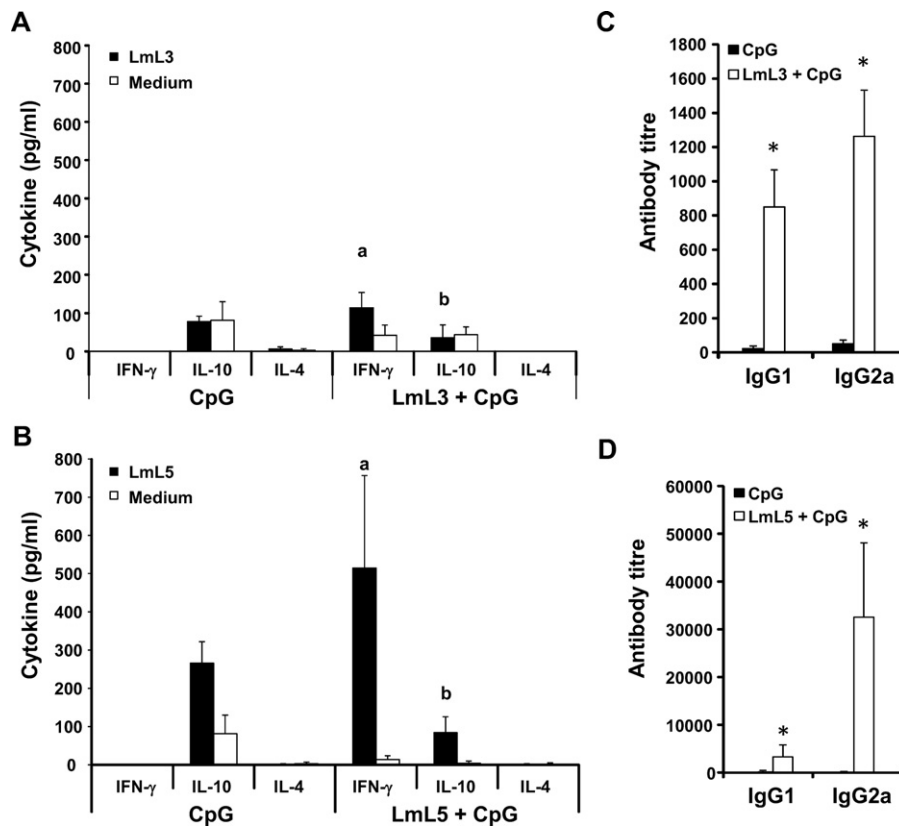
The immune response induced by vaccination with LmL3 and LmL5 was analyzed before challenge. Co-administration of the antigens with the CpG-ODN adjuvant induced a Th1-biased immune response, which was absent in mice immunized with the proteins alone. A significantly higher antigen-specific production of IFN- $\gamma$  was found after *in vitro* stimulation with the LmL3 ( $P = 0.0000146$ ;  $P = 0.00001468$ ) (Fig. 3A) and LmL5 ( $P = 0.000015$ ;  $P = 0.00029$ ) (Fig. 3B) proteins beside an IgG2a dominant antibody response against the vaccine antigen (Fig. 3C and D) when compared with saline and CpG-ODN mice group, respectively. Stimulation with SLA



**Fig. 3.** Immune response elicited in mice vaccinated with LmL3 and LmL5. Mice ( $n = 6$  per group) were vaccinated with LmL3 in the absence and presence of CpG-ODN (A) or LmL5 alone or plus CpG-ODN (B). Splenocytes from each mouse were independently cultured with medium alone or were *in vitro* stimulated with soluble *Leishmania* antigen (SLA), with mouse ribosomal proteins (MRP) and with the corresponding recombinant protein. Splenocytes from control mice, inoculated with saline or CpG-ODN, were stimulated with the same protein preparations in similar conditions. Culture supernatants were collected after 48 h and the levels of IFN- $\gamma$ , IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. (\* $P < 0.05$  indicates a statistically difference between vaccinated mice relative to saline and CpG-ODN controls groups). Anti-LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in the indicated mice groups ( $n = 6$  per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (\* $P < 0.05$  significant differences between vaccinated and control mice). Results in each panel are representative of  $\geq 2$  independent experiments.

or MRP did not induce cytokine secretion by spleen cells (Fig. 3A and B).

Immune responses were also studied after challenge. Upon *in vitro* stimulation with the recombinant proteins, LmL3 plus CpG-ODN (Fig. 4A) or LmL5 plus CpG-ODN (Fig. 4B) vaccinated mice displayed a significant increase in the LmL3 or LmL5 driven IFN- $\gamma$  production ( $P = 0.00009$  and  $P = 0.0005$ , respectively) paralleled by a decrease in IL-10 secretion ( $P = 0.009$  and  $P = 0.00001$ , respectively), relative to the CpG-ODN control group. These results are in accordance with the predominant IgG2a antigen-specific antibody response against LmL3 (Fig. 4C) and LmL5 (Fig. 4D), although

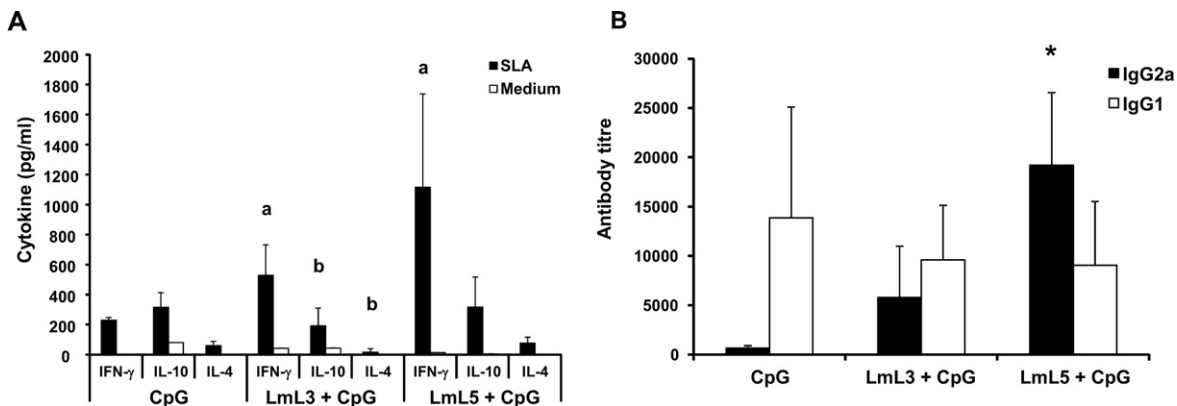


**Fig. 4.** Immune response elicited against LmL3 and LmL5 after *L. major* challenge in the protected mice. Mice ( $n = 6$  per group) vaccinated with LmL3 plus CpG-ODN (A) or LmL5 plus CpG-ODN (B) were latter challenged with *L. major*. Splenocytes obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with the corresponding recombinant protein. Control mice were inoculated with CpG-ODN and their splenocytes were stimulated as indicated. Culture supernatants were collected after 48 h and the level of IFN- $\gamma$ , IL-10 and IL-4 was independently measured by sandwich ELISA. Mean + SD are shown.  $P < 0.05$  indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection. Anti-LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ( $n = 6$  per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (\* $P < 0.05$  indicates significant differences between vaccinated and CpG-ODN control mice after infection). Results in each panel are representative of 3 independent experiments.

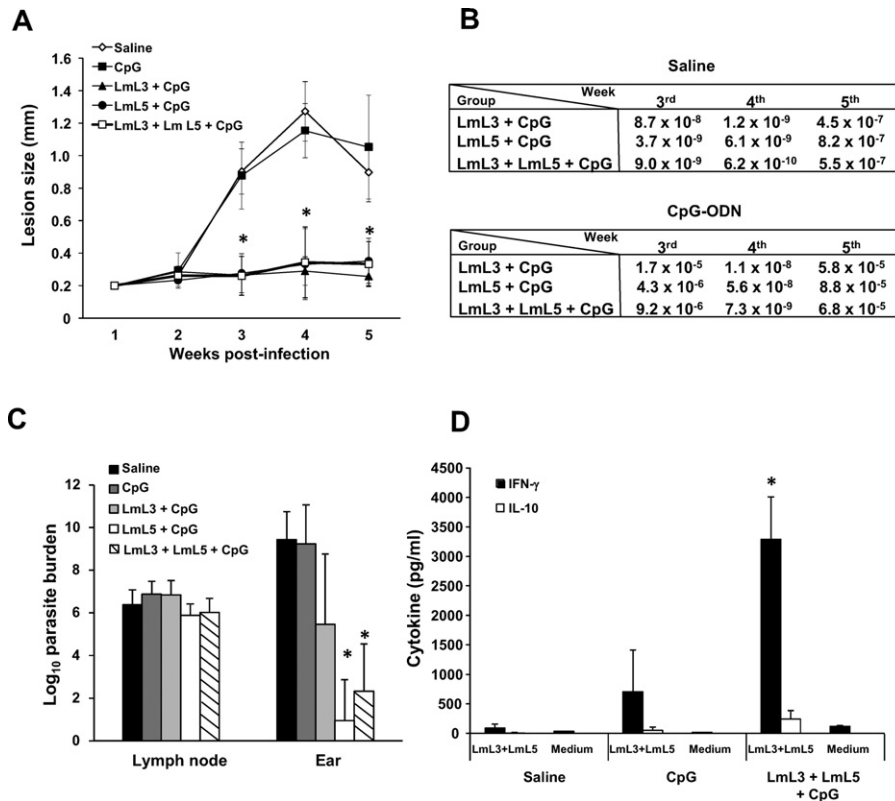
anti-LmL3 and anti-LmL5 IgG1 antibodies were also detected (Fig. 4C and D).

The cellular response against SLA was also analyzed in the protected mice after challenge. Secretion of IFN- $\gamma$  was significantly higher in LmL3 plus CpG-ODN or LmL5 plus CpG-ODN vaccinated

mice, when compared to mice inoculated with CpG-ODN ( $P = 0.014$  and  $P = 0.017$ , respectively) (Fig. 5A). In LmL3 plus CpG-ODN vaccinated mice a decrease in the SLA-dependent IL-10 production was also observed when compared with CpG-ODN immunized mice ( $P = 0.009$ ). An increment in the SLA-specific IgG2a antibodies was



**Fig. 5.** Immune response elicited against soluble leishmanial antigens (SLA) after *L. major* challenge in the protected mice. (A) Mice ( $n = 6$  per group) were vaccinated with LmL3 plus CpG-ODN or with LmL5 plus CpG-ODN. Mice were later challenged with *L. major* and splenocytes, obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with SLA. Control mice were immunized with CpG-ODN and their splenocytes were cultured as indicated in the presence or in the absence of SLA. Culture supernatants were collected after 48 h and the levels of IFN- $\gamma$ , IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. ( $P < 0.05$  indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection). (B) Anti-SLA IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ( $n = 6$ ) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (\* $P < 0.05$  indicates significant differences between vaccinated and CpG control mice after infection). Results in each panel are representative of 3 independent experiments.



**Fig. 6.** Course of *L. braziliensis* infection in BALB/c mice vaccinated with LmL3 and LmL5. Mice were vaccinated with LmL3 plus CpG-ODN, with LmL5 plus CpG-ODN or with LmL3 plus LmL5 plus CpG-ODN and challenged in the ear with  $1 \times 10^5$  *L. braziliensis* promastigotes plus salivary gland sonicate. (A) Course of CL development expressed as the mean  $\pm$  SD of the ear thickness from five mice ( $*P < 0.05$  indicates significant differences between vaccinated and saline or CpG-ODN controls). (B) *P* values (saline or CpG-ODN versus antigens plus CpG-ODN) from data shown in panel A. (C) Parasite burden in the ear dermis was independently quantified at 5th week five post-infection. Results are expressed as the mean  $\pm$  SD of five ears per group ( $*P < 0.05$  indicates a significant decrease between mice vaccinated with LmL5 plus CpG-ODN or LmL3 plus LmL5 plus CpG-ODN and control mice inoculated with CpG-ODN or with saline). (D) Production of IFN- $\gamma$  and IL-10 by lymph node cells following *in vitro* stimulation with a mixture of LmL3 and LmL5 ( $5 \mu\text{g}$  each protein) five weeks after challenge. Samples were independently processed and the level of cytokines are expressed as the mean plus SD of five mice per group ( $*P < 0.05$  indicates a significant increase in IFN- $\gamma$  levels between rLmL3 plus rLmL5 plus CpG-ODN group versus CpG-ODN or saline mice groups). Results in each panel are representative of 2 independent experiments.

observed in LmL3 plus CpG-ODN and LmL5 plus CpG-ODN groups relative to CpG-ODN group, only significant in LmL5 plus CpG-ODN group ( $P = 0.014$ ) (Fig. 5B). The IgG1 response to SLA was similar between the three groups (Fig. 5B).

### 3.3. Vaccination with LmL3 plus CpG-ODN and LmL5 plus CpG-ODN protects BALB/c mice against *L. braziliensis* challenge

We also investigated whether immunization with LmL3 or LmL5 was able to confer protection in an experimental model of New World CL. Mice were independently vaccinated with LmL3 or LmL5 combined with CpG-ODN or with a mixed formulation of LmL3 and LmL5 plus CpG-ODN. The outcome of infection with *L. braziliensis* inoculated in the presence of insect vector saliva was evaluated. Ear lesions of vaccinated mice groups were significantly smaller to control groups (saline or CpG-ODN) (Fig. 6A and B). Parasite burden in the ear dermis of the three vaccinated groups was lower than the parasite load of the control groups. Differences with both control groups were significant in the LmL5 plus CpG-ODN ( $P = 0.00016$ ) and in the LmL3 plus LmL5 plus CpG-ODN groups ( $P = 0.005$ ) (Fig. 6C). Similar parasite burden was detected in the DLN of controls and vaccinated mice (Fig. 6C). Finally, to analyze the cellular response elicited against the vaccine antigens, DLN cells from mice immunized with the mixed formulation and both control groups were stimulated with a mixture of the LmL3 and LmL5 recombinant proteins. A significant increase in antigen-specific IFN- $\gamma$  production was observed in the protected mice relative to both control groups ( $P = 0.015$  for saline and  $P = 0.007$  for CpG-ODN).

## 4. Discussion

Given that vaccines based on *Leishmania* ribosomal preparations have induced protection against disease development when immunized with Th1 inducing adjuvants [4–6] we have moved toward the identification of protective antigens in the *Leishmania* ribosome.

A few ribosome structural proteins have been described as antigenic in canine or human leishmaniasis, including the P0 [16,17], the L6 [18] and, recently, the L25 and L23a proteins [19]. The four ribosomal proteins identified herein (S4, S6, L3 and L5) are antigenic in different forms of the disease caused by distinct parasite species such as *L. chagasi* and *L. braziliensis* (in humans) and *L. infantum* (in dogs). Although the recombinant proteins were obtained from *L. major* DNA the high degree of sequence conservation existing between *Leishmania* parasite ribosomal orthologues may explain the observed cross-reactivity. Even though it was observed some variability in the recognition between human and canine VL sera (also between VL and MCL human patients) as occur with other parasite antigens [20], our data allow to conclude that the four studied proteins interact with the host immune system, in spite of differences in parasite species and disease forms.

Next, the protective capacities of the characterized antigens were evaluated. Previous reports have identified the prophylactic capacities of three structural proteins of the parasite ribosome: P0 [7], L22 and S19 [21]. Combination of the CpG-ODN adjuvant with the LmL3 or LmL5 proteins induced an immune state that was able to control CL disease due to *L. major* infection in susceptible

BALB/c mice. The immune correlate of protection was the induction of a Th1-like response specific for the recombinant LmL3 or LmL5 proteins. The magnitude of antigen dependent IFN- $\gamma$  secretion and antigen specific IgG2a titers were higher in the LmL5 plus CpG-ODN vaccinated mice than in mice immunized with LmL3 plus CpG-ODN based vaccine. Antigen specific Th1 responses induced upon vaccination were maintained after infection. Vaccines also controlled the LmL3 and LmL5-driven IL-10 responses induced after infection. However, the presence of IgG1 antibodies specific for LmL3, LmL5 is indicating that the Th2 immune response elicited against them by *L. major* infection was not completely abrogated by the vaccines. The Th2-biased response induced after infection against LmL3 and by LmL5 proteins occurring in human or dogs was also observed in mice vaccinated with the proteins without the adjuvant, since in these mice antibodies elicited against both antigens were of the IgG1 isotype (data not shown). This fact points out the importance of the adjuvant in ribosome-based vaccines. Thus, CpG-ODN motifs were able to redirect toward a protective Th1-like profile the response against LmL3 and LmL5 as also occur with other vaccine formulations like these based on LmSTI1 plus CpG-ODN soluble and particulate preparations [22].

Although the protective effects of the LmL3 or the LmL5 plus CpG-ODN documented here were analyzed in a model that employ a syringe-based challenge the results obtained allowed us to conclude that the protective effect of the immunization of LmL3 and LmL5 based vaccines is similar to that obtained with various parasite proteins assayed for protection under similar conditions [23].

Cross-prophylactic properties of the LmL3 or LmL5 based vaccines were also tested in an experimental model of CL caused by *L. braziliensis* [24]. Many of the proteins known to induce protection against *L. major* or *L. infantum* infection in BALB/c mice, were not able to control the CL caused by *L. braziliensis* [25,26] or only induce partial protection [27]. In this model, BALB/c mice show lesions in the challenge site (ear) that are resolved after induction of a Th1 type immune response that eliminates parasites from ears, maintaining a chronic infection in the DLN [24]. Since co-inoculation of vector saliva and *L. braziliensis* led to a significant exacerbation of both lesion size and parasites load in the mice experimental model [28] we have employed here a syringe-based challenge in which vector saliva and stationary parasites are co-inoculated. Interestingly, we found that ear inflammatory lesions were almost absent in vaccinated mice and a very low number of parasites was detected in the ears 5 weeks after challenge, especially in mice vaccinated with LmL5 plus CpG-ODN or with a combination of both ribosomal proteins and CpG-ODN. Given the antigen specific IFN- $\gamma$  mediated response was observed in protected mice, it can be suggested that IFN- $\gamma$ -secreting cells may have migrated to the infected ear early after challenge, promoting parasite killing in the absence of an inflammatory response of a high magnitude. These cells, however, are unable to destroy parasites in the DLNs as also occur in the infected controls in accordance to what it has been previously reported for this experimental model of infection [24]. Since results obtained by our group have shown that immunization with LmL3 or LmL5 ribosomal antigens combined with CpG-ODN also reduced parasite loads in BALB/c mice infected with *L. chagasi* (manuscript in preparation), we conclude that the LmL3 and LmL5 antigens, formulated with Th1 inducing adjuvants should be considered in the development of vaccines against leishmaniasis.

## 5. Conclusions

In this work four new antigenic proteins have been described in *Leishmania* ribosome: S4, S6, L3 and L5. Recombinant proteins obtained from *L. major* were recognized by the sera from individuals infected with different parasite species and suffering different

forms of the disease. Two of them, LmL3 or LmL5 were able to protect mice against CL caused by *L. major* and by *L. braziliensis* when administered in the presence of a Th1 inducing adjuvant. In both models, protection was associated with the induction of antigen-specific IFN- $\gamma$  mediated responses, but also with control of the antigen dependent production of IL-10 in some cases. Altogether, data presented here are indicating that LmL3 and LmL5 may be considered relevant antigens in the formulation of vaccines against leishmaniasis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2012.12.071>.

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