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Original article

Vaccination with the *Leishmania major* ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice

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Abstract

In the present work we analyze the antigenicity of *Leishmania major* ribosomal proteins (LRP) in infected BALB/c mice. We show that BALB/c mice vaccinated with LRP in the presence of CpG oligodeoxynucleotides (CpG-ODN) were protected against the development of dermal pathology and showed a reduction in the parasite load after challenge with *L. major*. This protection was associated with the induction of an IL-12 dependent specific-IFN-γ response mediated mainly by $CD4^+$ T cell, albeit a minor contribution of $CD8^+$ T cells cannot be ruled out. Induction of Th1 responses against LRP also resulted in a reversion of the Th2 responses associated with susceptibility. A marked reduction of IgG1 antibody titer against parasite antigens besides an impaired IL-4 and IL-10 cytokine production by parasite specific T cells was observed. In addition, we show that the administration of the LRP plus CpG-ODN preparation also conferred protection in the naturally resistant C57BL/6 mice. In this strain protection was associated with a LRP specific IFN-γ production in lymph nodes draining the challenge site. We believe that these evolutionary conserved proteins, combined with adjuvants that favor Th1 responses, may be relevant components of a pan-*Leishmania* vaccine.
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Keywords: Leishmania; BALB/C mice; C57BL/6 mice; Th1/Th2 immune responses; Ribosomal proteins; Vaccines

1. Introduction

Leishmaniases comprise several diseases caused by intracellular protozoan parasites belonging to the genus *Leishmania* that mainly infect macrophages of mammals including human and dogs. Outcome of infection is determined by interactions between the host immune system and different parasite molecules. A model for *Leishmania* virulence involving two different groups of parasite molecules has been proposed. The first one is formed by surface and secreted products that are necessary for the establishment of the infection, as a prerequisite for virulence. A second group is formed by highly conserved intracellular molecules referred as ‘‘pathoantigens’’ because an inadequate humoral response against them is thought to result in pathology mainly due to the adverse effects of the immune complexes formed [1,2].

Several lines of evidence suggest that *Leishmania* ribosomal proteins are immunologically relevant molecules during infection. In some cases, ribosomal proteins can contribute to the host immune system dysfunction through their capacity to modulate cell activities and cytokine release during infection [3−6]. Thus, injection of the *L. major* ribosomal protein S3a into BALB/c mice induce the polyclonal expansion of B-cell clones inhibiting T-cell proliferation [3]. In addition, parasite

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acidic ribosomal P proteins induce strong humoral responses in dogs and humans suffering from leishmaniasis (reviewed in ref. [7]). On the other hand, the administration of some ribosomal constituents using Th1 inducing adjuvants has been related to the generation of protective responses in mice models. Immunization with the *L. infantum* acidic ribosomal protein P0 as a DNA vaccine or as a combination of the recombinant protein and CpG-ODN protects C57BL/6 mice and induce partial protection in BALB/c mice after challenge with *L. major* [8,9]. Also, genetic immunization with two *L. major* ribosomal protein coding genes (L22 and S19) has been related with the generation of protective responses in BALB/c mice challenged with this parasite [10].

We decided to analyze the immunogenicity of leishmanial ribosome, because these intracellular ribonucleoprotein particles may be relevant either as pathoantigens, but also inducing protective responses. Here we show that during *L. major* infection, susceptible BALB/c mice develop a mixed Th1/Th2 response against several parasite ribosomal proteins. We also show that the Th1 immune response induced by the co-inoculation of LRP plus CpG-ODN confers protection against a challenge with *L. major* parasites in the BALB/c mouse strain. Protection correlates to a LRP specific IL-12 dependent production of IFN-γ and a diminished production of IL-4 and IL-10. Co-inoculation of LRP plus CpG-ODN also induces protection against a low dose challenge with metacyclic *L. major* parasites in C57BL/6 mouse strain, a model that more closely mimics the human disease in terms of route and infection dose [11].

2. Materials and methods

2.1. Mice strains and parasites

Female BALB/c and C57BL/6 mice (6–8 week old) were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). *L. major* parasites (WHOM/IR/-/173) and clone V1 (MHOM/IL/80(Friedlin)) were kept in a virulent state by passage in BALB/c mice. *L. major* amastigotes were obtained and transformed to promastigote by culturing at 26 °C in Schneider's medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 20% fetal calf serum. Metacyclic promastigotes of *L. major* (clone V1) were isolated from stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA).

2.2. CpG-ODN, leishmanial antigens and mouse ribosomal proteins

For preparation of *L. major* LRP, 10^9 promastigotes were harvested, washed twice in pre-chilled PBS and resuspended in 1 ml NP40 lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.5 mM $MgCl_2$ and 0.5% NP40) and pipetted up and down 10 times. After lyses, samples were microfuged at 3000 × g for 2 min at 4 °C to pellet the nuclei. Supernatant was twice microfuged at 13,000 × g for 15 min at 4 °C and the ribosomes were prepared from the cytosolic supernatant as described in ref. [12]. Briefly, cytosol was submitted to high speed centrifugation at 90,000 rpm for 30 min at 4 °C in a Beckman TL100.3 rotor. The crude ribosomal pellet was resuspended in buffer A (20 mM Tris–HCl, pH 7.4, 500 mM AcNH4, 100 mM $MgCl_2$, 5 mM β-mercaptoethanol) and centrifuged through a discontinuous sucrose gradient (20/40%) in buffer A at 90,000 rpm at 4 °C in a TL100.3 rotor. The pellet of washed ribosomes was dissolved in PBS and sonicated until complete ribosomal RNA degradation. Mouse ribosomal proteins extracts (MRP) were prepared from 5×10^7 RAW 264.7 murine macrophage cells using the same procedure.

Total proteins of *L. major* (soluble *Leishmania* antigen [SLA]) was prepared as described [9].

Phosphorothioate-modified CpG-ODN (5′-TCAACGTTGA-3′ and 5′-GCTAGCGTTAGCGT-3′) were synthesized by Isogen (TheNetherlands).

2.3. Immunizations, parasite challenge and parasite quantification

BALB/c mice (six per group) were subcutaneously (s.c.) inoculated in the right footpad with either 12 μg of *L. major* LRP alone or plus 25 μg of each CpG-ODN, CpG-ODN alone, or phosphate saline buffer (PBS). Each group was boosted 2 and 4 weeks later with the same dose used for priming. Parasite challenge was carried out by s.c. inoculation with 5×10^4 stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) into the left (untreated) footpad 4 weeks after the last inoculation. Footpad swelling was measured with a metric calliper and calculated as thickness of the left footpad minus thickness of the right footpad.

C57BL/6 mice (12 per group) were injected s.c. in the footpad with 12 μg of *L. major* LRP plus 25 μg of each CpG-ODN or with the CpG-ODN alone, and boosted 2 and 4 weeks later with the same immunization regime. The infection was performed 4 weeks after the last vaccination by intradermal (i.d.) inoculation of 300 metacyclic promastigotes of *L. major* (clone V1) in both ears. Indurations of the ear lesion were measured with a metric calliper.

The number of parasites was determined in the ears, draining lymph nodes (DLN) and spleen by limiting dilution assay as described [9].

2.4. Measurement of cytokines in supernatants

The release of IFN-γ, IL-10 and IL-4 was measured in the supernatants of splenocytes or DLN cells cultures stimulated with LRP (12 μg ml^{-1}) or SLA (12 μg ml^{-1}) or MRP (12 μg ml^{-1}) s described previously [9], using commercial ELISA kits (Diaclone, Besançon, France). When indicated, and in order to block IL-12, CD4 and CD8 mediated T cell cytokine release, DLN cells stimulated with 12 μg ml^{-1} of LRP were incubated in the presence of 10 μg ml^{-1} of monoclonal antibody (mAb) against either mouse IL-12 (C17.8) CD4 (GK 1.5), mouse CD8 (53–6.7) [13]. Appropriate isotype-matched controls were also analyzed in the assay. The antibodies (no azide/low endotoxin™) were purchased from BD (PharMingen, San Diego, CA, USA).

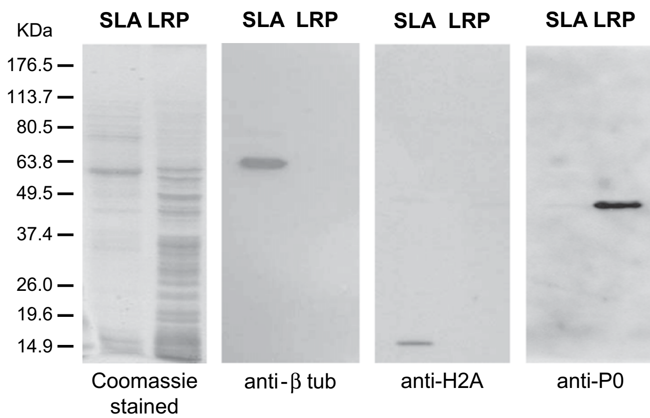

Fig. 1. Purity of the ribosomal fraction. *L. major* SLA and LRP were electrophoresed on linear 10–14% gradient SDS-PAGE gels, transferred onto nitrocellulose blots and incubated with anti-β tubulin, anti-*L. infantum* H2A and anti-*L. infantum* P0 ribosomal protein specific antibodies.

2.5. Analysis of the humoral responses

Reciprocal end-point titer (defined as the inverse of the highest serum dilution factor giving an absorbance >0.2) against LRP, SLA or MRP was determined by serial dilution of the sera assayed by ELISA using anti-IgG1 (1/1000) and anti-IgG2a (1/500) horseradish peroxidase-conjugated anti-mouse immunoglobulins as secondary antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). Plates were coated with 100 μl of LRP (5 μg ml^{-1} in PBS), SLA (2 μg ml^{-1} in PBS) or MRP (5 μg ml^{-1} in PBS). For Western blot (WB) analysis *L. major* indicated protein fractions were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Aylesbury, UK). Antibodies against *Leishmania* H2A histone were purified from dogs naturally infected with *L. infantum* by affinity chromatography as described [14]. Anti-β tubulin polyclonal antibody was purchased from Calbiochem (La Jolla, CA, USA). Anti-P0 antibodies were obtained from mice immunized with the recombinant *L. infantum* P0 ribosomal protein [9].

2.6. Statistical analysis

Statistical analysis was performed by a Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Antigenicity and immunogenicity of the LRP during L. major infection in BALB/c mice

The humoral and cellular responses of BALB/c mice experimentally infected with *L. major* against parasite LRP and SLA were first analyzed. To guarantee ribosomes purity, LRP fraction was analyzed by Western blot (WB). As shown in Fig. 1, sera against β-tubulin and histone H2A did not

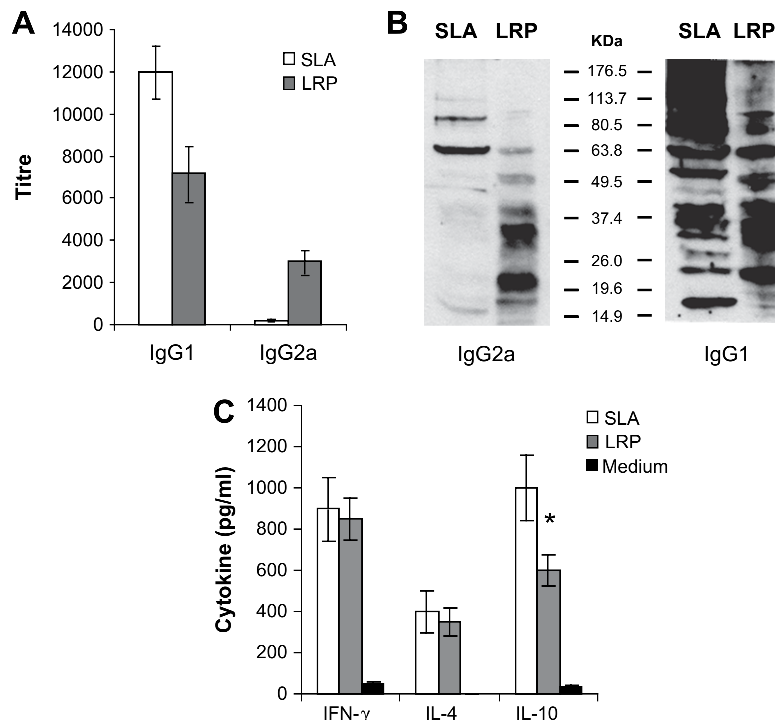

Fig. 2. Humoral and cellular responses against *L. major* LRP and SLA. Six BALB/c mice were s.c. infected with 5×10^4 *L. major* stationary-phase promastigotes in the left footpad and sera were obtained 8 weeks after challenge. Titers for IgG1 and IgG2a antibodies against LRP and SLA were determined individually by ELISA (A). Two μg of SLA and LRP were resolved on linear 10–12% gradient SDS-PAGE and transferred onto nitrocellulose blots. Western blots were incubated with the pooled sera at a 1/200 dilution. Antibody responses of both IgG1 and IgG2a isotype are shown (B). Popliteal DLN cells were obtained 8 weeks after infection and stimulated *in vitro* for 48 h with 12 μg ml^{-1} of SLA, LRP or medium alone. Levels of IFN-γ, IL-4 and IL-10 were assessed by ELISA in the culture supernatants. Each bar represents the means ± SD of data from individual mice (*$P < 0.05$) (C).

show reactivity against LRP whereas anti-P0 antibodies shown an increased reactivity in LRP relative to SLA. Next we analyzed the IgG1/IgG2a isotype polarization against LRP and SLA in *L. major* infected BALB/c mice. A mixed IgG1/IgG2a response against LRP was detected by ELISA (Fig. 2A) and WB assays (Fig. 2B). By contrast, antibodies against SLA were mainly of the IgG1 isotype. Similar levels of IFN-γ and IL-4 production were detected after *in vitro* stimulation of DLN cells with LRP and SLA, whereas the IL-10 production was significantly lower in the DLN cells stimulated with LRP (Fig. 2C).

The immunogenicity of the ribosomal proteins was evaluated in BALB/c mice after administration of the LRP in the absence or presence of CpG-ODN. After vaccination with LRP + CpG-ODN the anti-LRP humoral response was predominantly of the IgG2a isotype, whereas a lower titer of antibodies of the IgG1 isotype was detected in the sera from mice immunized with LRP alone (Fig. 3A). No reactivity against MRP was found in the sera for any of the groups (data not shown). Following *in vitro* stimulation with LRP, spleen cells from mice immunized with LRP + CpG-ODN secreted higher levels of IFN-γ than those secreted by spleen cells from controls and from mice immunized with LRP alone (Fig. 3B). No increase in IL-4 production was observed after stimulation with LRP in any experimental group (Fig. 3C). LRP specific IL-10 was also detected in the supernatant of cultures established from spleens of LRP + CpG-ODN vaccinated mice being the IFN-γ/IL-10 ratio ≈40 (Fig. 3D).

3.2. *Vaccination with LRP + CpG-ODN protects BALB/c mice against* L. major *challenge*

We analyzed whether LRP administration was able to induce protection against *L. major* infection. Footpad swelling of LRP + CpG-ODN vaccinated mice was significantly lower compared with controls and mice vaccinated with LRP alone (Fig. 4A). In addition, DLN from LRP + CpG-ODN immunized mice showed a 3-log reduction in parasite burden and no parasites could be detected in their spleens (Fig. 4B). It has been reported that CpG alone may protect BALB/c mice against *L. major* infection [15]. This unspecific effect was not observed in our study, indicating that the transient protection conferred by this adjuvant is not effective 4 weeks after inoculation.

To determine the immunological parameters protection, the SLA and the LRP-driven production of IL-4, IL-10 and IFN-γ was assayed. SLA or LRP specifically-induced IL-4 and IL-10 production was detected in DLN cells from controls (saline and CpG) and from mice immunized with LRP alone (Fig. 4C,D). In contrast, DLN cells from mice immunized with LRP + CpG-ODN produced higher amounts of IFN-γ than those detected in the other three groups (Fig. 4E). The contribution of $CD4^+$ and $CD8^+$ T cells and the dependence on IL-12 to the LRP specific production of IFN-γ was also analyzed (Fig. 4F). Production of IFN-γ was completely suppressed by anti-IL-12 or anti-CD4 monoclonal antibodies. The addition of anti-CD8 antibodies to the DLN cell cultures only partially reduced the amounts of this cytokine in the supernatants.

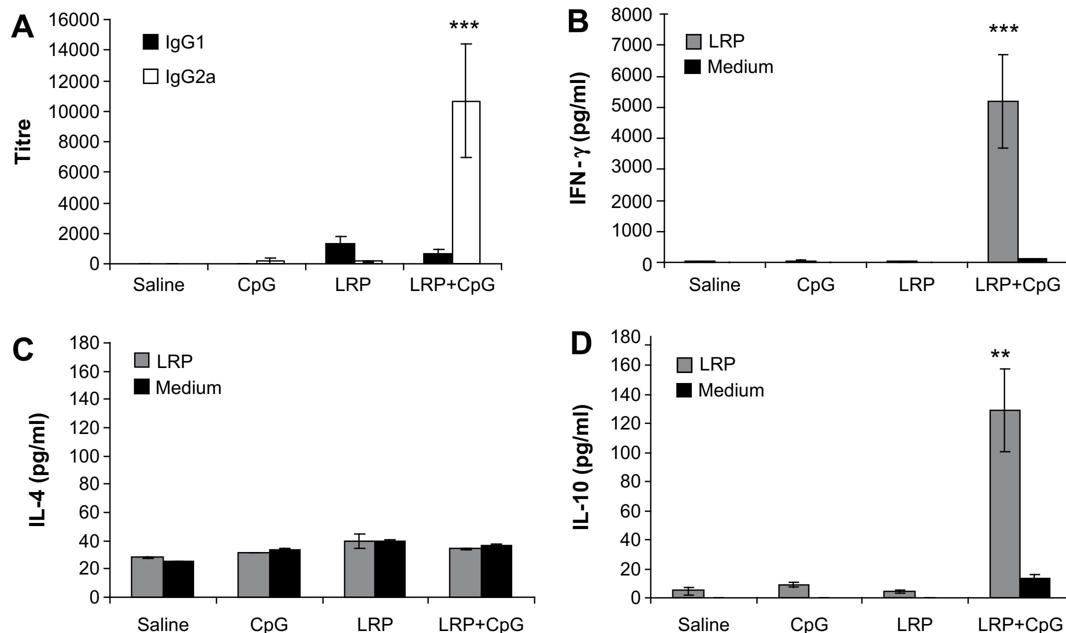

Fig. 3. Antibody responses and cytokine production induced by vaccination in BALB/c mice. Titer for IgG1 and IgG2a antibodies against LRP was determined individually by ELISA at week 4 after last dose (***$P < 0.001$ significant differences in IgG2a titer between mice vaccinated with LRP + CpG-ODN and the other three groups) (A). Spleen cells were obtained 4 weeks after vaccination and cultured *in vitro* for 48 h in the presence of LRP or medium. The levels of IFN-γ (B), IL-4 (C) and IL-10 (D) was assessed by ELISA in culture supernatants. Each bar represents the means ± SD of data from individual mice. Differences in the IFN-γ (***$P < 0.001$) and in the IL-10 (**$P < 0.01$) levels between LRP + CpG-ODN and the other three groups were statistically significant. This experiment was repeated with similar results.

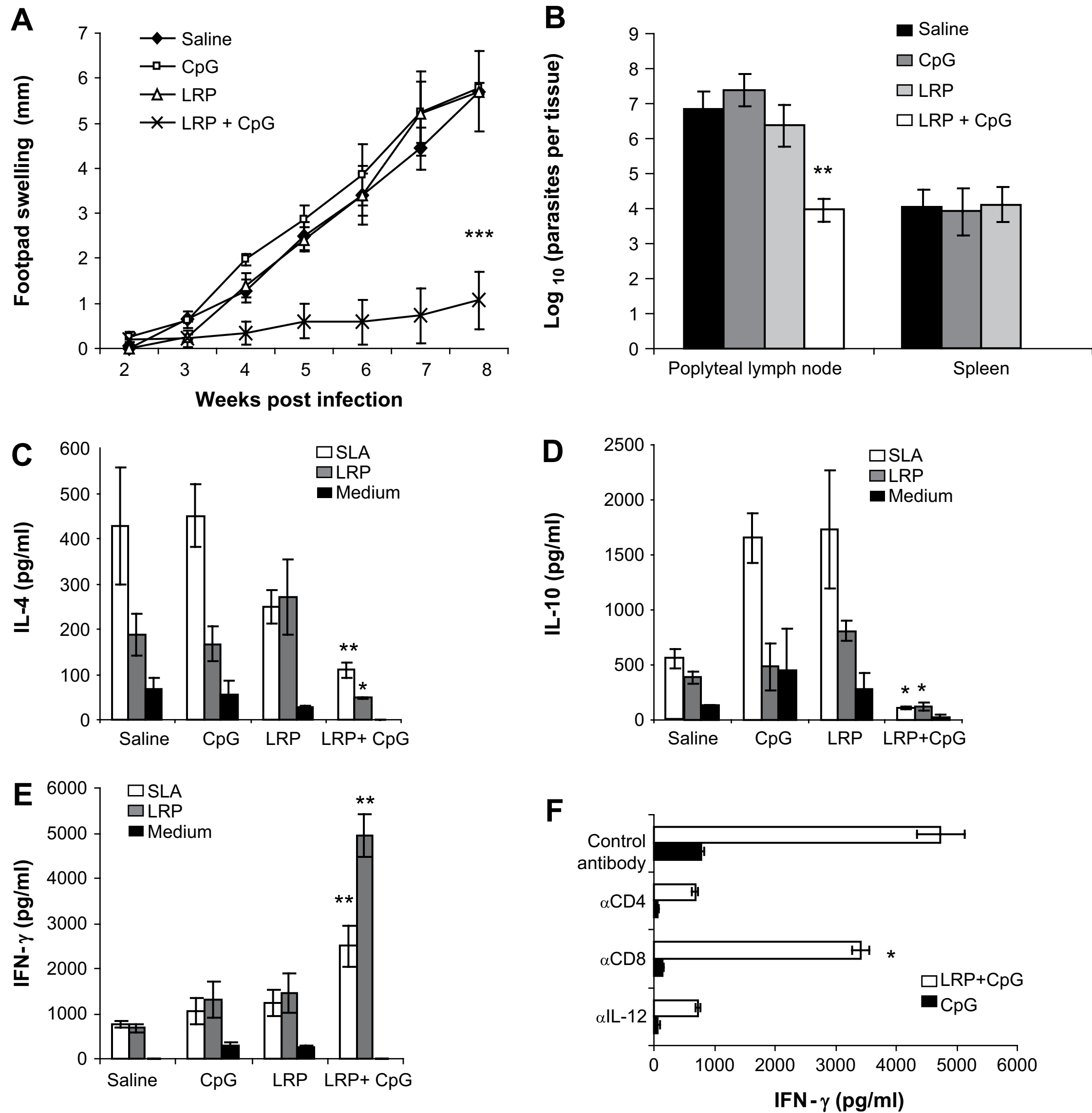

Fig. 4. Course of *L. major* infection in BALB/c vaccinated mice after challenge. Footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpads. Results represent the mean ± SD for two independent experiments (***$P < 0.001$ significant differences in inflammation for LRP + CpG-ODN vaccinated mice versus the CpG-ODN mice group at week 8 post-challenge) (A). The number of viable parasites in the popliteal DLN of the infected leg and spleen were individually determined by limiting dilution at week 8 post-challenge. Results represent the mean ± SD representative of two independent experiments (**$P < 0.01$ significant differences in popliteal parasite burden for LRP + CpG-ODN vaccinated mice versus the CpG-ODN mice group) (B). At week 8 post-challenge, the levels of IL-4 (C), IL-10 (D), and IFN-γ (E) were measured by ELISA in the supernatants of popliteal DLN cells cultures from the different mice groups 48 h following stimulation with SLA, LRP and medium alone. Each bar represents the means ± SD for levels of cytokine production representative of two independent experiments. Asterisks indicate the *P*-value with respect to the other three groups (*$P < 0.05$; **$P < 0.01$). Analysis of the involvement of IL-12 and T cells in the production of IFN-γ in CpG-ODN and LRP + CpG-ODN vaccinated mice 8 weeks after challenge. Levels of IFN-γ were measured by ELISA in the supernatants of popliteal DLN cell cultures 48 h following stimulation with LRP in the presence of either anti-IL-12, anti-CD4 or anti-CD8, and control monoclonal antibodies. Each bar represents the mean ± SD of data from individual mice. Differences in IFN-γ production between treatment with anti-CD8 monoclonal antibodies and treatment with control antibodies were statistically significant (*$P < 0.05$) (F). This experiment was repeated with similar results.

Given that in BALB/c mice the IL-4 dependent production of high titers of antibodies is associated with disease progression we analyzed the humoral responses elicited against LRP after infection in the different mice groups. The antibodies against LRP elicited by the parasite challenge in mice that had been immunized with LRP + CpG-ODN were mainly of the IgG2a isotype. Also, lower titer of anti-LRP antibodies of the IgG1 isotype were present in the sera of protected mice when compared to those immunized with LRP alone and in the two control groups (Fig. 5A, panel LRP). We also analyzed the humoral responses elicited against MRP after challenge. Anti-MRP antibodies of the IgG1 isotype were detected in control mice immunized with saline buffer and to a lesser extent in mice immunized with CpG-ODN alone. These anti-self ribosome antibodies were not detected in the sera from mice vaccinated with LRP or LRP + CpG-ODN after challenge (Fig. 5A, panel MRP).

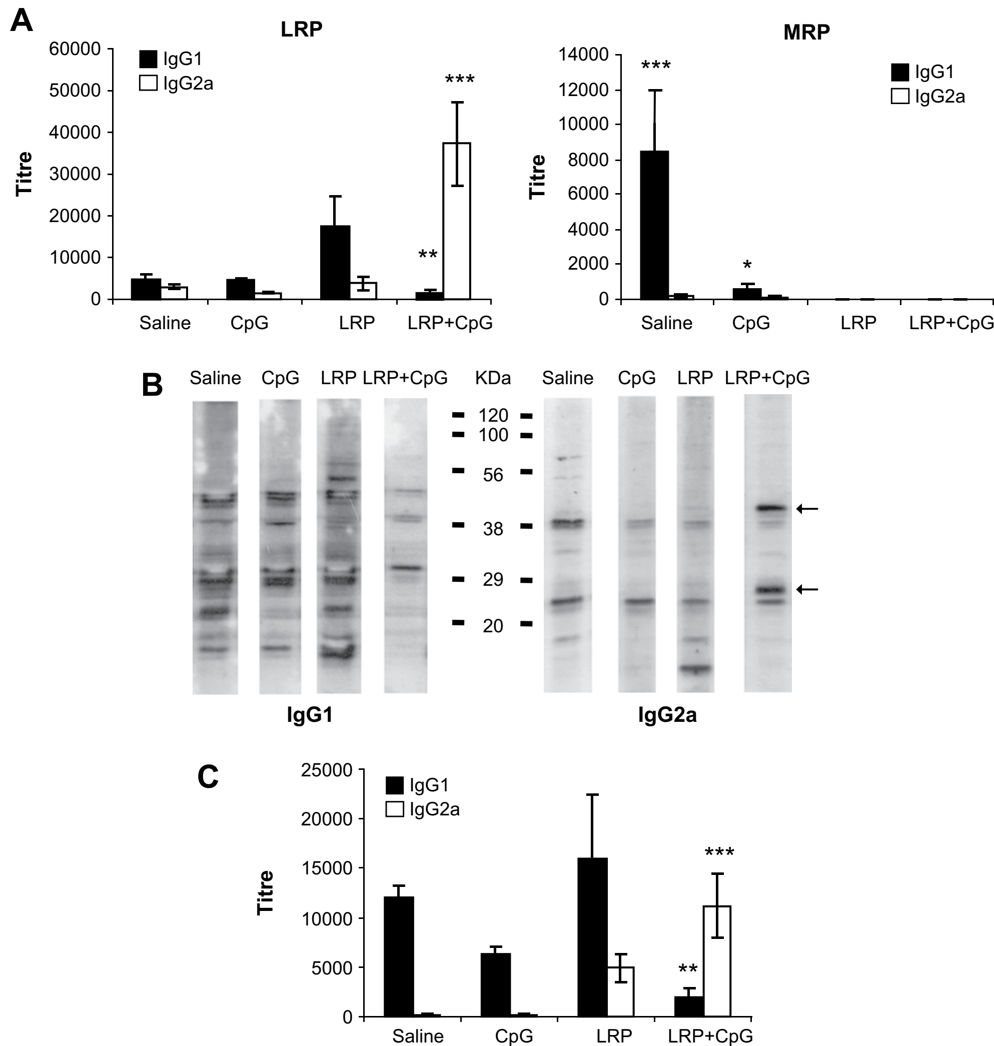

Fig. 5. Analysis of the IgG1/IgG2a polarization. Serum samples obtained 8 weeks after challenge were individually tested by ELISA to determine the presence of anti-LRP (Panel LRP) or anti-MRP (Panel MRP) IgG1 and IgG2a antibodies. Each bar represents the means ± SD of data from individual mice. Differences in the IgG1 (** $P < 0.01$) and IgG2a (*** $P < 0.001$) titer between mice vaccinated with LRP + CpG-ODN and the other three groups were statistically significant (panel LRP). Differences in the IgG1 (*** $P < 0.001$; * $P < 0.05$) titer between mice immunized with saline buffer or CpG-ODN and mice vaccinated with LRP or LRP + CpG-ODN were statistically significant (panel MRP) (A). *L. major* LRP were resolved on linear 10–14% gradient SDS-PAGE gel transferred onto nitrocellulose blots and incubated with the pooled sera from the indicated mice groups a 1/200 dilution. Antibody responses of both IgG1 and IgG2a isotypes are shown (B). The same sera were employed for the determination of the IgG1 and IgG2a titers against SLA. Each bar represents the means ± SD of data from individual mice. Differences in the IgG1 (** $P < 0.01$) and IgG2a (*** $P < 0.001$) titer between mice vaccinated with LRP + CpG-ODN and the other three groups were statistically significant (C).

The IgG1 isotype antibodies from sera of mice immunized with saline, CpG-ODN or LRP alone recognized a higher number of protein bands in LRP blots, whereas only a few bands were recognized by the IgG1 antibodies of the protected mice (Fig. 5B). Two protein bands were recognized with higher intensity by the IgG2a antibodies in the sera from LRP + CpG-ODN vaccinated mice when compared with the other three groups (Fig. 5B). Vaccination with LRP + CpG-ODN also conditioned the global anti-*Leishmania* humoral response induced by *L. major* infection. Antibodies produced by parasite-challenged mice immunized with LRP + CpG-ODN were mainly of the IgG2a isotype being the anti-SLA titer of the IgG1 isotype antibodies significantly lower than those detected in the other three groups (Fig. 5C).

3.3. Vaccination with LRP + CpG-ODN confers protection against dermal pathology due to L. major *challenge in C57BL/6 mice*

Given that vaccination with LRP + CpG-ODN protects against *L. major* infection in susceptible BALB/c mice by the redirection the Th2 immune response against LRP towards a Th1 response, we analyzed the effect of the administration of this vaccine in C57BL/6 mice. This resistant mice strain naturally develops Th1 responses against *Leishmania* antigens. LRP + CpG-ODN vaccinated C57BL/6 mice were protected against the development of dermal lesions since little or no pathology was observed (Fig. 6A). CpG-ODN immunized control mice developed lesions that reached a peak at week 7 and

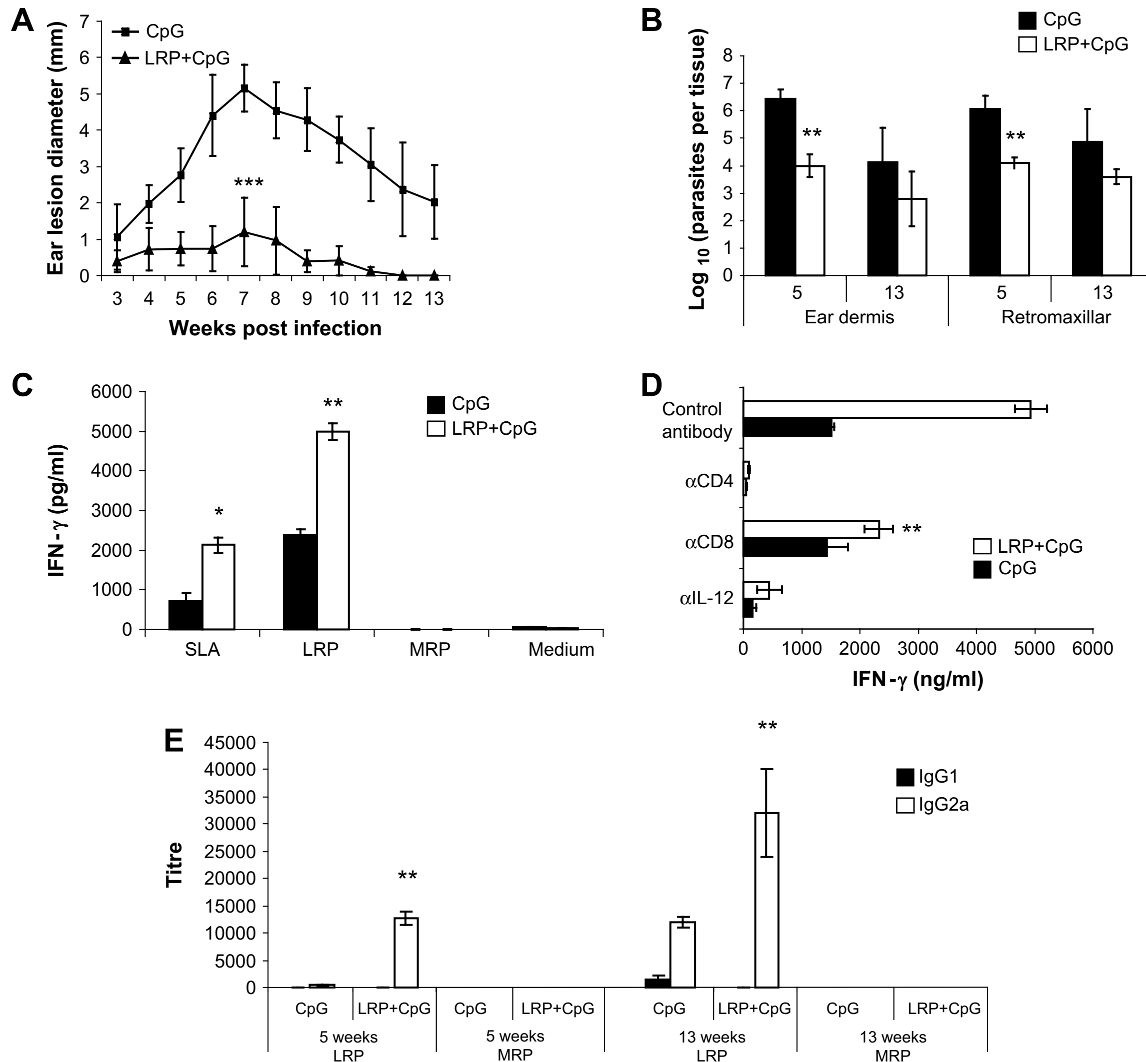

Fig. 6. Course of *L. major* infection in C57BL/6 mice. Values represent the mean lesion of ear diameter ± SD (***$P < 0.001$ significant decrease in the inflammation between mice vaccinated with CpG-ODN and LRP + CpG-ODN) (A). Parasite burden in the ear dermis and in the local DLN 5 and 13 weeks post-infection. Results are expressed as the mean ± SD of 12 ears and DLN (**$P < 0.01$ significant decrease between groups) (B). IFN-γ level was measured at week 5 after challenge in the supernatant of control and vaccinated retromaxillar DLN cells cultures following stimulation with SLA, LRP, MRP and medium alone. Each bar represents the means ± SD of data from individual mice. Asterisk indicate the *P*-value with respect to control mice (*$P < 0.05$ for SLA and **$P < 0.01$ for LRP) (C). IFN-γ level was also measured at week 5 after challenge in the supernatant of control and vaccinated retromaxillar DLN cells cultures following stimulation with LRP in the presence of anti-IL-12, anti-CD4, anti-CD8, and control monoclonal antibodies. Each bar represents the means ± SD of data from individual mice. Differences in IFN-γ production between treatment with anti-CD8 monoclonal antibodies and treatment with control antibodies were statistically significant (**$P < 0.01$) (D). Titers for IgG1 and IgG2a antibodies against LRP and MRP were determined individually by ELISA at week 5 and 13 after challenge. Each bar represents the means ± SD of data from individual mice. Differences in the anti-LRP IgG2a titer between the two mice groups were statistically significant (**$P < 0.01$) (E).

were almost completely healed at week 13. Since in this model the number of parasites in the infected site peaks just before the development of lesion [16], we determined the parasite load in the ear and in the local DLN (retromaxilar) at week 5. Parasite burden of LRP + CpG-ODN vaccinated mice had a 300-fold reduction in the ear dermis and a 40-fold reduction in the DLN (Fig. 6B, week 5). Chronic infection was patent in both groups of mice after healing (Fig. 6B, week 13). Notably, the number of parasites in these healed mice were quite similar to the levels observed in LRP-CpG vaccinated mice at week 5, confirming that LRP + CpG-ODN vaccine induce a robust protective response against *L. major* in C57BL/6 mice.

To determine the immunological parameters associated with protection the IFN-γ production driven by the LRP and SLA was assayed. DLN cells from vaccinated mice produced more SLA and LRP specific IFN-γ than those from control mice at week 5 after challenge (Fig. 6C). The IFN-γ production was found to be induced specifically by *Leishmania* ribosomal proteins, since stimulation of DLN cells cultures with MRP did not resulted in the production of this cytokine (Fig. 6C). LRP specific secretion of IFN-γ was inhibited by anti-IL-12 or anti-CD4 monoclonal antibodies whereas the anti-CD8 antibodies treatment only partially reduced the level of this cytokine (Fig. 6D). In addition, IgG2a anti-LRP

specific antibodies were detected earlier presenting higher titers in vaccinated mice (Fig. 6E). Anti-self ribosome antibodies were not detected in the sera from both mice groups (Fig. 6E).

4. Discussion

Research for the development of second generation vaccines based on *Leishmania* crude parasite fractions or based on defined parasite antigens was addressed to the identification of different parasite molecules that have been tested as vaccine candidates in several experimental models. Some of them are surface or secreted parasite antigens and others have been considered as good vaccine candidates because they elicit primarily a Th1 immune response in infected mice or human patient cells (reviewed in ref. [17]). On the other hand, some other parasite antigens presented to the immune system during the natural course of infection are conserved intracellular proteins like heat shock proteins, histones or ribosomal proteins. These proteins predominantly stimulate specific humoral responses in human or dogs suffering VL or Th2-mediated humoral responses in experimentally infected mice (reviewed in ref. [2]).

Data presented here show that in the sera from infected BALB/c mice there are IgG1 and IgG2a anti-LRP antibodies. Since the induction of IgG1 and IgG2a antibodies is considered a marker of Th2- and Th1-type responses, respectively [18], it can be concluded that a predominant Th2 response is mounted against parasite ribosomal proteins. In addition, in spite of the strongly biased Th2 responses developed in BALB/c infected mice some ribosomal antigens were capable to elicit a Th1 response. In accordance, after *in vitro* stimulation of the DLN cells from infected mice with LRP, high amounts of both IFN-γ and IL-4 were detected in the culture supernatants. Also, it was observed comparable levels of IL-10, a pleiotropic anti-inflammatory cytokine that renders infected macrophages unresponsive to the activation signals for parasite destruction [19]. Furthermore, given that the effects of IL-4 and IL-10 in promoting disease in BALB/c mice appear to be additive (reviewed in ref. [20]), stimulation of these cytokines by LRP would indicate that the host responses against ribosomal antigens might favor parasite expansion and persistence in BALB/c mice. We may assume that early after *Leishmania* infection, the host immune system is primed by the high abundance of ribosomal proteins released by parasite cytolysis that afterwards, may be boosted as a result of parasite proliferation. Thus, the strong immunogenicity of LRP and their pathoantigenic role probably relies on their high abundance and antigenic specificity in spite of their evolutionary conserved character.

In BALB/c mice the administration of LRP without adjuvants induced only weak IgG1 humoral responses, but co-administration with CpG-ODN an adjuvant that confers a Th1-related long term immunity and protection when used with different leishmanial antigens [21] and that also can suppress some parasite specific Th2 responses in mouse [22,23], boosted a Th1-like response against these antigens. Thus, LRP plus CpG-ODN vaccinated mice developed anti-LRP antibodies of the IgG2a isotype and their splenocytes produced high amounts of IFN-γ, but not IL-4, after *in vitro* stimulation with LRP. The LRP-driven production of IL-10 observed might be related to the homeostatic control of the Th1 responses since it has been recently reported that the IFN-γ producing Th1 cells can also be implicated in the production of IL-10 as a mechanism of feedback control [24]. Moreover, the high IFN-γ/IL-10 ratio values obtained might provide a good prediction of vaccine outcome [4].

Co-administration of LRP + CpG-ODN induce protection in two different models of cutaneous experimental leishmaniasis: high dose inocula in the footpad of BALB/c mice (widely used in cutaneous leishmaniasis vaccine assays), and low dose in the ear of C57BL/6 mice (a model that more closely mimics the human disease in terms of route and infectious dose). In both strains LRP plus CpG-ODN vaccinated mice showed reduction on parasite burden and were protected against pathology. Protection is correlated to the generation of Th1 specific immune responses against LRP being the IFN-γ response IL-12 dependent and mainly produced by $CD4^+$ T cell. Generation of the Th1 responses in the protected mice correlates to the generation of predominant IgG2a specific antibodies against LRP in both mice strains.

Remarkably, we have found that protection showed in the BALB/c mice after vaccination with LRP + CpG-ODN is also related to a significant reduction in the production of antigen driven IL-4 and IL-10 after stimulation *in vitro* with LRP or SLA. These cellular responses correlated *in vivo* with the reversion of the Th2-mediated antibody responses against ribosomal proteins. Thus, sera from protected BALB/c mice presented a significant decrease in the titer and, notably, in the number of antigens recognized by IgG1 antibodies specific for LRP. In addition, immunization of BALB/c mice with LRP CpG-ODN also had a clear effect on the global humoral response elicited in mice by the *L. major* infection. Thus, the infection of vaccinated mice induced limited IgG1 anti-*Leishmania* specific antibodies, whereas the humoral response in the control groups was higher and with a predominance of Th2-type antibodies (i.e., IgG1 isotype).

In our opinion, generation of vaccines against such a complex parasite as *Leishmania*, would be optimized by incorporating different target antigens in the vaccine formulation, taking advantage of these antigens that induce the required immunity (mainly $CD4^+$ and $CD8^+$ IFN-γ mediated responses), and redirecting towards a Th1 bias the immune responses that result in pathology (IL-4 Th2-driven and IL-10 deactivating responses). Notwithstanding, it should be taken into account that the Th2-response against some of these antigens may not be redirected by the usual Th1-inducers, as occur with the meta-1 antigen of *L. major* [25] or the parasite P2a and P2b acidic ribosomal proteins [26]. In this work we show that vaccination with LRP + CpG-ODN has direct influences on the immune system decisions at the time of *Leishmania* infection in both, resistant and susceptible mice, employing a pure preparation of *Leishmania* intracellular ribosomal proteins. Although these proteins are highly conserved in evolution, we have not detected IgG1 or IgG2a antibodies

against murine ribosomal proteins in the LRP or LRP + CpG-ODN vaccinated mice. In addition, DLN cells from C57BL/6 vaccinated and infected mice did not produce IFN-γ after *in vitro* stimulation with MRP. These data support the idea that vaccination with LRP induces an immune response specific for *Leishmania*, since no humoral or cellular responses were found against self-ribosomal proteins. Our results indicate that the parasite ribosomal proteins may be useful as components in a pan-Leishmania vaccine. Further characterization of the ribosomal constituents implicated in both type of responses should contribute to a more rational development of effective molecular defined vaccines against leishmaniasis.

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