

## Research Article

# BALB/c Mice Vaccinated with *Leishmania major* Ribosomal Proteins Extracts Combined with CpG Oligodeoxynucleotides Become Resistant to Disease Caused by a Secondary Parasite Challenge

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Leishmaniasis is an increasing public health problem and effective vaccines are not currently available. We have previously demonstrated that vaccination with ribosomal proteins extracts administered in combination of CpG oligodeoxynucleotides protects susceptible BALB/c mice against primary *Leishmania major* infection. Here, we evaluate the long-term immunity to secondary infection conferred by this vaccine. We show that vaccinated and infected BALB/c mice were able to control a secondary *Leishmania major* challenge, since no inflammation and very low number of parasites were observed in the site of reinfection. In addition, although an increment in the parasite burden was observed in the draining lymph nodes of the primary site of infection we did not detect inflammatory lesions at that site. Resistance against reinfection correlated to a predominant Th1 response against parasite antigens. Thus, cell cultures established from spleens and the draining lymph node of the secondary site of infection produced high levels of parasite specific IFN- $\gamma$  in the absence of IL-4 and IL-10 cytokine production. In addition, reinfected mice showed a high IgG2a/IgG1 ratio for anti-*Leishmania* antibodies. Our results suggest that ribosomal vaccine, which prevents pathology in a primary challenge, in combination with parasite persistence might be effective for long term maintenance of immunity.

## 1. Introduction

Protozoa of the genus *Leishmania* are obligate intracellular parasites of the mononuclear phagocytic lineage. *Leishmania* infection causes a group of diseases ranging from self-healing cutaneous ulcers to potentially lethal fatal visceral infection, globally known as leishmaniasis [1]. *L. major* is the main causative agent of cutaneous leishmaniasis (CL) in the Old World. In humans, CL due to *L. major* infection is self-limiting and healing is associated with resistance to reinfection. This acquired immunity to reinfection in natural

*Leishmania* hosts suggests that a vaccine is feasible. However, there are no available vaccines against human leishmaniasis [2].

Effective primary immunity against *L. major* in mouse requires an IL-12 dependent production of IFN- $\gamma$  from CD4<sup>+</sup>T cells (Th1 response) and CD8<sup>+</sup> T cells that mediates a nitric oxide-dependent killing by infected macrophages [3, 4]. In contrast, susceptibility correlates with the dominance of an IL-4 driven Th2 response, as it has been observed in certain mice strains like BALB/c [3, 4]. Subcutaneous (s.c.) experimental infection of BALB/c mice with a high

dose inoculum of stationary phase promastigotes induces rapidly evolving lesions that correlated with the generation of strong Th2 responses [5]. This model of experimental CL has been extensively used to explore the protective role of several parasite antigens combined with different adjuvants [2, 6, 7]. The immunization with certain parasite proteins, irrespective of their cellular location (surface or intracellular parasite antigens), inoculated with Th1 modulating adjuvants can induce immune responses that resulted in protection [8, 9]. The production of parasite specific IFN- $\gamma$  combined with the control of the production of the disease associated IL-4 and IL-10 cytokines has been correlated to protection against the development of CL in vaccinated BALB/c mice [10]. Protective cell mediated immunity can also be induced in BALB/c mice after s.c. infection using a nonpathogenic challenge of *L. major* promastigotes (leishmanization) [11–14]. Leishmanized mice developed very low or no pathology after primary infection and acquired resistance against a pathogenic rechallenge [11, 13, 14]. Leishmanization induced parasite specific Th1 responses that were able to control the secondary challenge made in a distant site [13, 14].

In a previous work, we have shown that during *L. major* infection, susceptible BALB/c mice develop a Th2 response against parasite ribosomal crude extracts purified from promastigotes [15]. Vaccination with the parasite ribosomal proteins (LRP) combined with CpG oligodeoxynucleotides (CpG ODN) as adjuvant induced a specific Th1 response, since vaccinated mice developed anti-LRP antibodies of the IgG2a isotype and their splenocytes produced high amounts of IFN- $\gamma$ , but not IL-4, after in vitro stimulation with LRP [15]. The immune state induced by vaccination conferred protection against a primary challenge with *L. major* parasites in the footpad. After infection, a *Leishmania* specific IL-12 dependent production of IFN- $\gamma$  and a reduced production of IL-4 and IL-10 were associated to protection [15].

In this work, we have analyzed whether or not vaccinated and protected mice were able to control the development of CL after a secondary challenge. To this end, mice vaccinated with LRP + CpG ODN were infected in the footpad with a pathogenic challenge of *L. major* parasites. The development of footpad swelling was analyzed over a period of 18 weeks as a stringent test of vaccine induced protection. Since no CL pathology was found during the follow up, mice were reinfected into the ear dermis with a low dose pathogenic challenge of *L. major* metacyclic promastigotes. Our results showed that vaccinated and infected mice developed a resistant phenotype to parasite associated disease at a secondary site of infection.

## 2. Materials and Methods

**2.1. Animals and Parasites.** Female BALB/c mice (4–6 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% foetal calf serum. Metacyclic promastigotes of *L. major* (clone V1) were isolated from stationary cultures by negative selection as described in [16] using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA).

**2.2. Parasite Antigens, Adjuvant and Immunizations .** Soluble *Leishmania major* antigen (SLA) was prepared as described [17]. Briefly, *L. major* promastigotes were harvested from culture and washed four times in phosphate-buffered saline (PBS). The parasites were suspended in PBS and subjected to three freezing and thawing cycles and sonicated with five cycles of 30 seconds at 38 MHz. After cell lysis, soluble antigens were separated from the insoluble fraction by centrifugation for 15 minutes at 12,000  $\times$  g using a microcentrifuge. *L. major* ribosomal proteins (LRP) were prepared as described [15]. Phosphorothioate-modified CpG ODN (5'-TCAACGTTGA-3' and 5'-GCTAGCGTTAGCGT-3') were synthesized by Isogen (The Netherlands).

Six mice were s.c. immunized in the right footpad with 12  $\mu$ g of *L. major* LRP combined with 25  $\mu$ g of each CpG ODN in a volume of 30  $\mu$ l. Control groups ( $n = 6$ ) received either CpG ODN or phosphate saline buffer PBS. Mice were immunized three times at two-week intervals.

**2.3. Parasite Challenge.** The primary parasite challenge was done by s.c. inoculation in the left footpad with  $5 \times 10^4$  stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) in a volume of 30  $\mu$ l, four weeks after the last vaccine inoculation. The secondary infection was done at week 18 after primary infection with 1000 metacyclic promastigote of *L. major* (clone V1) isolated from stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA). Metacyclic forms were injected into the dermis (i.d.) of both ears of each mouse in a volume of 10  $\mu$ l.

Footpad swelling was measured with a metric calliper and calculated as thickness of the left footpad minus thickness of the right footpad. Evolution of the ear lesion was monitored by measuring the diameter of the indurations with a metric calliper.

**2.4. Estimation of Parasitic Load.** The number of parasites was determined by limiting dilution assay [18]. Briefly, ears were recovered from infected mice and the ventral and dorsal sheets were separated. Ear sheets were deposited in RPMI medium containing Liberase CI enzyme blend (50  $\mu$ g ml<sup>-1</sup>) (Roche, Mannheim, Germany). After an incubation period of 2 hours at 37°C, the tissues were cut into small pieces, homogenized and filtered using a cell strainer (70  $\mu$ m-pore size). The homogenized tissue was serially diluted in a 96-well flat-bottomed microtiter plate containing Schneider's medium plus 20% FCS. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown up to 7-day incubation at 26°C. The number of parasites was also determined in the local draining

lymph nodes (DLN) of infected ears (retromaxillar) and footpad (popliteal) and in the spleen. Organs were recovered, mechanically dissociated, homogenized and filtered and then serially diluted as above. Parasite load is expressed as the number of parasites in the whole organ.

**2.5. Measurement of Cytokines in Supernatants.** Splenocytes and DLN cells suspensions were seeded in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 10 mM 2-mercaptoethanol).  $3 \times 10^6$  cells were seeded in 48-well plates during 48-hour at 37°C in the presence of LRP ( $12 \mu\text{g ml}^{-1}$ ) or SLA ( $12 \mu\text{g ml}^{-1}$ ). The release of IFN- $\gamma$ , IL-10, and IL-4 was measured in the supernatants of splenocytes and DLN cell cultures using commercial ELISA kits (Diaclone, Besançon, France).

**2.6. Analysis of the Humoral Responses.** Reciprocal end-point titre (defined as the inverse of the highest serum dilution factor giving an absorbance  $>0.2$ ) against LRP and SLA 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  $100 \mu\text{l}$  of LRP ( $5 \mu\text{g ml}^{-1}$  in PBS) or SLA ( $2 \mu\text{g ml}^{-1}$  in PBS).

**2.7. Statistical Analysis.** Statistical analysis was performed by a Student's *t*-test. Differences were considered significant when  $P < .05$ .

### 3. Results and Discussion

**3.1. Protective Immunity Generated by s.c. Vaccination with LRP + CpG ODN in the Footpad.** In a previous work we showed that BALB/c mice vaccinated with LRP combined with CpG ODN were protected against the development of cutaneous lesions in the footpad 8 weeks after parasite challenge [15]. The absence of footpad swelling was correlated with a 3-log reduction in parasite burden in the ipsilateral popliteal DLN when compared with mice immunized with the adjuvant or the excipient (control groups) [15]. In addition, no parasites were found in the spleen of the LRP + CpG ODN vaccinated animals whereas control groups contained approximately  $10^4$  parasites. It was concluded that the Th1 immune response induced in BALB/c mice by the vaccination of the LRP combined with CpG ODN resulted in a solid immunity that efficiently controlled parasite induced cutaneous disease maintaining a chronic infection in the local DLN [15]. In this work, we decided to analyze the footpad swelling of LRP + CpG ODN vaccinated mice after a longer period of time. After parasite challenge, vaccinated mice did not develop lesion for up to eighteen weeks (Figure 1(a)). Since control groups were sacrificed at week seven after challenge (because they began to develop severe necrotic lesions) a comparative analysis between controls and LRP + CpG ODN vaccinated mice was not possible. However, the parasite burden in the spleen and in the popliteal DLN of the LRP + CpG ODN vaccinated mice was

analyzed at week 18 after parasite challenge in the footpad. As it is shown in Figure 1(b), no parasites could be detected in the spleen of the vaccinated mice. The number of parasites located at the popliteal DLN at week 18 after challenge ( $5.41 \pm 0.99$ ;  $\log_{10}$  scale) represents a 1.12-log increment ( $P = .23$ ) when compared with the number of parasites detected in the same organ in LRP + CpG ODN vaccinated mice 8 weeks after challenge ( $4.84 \pm 0.26$ ;  $\log_{10}$  scale) [15]. Although a slightly increment in the number of parasites was detected, the presence of high levels of IFN- $\gamma$  measured in the supernatants of DLN cells cultures after stimulation with SLA and LRP in the absence of detectable levels of IL-4 and IL-10 (Figure 1(c)), should be taken as an indication that the parasite-specific Th1 response observed at week 8 [15] was maintained at week 18 after challenge. Thus, the Th1 response elicited by LRP + CpG ODN vaccination was able to induce an immunological status that protects mice against the development of cutaneous lesions during the 18 weeks of follow up. In addition, a chronic infection was patent in the vaccinated mice, being the parasites maintained located in the local DLN without dissemination to the internal organs.

This study reinforces that CpG ODN provides protection when used in combination with LRP extracts. Previous studies using this adjuvant in combination with parasite lysates showed a different degree of protection against *L. major* infection in the susceptible BALB/c [19–21] and in the resistant C57BL/6 [19] mice strains. The identification of a protein fraction composed by ribosomal proteins that provide protection against the development of cutaneous leishmaniasis lesions represents a substantial step in defining the protective immunogens within SLA, helping to identify new protective parasite antigens for the development of molecularly defined vaccines against leishmaniasis.

**3.2. Protected Mice Became Resistant to Disease Caused by a *L. major* Rechallenge in the Ear Dermis.** Next, we analyzed if protected mice were able to control a second parasite challenge. For that purpose, vaccinated and protected mice ( $n = 6$ ) were rechallenged in the ear dermis with 1000 *L. major* metacyclic promastigotes, parasite infective forms that seem to be similar to the promastigotes that are inoculated during the insect vector blood feeding [22]. Since vaccines were inoculated in the contralateral footpad of the primary infection site, rechallenge was made by i.d. infection in the ears. BALB/c mice infected with a low dose of *L. major* metacyclic promastigotes develop progressive inflammatory lesion in the spot of infection that increased in size, accompanied by ulceration and tissue necrosis [23–26] as occurred in mice challenged in the footpad with a high dose inoculum of stationary promastigotes [3, 4]. As control six naïve BALB/c mice were also infected in the ear dermis with the same dose of parasites.

Very low dermal lesion development was observed in reinfected mice (Figure 2(a)). In some cases (in four of six mice) a complete absence of inflammation in the ears was observed for up to seven weeks. Two mice developed low dermal lesions ( $<1$  mm) that reached a peak at week 5 and were almost completely healed at week seven. On

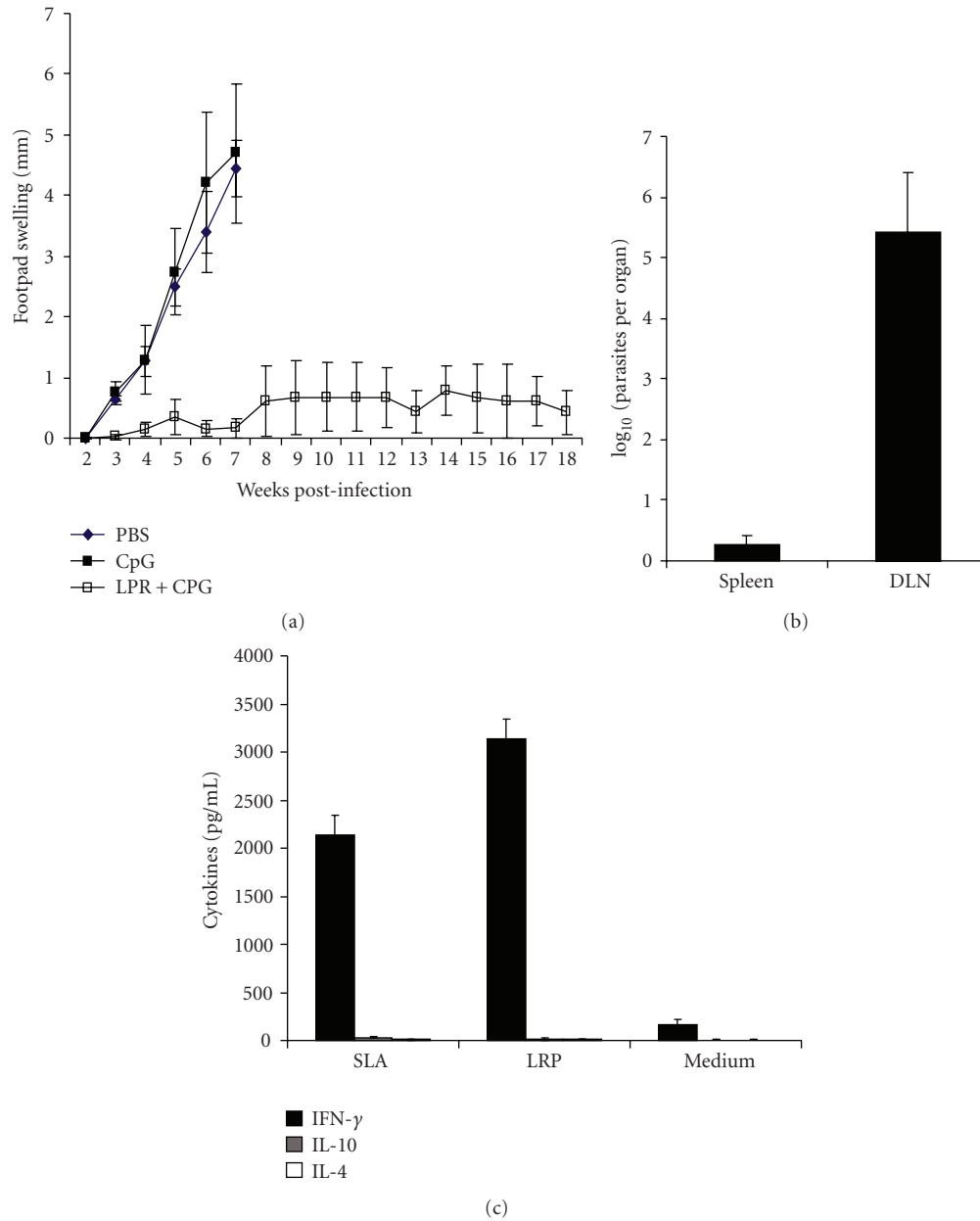


FIGURE 1: Course of *L. major* infection in BALB/c vaccinated mice. Mice (six per group) were s.c. immunized in the right footpad with three doses of the LRP adjuvanted with CpG ODN (LRP + CpG), with the CpG ODN adjuvant (CpG) or with PBS. One month after the last immunization, the animals were infected in the left hind footpad with  $5 \times 10^4$  *L. major* stationary phase promastigotes. (a) Footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. (b) The number of viable parasites in the spleen and the popliteal DLN of the LRP + CpG ODN vaccinated were individually determined by limiting dilution at week eighteen post challenge. Results are expressed as the mean  $\pm$  SD of six spleens and popliteal DLN. (c) At week eighteen after footpad infection the level of IFN- $\gamma$ , IL-10 and IL-4 was measure by ELISA in the supernatants of popliteal lymph node cells cultures from LRP + CpG ODN vaccinated mice. Cells were in vitro stimulated for 48 hours with 12  $\mu$ g/ml of SLA or LRP and medium alone. Results are expressed as the mean  $\pm$  SD.

the other hand, infection in all control naïve mice leads to the development of progressive inflammatory lesions in the ears (Figure 2(a)). The parasite load in the ear dermis and retromaxillar DLN was analyzed at week seven after challenge. Vaccinated and reinfected mice showed very low parasite loads in the ears and in the retromaxillar DLN, correlating to the absence of parasite in the spleens

(Figure 2(b)). These data contrast with the parasite burdens found in the ear dermis and in the retromaxillar DLN in the control group mice. Also, as an indication of parasite dissemination, parasites were detected in the spleen of the control mice (Figure 2(b)). These data indicate that the immune state generated after the first infection in the LRP + CpG ODN vaccinated mice is extremely potent, leading

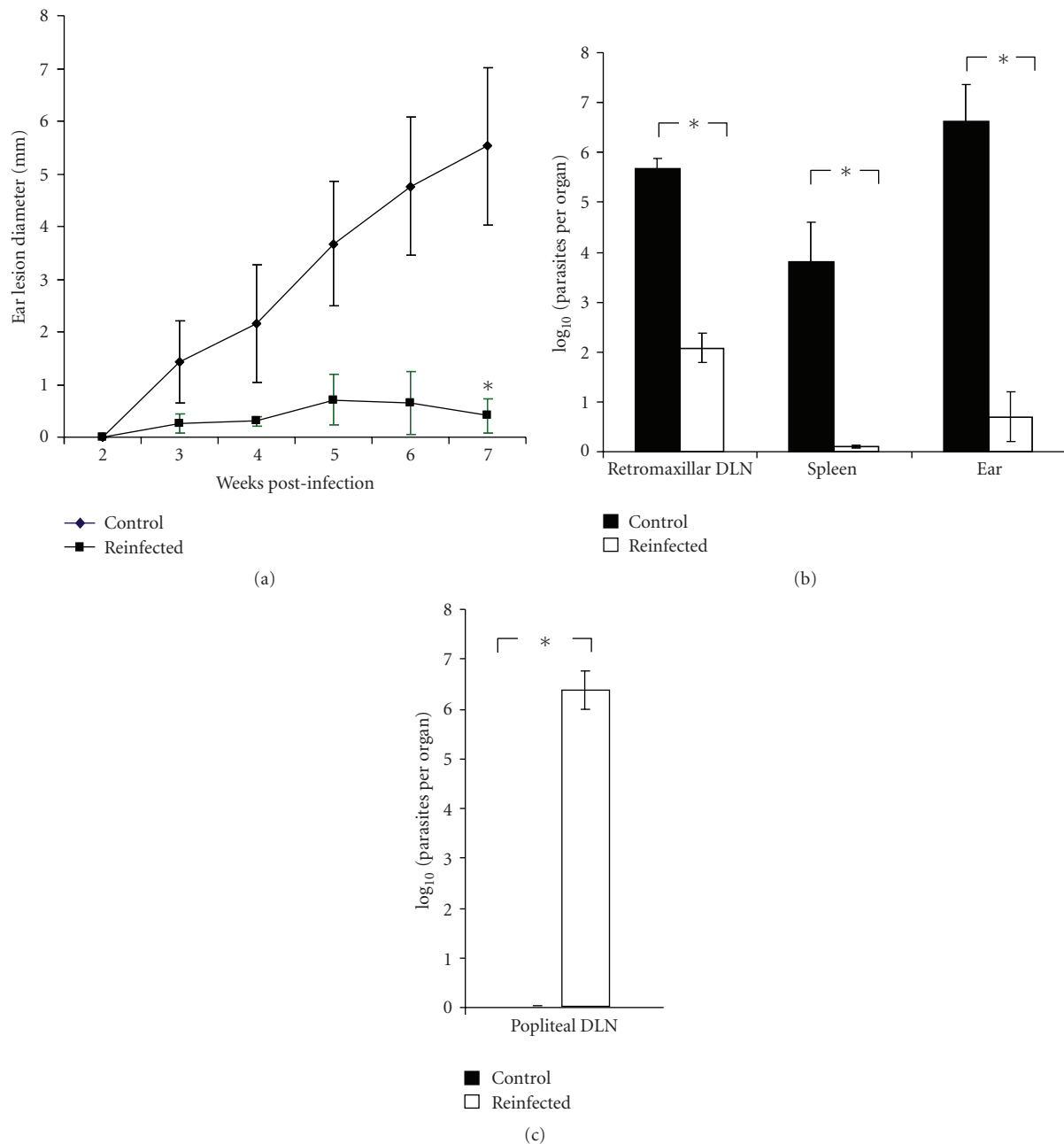


FIGURE 2: (a) Course of *L. major* infection in protected and reinfected BALB/c mice. Values represent the mean lesion diameter  $\pm$  standard deviation (SD). \* $P < .001$ : significant differences in inflammation for protected versus control mice at week seven postchallenge. (b) Seven weeks after reinfection, mice were euthanized and parasite burden in the ear dermis, spleen and in the retromaxillar DLN was individually quantitated. Results are expressed as the mean  $\pm$  SD of twelve ears and six spleens and DLN. \* $P < .001$ : significant decrease for reinfected versus control mice. (c) The parasite burden at week seven after rechallenge was individually quantitated in the popliteal lymph nodes of control and reinfected mice. Results are expressed as the mean  $\pm$  SD of six DLN. \* $P < .001$ : significant decrease for reinfected versus control mice.

to a rapid and efficient control of parasite growth in the site of reinfection, that resulted in the generation of a moderate dermal pathology.

In the vaccinated reinfected mice the primary challenge site was also analyzed, since in immune genetically resistant mice an *L. major* secondary challenge can cause disease reactivation in the primary site despite efficient parasite

clearance in the site of reinfection [27, 28]. The parasite load found in the popliteal DLN of the reinfected mice ( $6.68 \pm 0.63$ ; log<sub>10</sub> scale) (Figure 2(c)) represents an increment of 1.23-log ( $P = .028$ ) when compared with the number of parasites detected at the moment of the secondary challenge. Since no parasites were found in the popliteal DLN of control mice (Figure 2(c)), parasite dissemination from the ear to

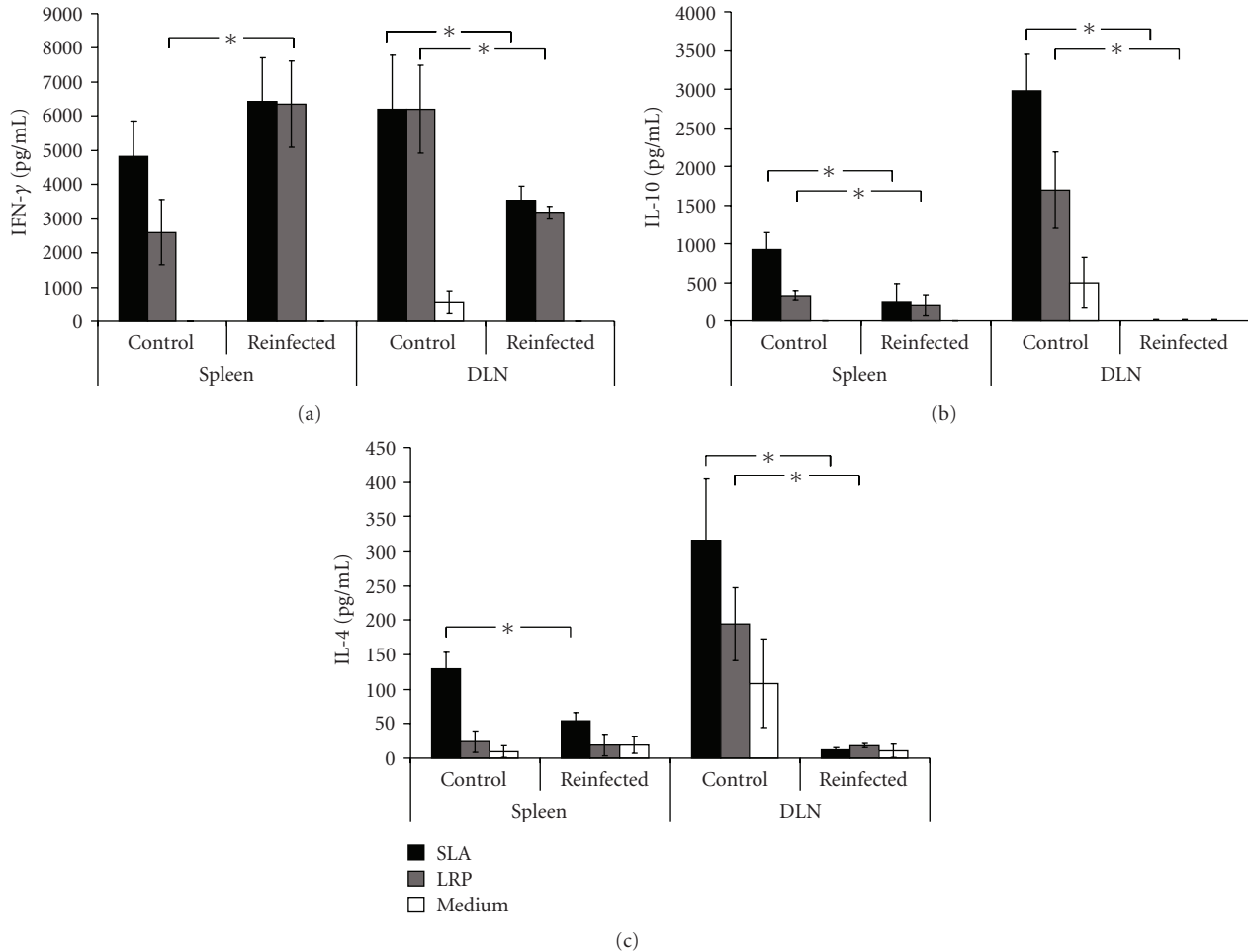


FIGURE 3: Analysis of the cellular responses. At week seven after ear infection the level of IFN- $\gamma$  (a), IL-10 (b), and IL-4 (c) was measured by ELISA in the supernatants of spleen and retromaxillar lymph node cells cultures from both mice groups. Cells were in vitro stimulated for 48 hours with 12  $\mu$ g/ml of SLA or LRP and medium alone. Results are expressed as the mean  $\pm$  SD of twelve ears and DLN. ( $*P < .001$ ).

these lymph nodes seems to be unlikely and the increase in the number of parasites found in the popliteal DLN may be indicating that the secondary challenge induced some parasite replication. However, we did not detect an increment in the footpad swelling in the vaccinated reinfected BALB/c mice for up to seven weeks after secondary challenge (not shown). Thus, we conclude that secondary challenge in the ear dermis did not produce a disease reactivation in these vaccinated mice.

**3.3. Analysis of the Cellular Immune Response.** To determine which immunological parameters are related to resistance after the secondary challenge, the SLA and the LRP driven production of IL-4, IL-10, and IFN- $\gamma$  was assayed at week seven after ear infection. Spleen cell cultures from control and reinfected mice were established to analyze the systemic response and DLN cells (retromaxillar) were cultured to analyze the local response induced by the ear infection. Spleen cells from reinfected mice produced higher amounts of IFN- $\gamma$  after SLA or LRP stimulation than control mice, but only the level of LRP specific IFN- $\gamma$  was significantly

different between the two groups. We observed that the level of SLA- and LRP-specific IFN- $\gamma$  detected in the DLN cell cultures was higher in control than in the reinfected mice (Figure 3(a)). Most likely, the high level of IFN- $\gamma$  detected in retromaxillar DLN could be related to the high number of parasites found in control animals (Figure 2(b)) that may be stimulating the production of IFN- $\gamma$  by Th1 cells, since in this model of infection the presence of parasites is correlated with IFN- $\gamma$  production [26]. The IL-10 and IL-4 production after stimulation with SLA or LRP was barely detected in the spleen and DLN cells from reinfected mice whereas in the spleen cell cultures and especially in the DLN cell culture supernatants from control mice high levels of these cytokines were measured (Figures 3(b) and 3(c)).

Those data are compatible with the fact that Th1/Th2 mixed responses were elicited after infection in control mice, characterized by the production of parasite specific IFN- $\gamma$  and IL-4 cytokines. In addition, the presence of high levels of parasite specific IL-10 may be also implicated in the progression of the disease, since the inactivating effect of this cytokine in infected macrophages has been related

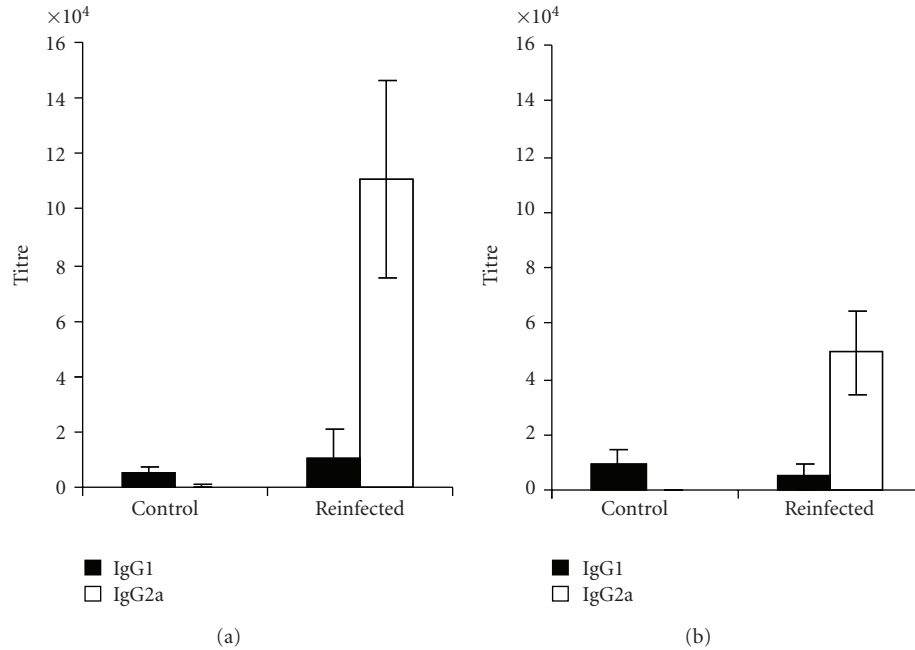


FIGURE 4: Analysis of the humoral responses. Serum samples from control and vaccinated reinfected mice were obtained seven weeks after challenge in the ear dermis. The titre for IgG1 and IgG2a antibodies against LRP (a) and SLA (b) was determined individually by ELISA. Results are expressed as the mean  $\pm$  SD.

TABLE 1: Cytokine production by popliteal DLN cells from vaccinated reinfected mice at week seven after secondary challenge.

	SLA	LRP	Medium
IFN- $\gamma$	5837.16 $\pm$ 834.82	5773.52 $\pm$ 1411, 14	1635, 11 $\pm$ 607, 89
IL-10	526.31 $\pm$ 214.98	408,92 $\pm$ 233.36	104, 39 $\pm$ 57, 13
IL-4	229.54 $\pm$ 58.78	44.36 $\pm$ 31.22	54.38 $\pm$ 37.89

The level of cytokines was determined by ELISA in the supernatant of popliteal DLN cells obtained from reinfected mice at week seven post rechallenge, after in vitro stimulation with 12  $\mu$ g/ml of SLA and LRP. Mean  $\pm$  SD of samples from six mice is shown (pg/ml).

with BALB/c mice susceptibility against *L. major* infection [29–32]. The pattern of cytokine production observed in infected control mice, with detectable level of parasite specific production of IFN- $\gamma$ , IL-10 and IL-4 was previously observed after infection with a pathogenic challenge of *L. major* in BALB/c ears [25, 26, 33]. On the contrary, a Th1-mediated IFN- $\gamma$  production was elicited in the reinfected mice group in the absence of Th2 responses and IL-10 mediated regulatory responses.

The SLA and the LRP driven production of IL-4, IL-10, and IFN- $\gamma$  was also assayed in the popliteal DLN of the reinfected mice. Although detectable levels of the three cytokines were observed, the level of IFN- $\gamma$  was higher than the levels of IL-10 and IL-4 (Table 1). A high ratio of IFN- $\gamma$ /IL-10 and IFN- $\gamma$ /IL-4 for both parasite proteins preparations (11.1 and 25.5 for SLA; 14.1 and 130.15 for LRP, respectively) was obtained, indicating that a parasite-specific IFN- $\gamma$  response was still maintained at week seven after secondary challenge in the popliteal DLN, yet in the presence of IL-4 and IL-10 cytokines. This Th1/Th2 mixed response may account for the increment observed in the number of parasites after secondary infection in the popliteal DLN.

**3.4. Analysis of the Humoral Responses.** The humoral response elicited in control mice and in the reinfected mice was analyzed at week seven after parasite challenge in the ear dermis. The titre of anti-LRP and anti-SLA specific IgG1 and IgG2a antibodies were determined, since the presence of IgG1 and IgG2a antibodies is considered a marker of Th2 and Th1 type responses, respectively [34]. In the sera from control mice the anti-*Leishmania* predominant antibodies were of the IgG1 isotype and very low but detectable levels of IgG2a were observed (Figures 4(a) and 4(b)). On the contrary, vaccinated reinfected mice showed high titres of IgG2a antibodies against LRP (Figure 4(a)) and SLA (Figure 4(b)). These humoral responses are in agreement with the nature of cellular responses observed after in vitro stimulation with both antigenic preparations. A strong Th1 response was elicited in vaccinated reinfected mice after parasite rechallenge having a resistant phenotype. On the contrary, antibodies found in the sera from mice of the control group were mainly of the IgG1 isotype as expected for their nonhealing phenotypes.

Altogether, our data showed that the immune response elicited in the LRP + CpG ODN vaccinated mice after

the primary infection was able to control a secondary challenge. Acquisition of the resistant phenotype was correlated to the capacity to induce a Th1 response (large amounts of parasite specific production of IFN- $\gamma$  and a high anti-leishmanial proteins IgG2a/IgG1 ratio) in the absence of Th2 or IL-10 mediated responses. The immune responses associated with the resistance after secondary infection in the vaccinated-infected mice were similar to that obtained in BALB/c mice that controlled a secondary infection in the ear after a primary infection in the contralateral ear, showing a Th1 response after rechallenge [26]. It is important to note that protection in these mice only occurred when lesions were developed in the primary site of infection [26], whereas LRP + CpG ODN vaccinated mice became resistant after primary challenge without the development of dermal lesions. Also, protection to reinfection achieved by BALB/c mice infected with a low infection dose in the footpad was dependent on the induction of Th1 responses [11, 35]. Here we show that that the immune response elicited by the LRP + CpG ODN vaccine after primary challenge was able to control the development of lesions and generated a long-term immune state necessary for the maintenance of immunity to further infection. The presence of parasites in the popliteal DLN may be related with this Th1 response, since it has been demonstrated that the presence of parasite antigens is necessary for the maintenance of cell mediated immunity in BALB/c mice [14, 36].

#### 4. Conclusion

The data reported here provided evidence that BALB/c mice protected against the development of dermal pathology due to *L. major* s.c challenge after LRP + CpG ODN vaccination have acquired an immunological status which conferred them the capacity to resist a further infection (an appealing feature for a vaccine that might be employed in endemic areas, where reexposure to the parasite would be very frequent). After a secondary challenge in the ear dermis, these mice showed a robust protection against *L. major* infection. Very low parasite burdens and development of dermal lesions in the site of reinfection were found. A specific Th1 protective response after the secondary challenge was correlated to resistance to reinfection. Thus, the immune state generated by the combination of vaccination with LRP + CpG ODN and the primary infection is extremely potent, leading to a rapid and efficient elimination of the parasite from the site of reinfection. Despite extensive research efforts, leishmanization with viable *Leishmania* parasites is the only vaccine with proven efficacy in humans [2, 37]. The induction of long-term immune responses by vaccines based on parasite extracts or recombinant parasite products that protected mice against the development of leishmaniasis after a primary challenge has been extensively reported [8, 38]. However, there is scarcity of studies analyzing the long-term maintenance of resistance to reinfection of vaccinated mice. Extrapolation of this approach to other animal or human models is hazardous but our findings may be relevant to develop effective tools against leishmaniasis based on defined *Leishmania* subunits.

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