

Anti-leishmanial Vaccination with Parasite Antigens in Liposomes Relies on the Effective Induction of T cell Responses and Inhibits Parasite Metastasis in Mice

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Abstract

In this work we vaccinated CBA/ca and C57BL/10 mice presenting different clinical forms of leishmaniasis after infection with *L. mexicana* parasites. CBA/ca mice develop localized cutaneous leishmaniasis while C57BL/10 mice develop disseminated cutaneous leishmaniasis 14 weeks postinfection. We used an electrophoretic procedure to record parasite molecules present in our vaccine preparation. Vaccination was effective to prevent disease development in CBA/ca but not C57BL/10 animals. There were not differences in the number of T cell subsets among normal and vaccinated animals of either strain as determined by flow cytometry. DTH response and serum INF-gamma [IFN] levels were induced in vaccinated CBA/ca but C57BL/10 animals. Vaccine induced protection was adoptively transferred by CD4⁺ and CD8⁺ T cells into CBA/ca [not in C57BL/10 mice] naïve syngeneic recipient mice challenged with virulent parasites. We concluded that effective protection through vaccination of CBA/ca but not C57BL/10 mice correlates with the induction of protective CD4⁺ and CD8⁺ T cells, specific DTH response and production of IFN while the synthesis of specific IgG anti-Leishmania is induced in both strains of vaccinated animals and vaccination prevents parasite metastasis to different skin locations in C57BL/10 mice.

Keywords: T cell-subpopulations; Interferon gamma; *Leishmania mexicana*; Liposomes; Monoclonal antibodies

Introduction

L. mexicana is a protozoan parasite responsible for a number of human cases of localized cutaneous leishmaniasis [LCL] in Southern USA, Mexico and Central America and very few cases of disseminated leishmaniasis [LCD] in Southern Mexico [1]. Different vaccine strategies have been tested in an attempt to prevent different forms of experimental leishmaniasis. Experimental murine infection induced with different *Leishmania* species could be prevented by previous inoculation of radio-attenuated or chemically attenuated parasites [2,3]. Different carriers for antigen release have been developed to construct vaccines against murine leishmaniasis. Liposomes are efficient adjuvant for vaccination and good vehicles for slow release of antigens or drugs to treat leishmaniasis and they also avoid the use of living organisms as vaccines [3]. Immunization of mice using microencapsulated purified glycoproteins from *L. donovani*, *L. major* and *L. mexicana* was reported to induce protection [4-9]. Previous reports have indicated that certain strains of mice can be fully protected against *L. mexicana* infection by intraperitoneal immunization with liposomes [8,10] or niosomes [9] containing gp63 or whole membrane antigens from *L. mexicana* [8,9]. Recently, a synthetic peptide derived from the glycosomal enzyme of *Leishmania* has been reported to protect mice against different forms of leishmaniasis [10]. T helper (Th) cells, dendritic cells and cytokines have shown to be central players in leishmaniasis [11,12]. Therefore, identification of immuno-prophylactic molecules that could induce growth and expansion of protective cells could represent the basis for a rational design of new vaccines to prevent leishmaniasis. This work

was designed to determine the role of specific antibodies, Delayed Type Hypersensitivity [DTH] responses, production of interferon-gamma [IFN] and the role of T cell subpopulations associated with successful vaccination. In this report we present evidence of the generation of protective CD4⁺, CD8⁺ T cells, the induction of a specific DTH response and production of IFN only after effective vaccination of CBA/ca but C57BL/10 mice. Furthermore, this vaccine prevents parasite metastasis in C57BL/10 animals.

Material and Methods

Parasites

L. mexicana promastigotes [MNYC/BZ/62/M379] were maintained by serial passage of amastigotes inoculated subcutaneously into the shaven rumps of BALB/c and CBA/ca mice. Stationary phase promastigotes were produced by *in vitro* culture from amastigotes in RPMI-1640 [pH=7.1; from Gibco], L- glutamine [2 mM, Gibco], penicillin G potassium [100 IU/ml, Gibco] and streptomycin [50 µg/ml] supplemented with 10% fetal calf serum [FBS Gibco]. Culture media was seeded with parasites recovered from the inside of fully grown lesions in infected mice. Culture of parasites was carried out at room temperature for no more than a month to avoid loss of parasite infectivity. Parasite stock was maintained in BALB/c mice following guidelines approved by The University of Campeche.

Animals

Ten to fifteen weeks old sex matched CBA/ca, C57BL/10 and BALB/c mice were used in this work. All animal experiments

performed throughout this work followed the accepted Bioethical regulations of the University of Campeche and the local government.

Antigen preparation and encapsulation into liposomes

Parasite membrane antigens were extracted from *L. mexicana* stationary phase promastigotes following the procedure we previously described [8,9]. Stationary phase promastigotes ($2-5 \times 10^{10}$) were washed a few times with phosphate buffered saline [PBS]. Next, parasites were suspended in 40-50 ml of hypotonic buffer [10 mM Tris-HCl; 2 mM ethylenediaminetetraacetic acid (EDTA), pH=7.8; 0.050 mM of Tosyl-L-lysyl-chloromethane hydrochloride (TLCK); 0.015 mM of leupeptin] and left in ice for 15 min. Parasites suspension was disrupted by several rounds of freeze-thawing. Disrupted parasites were examined under light microscopy to ensure for cell lysis, thereafter they were spun down at 10,000 xg for 30 min at 4°C. Pellet so obtained was resuspended in 5-10 mL of PBS containing enzyme inhibitors [50 µM TLCK, 15 µM leupeptide and 2% w/v octylglucoside, pH=7.1], left in ice for about 20-30 min and spun down for 1 h at 100,000 × g in a Beckman ultracentrifuge. The supernatant containing parasite membrane antigens was tested for protein content and was frozen and stored until use. Glycoproteins were separated and their molecular weight was identified by SDS-PAGE using a cocktail of molecular weight markers containing: Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36,000 D), Ovalbumin, chicken egg (45,000 D), Albumin, bovine serum (66,000 D), β-Galactosidase, *E. coli* (116,000 D), Myosin, porcine heart (200,000 D) from Sigma followed by silver stain as originally was described by Laemmli [13] and Wray [14] respectively. Protocol for reconstitution of antigen into liposomes is as follows: A suspension was produced by mixing 32 mg soybean lecithin, 2 mg diacetyl phosphate and 6 mg of cholesterol with 2 ml of chloroform in a 500 ml conical flask [these chemicals were purchased from Sigma]. The mixture was dried under a N₂ steam to produce a thin film and incubated for 1 h at 37°C. Parasite molecules prepared as described before were added to the lipid film and sonicated in an ice bath for 5 min. A ratio of 2 µg of protein of parasite sample to a 1 mg of phospholipid and cholesterol mixture was maintained in all experiments. The detergent was removed by overnight dialysis against PBS at 4°C and the resulting vesicles were washed with PBS, centrifuged for 1 h at 100,000 × g and were frozen and stored at -20°C until use. The same protocol was applied for the preparation of empty liposomes, except that PBS without parasite antigen was used.

Vaccination protocol

Vaccination protocol was performed as we previously described [8,9]: 8 µg of protein contained in membrane antigens reconstituted into unilamellar liposomes [as described previously] were intraperitoneally [ip] inoculated into mice twice at two weeks interval. One month after the last immunization, both controls and experimental CBA/ca or C57BL/10 mice were infected in the shaven rump with 3×10^6 *L. mexicana* virulent promastigotes and lesion growth was recorded for 14 weeks. Control groups included mice inoculated only with antigen/PBS and those inoculated with empty liposomes. At the end of the experiment all CBA/ca and C57BL/10 animals were individually inspected for dissemination of lesions throughout the body including skin, spleen and liver.

ELISA assay for IgG and IFN determinations

A solid phase ELISA was performed as originally described by Ngo to measure specific antibodies [15]: *L. mexicana* membrane antigens [one µg of protein/well] were dried onto 96 wells polyvinyl microtiter plates previously covered with 1% BSA. Serum dilutions (1:100) were added to each microtiter plate well in duplicate, including a pool of sera from chronically infected and normal mice used as positive and negative controls, respectively. Fifty µL of each sample were added and incubated at 37°C for 1.5 h and then washed [0.05% Tween 20 in PBS, pH=7.4]. One hundred µL of peroxidase conjugate [1:5000 (BioLegend) diluted with 1% egg albumin in 20 mM Tris, pH=7.4] were added and incubated for 1.5 h at 37°C followed by washing. Fifty µL of substrate [tetramethylbenzidine in 0.1 M acetate-citrate buffer, pH=5.5] were added to each well and incubated in the dark at room temperature for 30 min. Finally, the reaction was stopped by adding 50 µL of 2.5 M of sulfuric acid and read in an ELISA reader [Titrek multiscan] at 450 nm. Results are calculated by dividing the optical density of experimental samples by the optical density of negative controls. A sandwich ELISA was used to quantify interferon-gamma [IFN] in serum samples [16]. A 96 ELISA plate was coated with anti-IFN capture antibody [Ab, from BioLegend]. Next, washed plates [0.05% Tween 20 in PBS, pH=7.4] were treated with PBS [10% FBS] and after this 50 µL of unknown samples or serially diluted standards [recombinant IFN from BioLegend] were added and incubated overnight. After thorough washing plates was incubated 1 h with detection Ab [biotinylated anti-IFN Ab from BioLegend]. Next, plates were incubated for 30 min with streptavidin conjugated alkaline phosphatase and then washed before adding p-nitrophenyl phosphate substrate (PNPP) followed by reading in an ELISA reader at 405 nm. IFN concentrations were obtained by extrapolation from a standard curve.

Flow cytometry

Flow cytometry analysis of T cell subpopulations was performed as previously described [19]: Spleen cells [5×10^5] were obtained from either vaccinated and infected or only infected CBA/ca or C57BL/10 mice, we also included spleen cells from normal mice for flow cytometry testing. Cells were dispensed into conical microtitration wells and mixed with 10 µL of MoAbs diluted 1:500 [anti CD3 (Thy 1.1), anti CD4 (L3T4) and anti CD8 (Lyt-2)], or 1:10 anti CD19 [B220] from BioLegend, incubated for 45 min at 4°C and washed with a solution of 1% BSA in PBS-Azide (0.01%). A second antibody [fluorescein isothiocyanate (FITC)-goat anti rat IgG from BioLegend] was added to the wells, incubated at 4°C and washed. Cells were spun down and pellet was resuspended in PBS-formaldehyde [1%]. Preparations were analyzed by flow cytometry in a Becton Dickinson Cytofluorograph. Results are expressed as percentage of CD3 [Thy 1.1], CD4 [L3T4], CD8 [Lyt-2] or CD19 [B220] expressing spleen cells.

Delayed type hypersensitivity response [DTH]

The induction and evaluation of a DTH response was performed as follows: DTH responses were induced in all CBA/ca or C57BL/10 mice by inoculation in the left footpads of a formaldehyde fixed suspension of *L. mexicana* promastigotes. Approximately 10^8 promastigotes were suspended in PBS containing 1% formaldehyde and parasite mobility assessed under light microscopy using a 40x objective. Killed parasites were washed three times with sterile PBS and adjusted to a density of 5×10^7 parasites/ml. Twenty µL of this suspension was inoculated into the left footpad of each mouse and 20 µL of sterile PBS was inoculated

into the contralateral footpad (right footpad). Footpad thickness of rear left and right footpads were measured with a Vernier caliper before challenge and 24 h afterwards. We recorded differences in footpad swelling (mm of rear left–rear right footpad thickness).

Preparation of T-cell subsets for adoptive transfer experiments [negative selection]. Pooled spleen cell suspensions from vaccinated or infected mice were passed through a nylon wool column filled with warmth culture media and T cells were obtained as described somewhere else [19]. Isolated T cells were adjusted to 10^7 cells/ml and treated with 50 μ L of a 1:500 dilution of anti CD4 [L3T4] or anti CD8 [Lyt-2] MoAbs [Seralab] for 60 min at 4°C. Following removal of excess antibody, cells were treated twice with 100 μ L [1:30 dilution] of rabbit serum [complement source] for 60 min at room temperature. Dead cells were removed by low speed centrifugation [400 \times g, 20 min at room temperature] using culture media. After washing pelleted viable cells were counted using the trypan blue [Sigma] exclusion test and adjusted to inoculate 3×10^6 cells/mouse by intraperitoneal administration into naive syngeneic recipients [CBA/ca or C57BL/10 naive mice]. Next all groups of animals were infected with 3×10^6 *L. mexicana* promastigotes and disease progression was recorded for 8 weeks. Efficiency of single depletions [negative selection] using anti CD4 or anti CD8 MoAbs plus rabbit complement was monitored by flow cytometry in a Becton Dickinson instrument. Purity and viability of transferred cells were more than 95% in all cases.

Statistical analysis

The t test of Student and Dunnett's test were used in all statistical analysis.

Results

Vaccine preparation

Parasite antigens were prepared using a non-ionic detergent [octylglucoside from Sigma] as described in material and methods. Electrophoretic analysis [SDS-PAGE] of this material presented a few strong bands at approximately 200 kD and several weaker bands at 15, 60 and 65 kD [Figure 1]. Antigens so prepared were encapsulated into liposomes and used to vaccinate CBA/ca and C57BL/10 mice.

Vaccination of CBA/ca and C57BL/10 mice

To determine effectiveness of vaccination, groups of CBA/ca and C57BL/10 mice were intraperitoneally vaccinated twice at two weeks interval using membrane antigens [8 μ g of protein/mouse] reconstituted into liposomes. Groups of 6 unimmunized controls and 6 vaccinated animals were challenged with 3×10^6 stationary phase *L. mexicana* parasites and lesion growth was recorded for 14 weeks. All vaccinated CBA/ca mice proved to be protected developing no lesions at the site of infection while all control mice developed uncuring single nodular lesions without evidence of parasite dissemination throughout the skin, spleen or liver by week 14 after infection. Lesion measurements between control and vaccinated CBA/ca mice were significantly different from week 6 after infection and onwards [$p < 0.05$; Figure 2A]. In contrast, vaccinated C57BL/10 mice did not developed protection against a homologous challenge with virulent parasites and lesion growth was not different than control mice throughout the experiment [$p > 0.05$; Figure 2B].

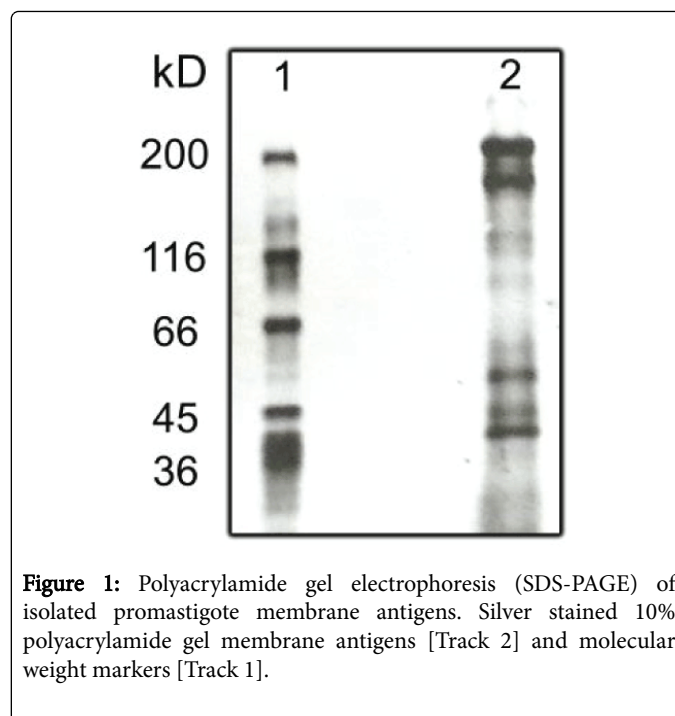


Figure 1: Polyacrylamide gel electrophoresis (SDS-PAGE) of isolated promastigote membrane antigens. Silver stained 10% polyacrylamide gel membrane antigens [Track 2] and molecular weight markers [Track 1].

However, none of vaccinated C57BL/10 mice developed metastatic lesions (Table 1) while all control C57BL/10 animals developed lesions at the infection site and metastasized to other skin locations such as snout, tail and footpads (Figure 2B, Table 1).

Type of lesions:	Single lesion	Multiple lesions	metastatic	skin
Control CBA mice	6/6 mice	0/6 mice		
Vaccinated CBA mice	0/6 mice	0/6 mice		
Control C57BL/10 mice	6/6 mice	6/6#		
Vaccinated C57BL/10 mice	6/6 mice	0/6##		

All animals developed 2 or more metastatic lesions on snout, tail or footpads.
none of all 6 mice (0/6 mice) developed metastatic lesions 14 weeks post-infection. This data represents a replica of two different experiments.

Table 1: Lesion development in vaccinated mice. Development of single or metastatic lesions disseminated throughout the skin.

We did not find evidence of parasite spread to spleen or liver in control or vaccinated C57BL/10 mice by week 14. Additional control groups of CBA/ca and C57BL/10 mice were treated with two intraperitoneal injections of membrane antigens alone or empty liposomes developing un-curing lesions not different from those growing in controls by week 14 after infection [Figure 2].

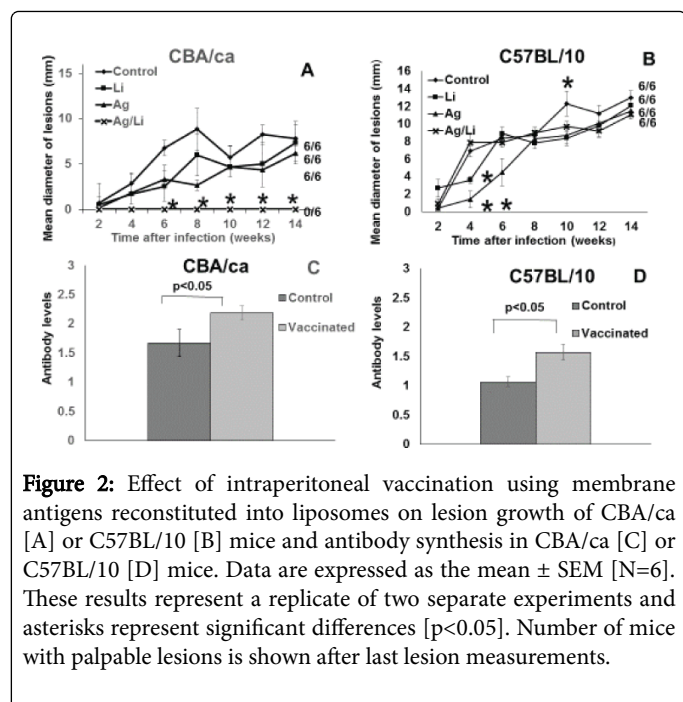


Figure 2: Effect of intraperitoneal vaccination using membrane antigens reconstituted in liposomes on lesion growth of CBA/ca [A] or C57BL/10 [B] mice and antibody synthesis in CBA/ca [C] or C57BL/10 [D] mice. Data are expressed as the mean \pm SEM [N=6]. These results represent a replicate of two separate experiments and asterisks represent significant differences [$p<0.05$]. Number of mice with palpable lesions is shown after last lesion measurements.

Production of IgG anti Leishmania in vaccinated mice

Specific antibodies were measured in vaccinated and infected and only infected (controls) CBA/ca or C57BL/10 mice two weeks after last vaccination or infection with living parasites of both strains of mice. The IgG response in vaccinated CBA/ca mice showed a significant increase as compared to control CBA/ca mice inoculated with living parasites [$p<0.05$; Figure 2C]. Specific IgG tested in C57BL/10 mice induced less, as compared to CBA/ca mice, but still significantly higher [$p<0.05$] synthesis of Abs when compared to C57BL/10 control mice [Figure 2D].

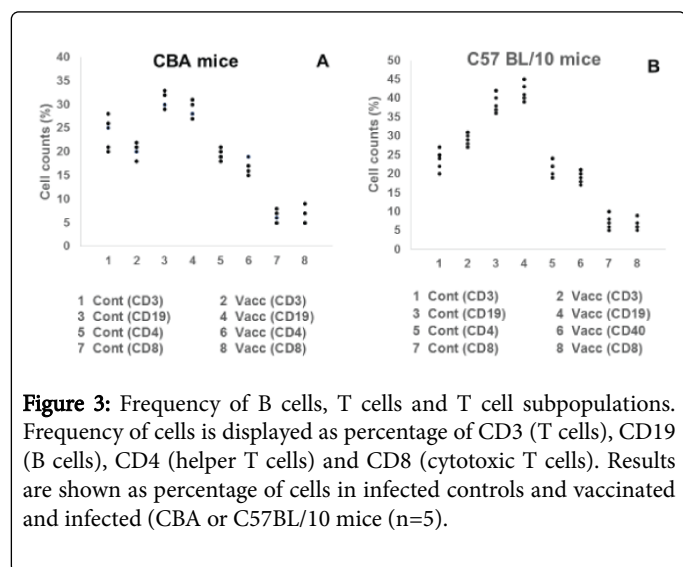


Figure 3: Frequency of B cells, T cells and T cell subpopulations. Frequency of cells is displayed as percentage of CD3 (T cells), CD19 (B cells), CD4 (helper T cells) and CD8 (cytotoxic T cells). Results are shown as percentage of cells in infected controls and vaccinated and infected (CBA or C57BL/10 mice (n=5).

Flow cytometry analysis of lymphocyte-subpopulations in vaccinated mice

Flow cytometry analysis of murine T cells subpopulations and whole T and B cells was performed to assess the frequency of these cells in spleens of CBA/ca and C57BL/10 mice. The analysis of spleen cells revealed that the percentage of CD3⁺ [Thy-1.1⁺], CD4⁺ [L3T4⁺], CD8⁺ [Lyt-2⁺] and CD19⁺ [B220⁺] cells did not change appreciably among vaccinated CBA/ca or C57BL/10 mice and their corresponding controls [Figures 3A and 3B respectively].

Adoptive transfer of T cell subpopulations from vaccinated mice

Adoptive transfer of T cell subpopulations showed that vaccinated and fully protected CBA/ca mice transfer vaccine-induced protection to naive recipients infected with virulent *L. mexicana* parasites (Figure 4A). Interestingly adoptive transfer of these cells from vaccinated and unprotected C57BL/10 mice fail to adoptively transfer protection to naive syngeneic mice challenged with virulent *L. mexicana* parasites (Figure 4B).

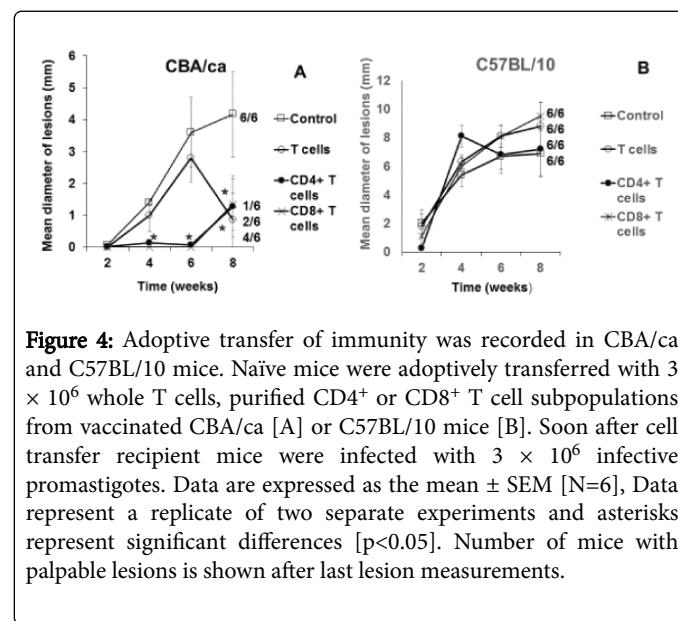


Figure 4: Adoptive transfer of immunity was recorded in CBA/ca and C57BL/10 mice. Naïve mice were adoptively transferred with 3×10^6 whole T cells, purified CD4⁺ or CD8⁺ T cell subpopulations from vaccinated CBA/ca [A] or C57BL/10 mice [B]. Soon after cell transfer recipient mice were infected with 3×10^6 infective promastigotes. Data are expressed as the mean \pm SEM [N=6], Data represent a replicate of two separate experiments and asterisks represent significant differences [$p<0.05$]. Number of mice with palpable lesions is shown after last lesion measurements.

Induction of delayed type hypersensitivity response [DTH] response and interferon-gamma [IFN] serum levels in vaccinated mice

To better understand cellular responses mediated by T lymphocytes we undertook a series of experiments. One of them is the induction and measurement of a delayed type hypersensitivity reaction in vaccinated or infected CBA/ca and C57BL/10 mice and their corresponding syngeneic controls. DTH response was induced and measured in the footpad of intraperitoneally vaccinated mice and their infected controls. Results of these data set show that CBA/ca animals developed a macroscopically apparent and specific DTH response in the footpad after 24 h of inoculation of formaldehyde fixed parasites. Footpad sizes between control and vaccinated CBA/ca mice were statistically significant ($p<0.05$, Table 2).

	CBA/ca		C57BL/10	
	DTH [mm]	Serum levels of IFN [pg/mL]	DTH [mm]	Serum levels of IFN [pg/mL]
Control	0.17+0.018	97.5+2.14	0.27+0.03	103.3+4.02
Vaccinated	0.27+0.014**	410+6.48**	0.24+0.05	101.6+10.17

DTH responses were induced with formalized parasites and recorded 24 h later. Sera IFN were measured by ELISA.
**p<0.05.

Table 2: Delayed type hypersensitivity and sera interferon levels in vaccinated mice.

After inoculation of formaldehyde treated parasites, vaccinated C57BL/10 mice did not developed a DTH and produce a slight but not significant reduction in footpad sizes compared to their syngeneic controls [p>0.05, Table II]. Interferon-gamma concentrations were recorded in sera from vaccinated and infected controls and we found significantly higher concentration in successfully vaccinated CBA/ca mice as compared to their syngeneic controls [Table 2, p<0.05]. However, unsuccessfully vaccinated C57BL/10 animals did not produce significantly different amounts of IFN as compared to their syngeneic infected controls (Table 2, p>0.05).

Discussion

For a long time considerable scientific effort has been made to develop an effective vaccine to prevent human leishmaniasis. Unfortunately, there are currently not commercial vaccines to prevent this disease and the use of virulent parasites for leishmanization is a dangerous practice and has been discontinued [1,3]. Immunogenetics of cutaneous leishmaniasis is an important factor in the regulation of healing or susceptibility of the disease [17,18]. Animals that express the H-2k or H-2b haplotypes such as CBA/ca or C57BL/10 mice respectively are susceptible to *L. mexicana* infection and develop a lesion at the site of infection [17,18]. Furthermore, C57BL/10 mice present parasite metastasis of chronic infections [17] while CBA/ca mice develop a single non-healing lesion at the site of infection and do not present metastasis during the time period of our experiments [17,19]. In this work, we compared some immunological and clinical outcomes of our vaccine in these two animal strains. Our vaccine fully protected CBA/ca but not C57BL/10 mice against a future challenge with virulent parasites. Furthermore this vaccine prevented parasite dissemination in C57BL/10 mice away from the site of initial infection. We also present evidence suggesting that effective vaccination against leishmaniasis would be associated to development of activated CD4⁺ and CD8⁺ T cells that produce IFN and induce strong DTH responses. Interferon-gamma is a key cytokine in the induction of protective responses against different species of *Leishmania* and has been used in the successful treatment of different clinical forms of the disease [1,3]. Therefore it is not surprising that only successfully vaccinated CBA/ca mice produce significantly more IFN than infected syngeneic controls. Other cell lineages such as natural killer cells, eosinophils, mast cells, Langerhans and CD4- 8- T cells (double negative T cells) would be involved in vaccine induced protection [19]. In a previous publication from our group we only reported the effective induction of CD4⁺, CD8⁺, CD4-CD8- T cells in intraperitoneally vaccinated CBA/ca mice and we did not studied the role of IFN after vaccination [19]. Furthermore we also reported on effectiveness of subcutaneous and

intraperitoneal vaccination of different animal's strains including CBA/ca, C57BL/6 and C57BL/10 mice and only recorded specific IgG levels [8,9]. The present work extends our previous observations to additional clinical and immunological parameters described above such as the production of IFN in both animal strains and inhibition of parasite metastasis in C57BL/10 mice. Although the mechanism for vaccine induced protection is still to be fully understood, perhaps parasite-specific T cell anergy showed by C57BL/10 mice could be an important part of the mechanism of vaccine failure in this strain of mice. Ongoing experiments in different laboratories are devoted to discover new strategies for experimental vaccination. One of them includes mining the *Leishmania* genome to develop DNA based vaccines [20]. Other groups develop new carriers for synthetic epitopes that bind class I and II MHC molecules and are used to protect mice from an intravenous challenge with *L. chagasi* [21,22]. Finally, we compared for the first time effectiveness of a vaccine preparation in mice with different clinical and immunological outcomes of cutaneous leishmaniasis. This vaccination correlates with protection and prevents parasite metastasis. It is clear that working on different scientific strategies would lead to the development of an effective anti-leishmanial vaccine for human application.

Declaration of Interest

This work was financially supported by CONCYT (Fondo Sectorial Salud 140091).

Conflict of Interest

Authors declare they do not have any conflict of interest.

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CMLD Designed and performed experiments reported in this work and wrote the manuscript.

APIM Assisted in design and perform experiments reported in this work and prepared some art work.

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