

## Vaccine-induced protection against *Leishmania amazonensis* is obtained in the absence of IL-12/23p40

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### Abstract

Protozoa of the genus *Leishmania* are intracellular parasites of macrophages and may cause diverse clinical forms of leishmaniasis, including cutaneous, diffuse cutaneous, mucocutaneous and visceral leishmaniasis. Infection with *L. major* in mice indicates that a protective immune response is achieved when Th1 cells are developed. Thus, adoptive or vaccine-induced protection against leishmaniasis is largely dependent on cell-mediated immunity and IFN- $\gamma$  production. Induction of a Th1 response is dependent on the presence of IL-12 whilst lymphocytes are activated. This study was aimed at evaluating the role of IL-12 during infection with *L. amazonensis* and after vaccination with Leishvacin® (killed *Leishmania amazonensis* promastigotes), since the role of this cytokine in vaccine-induced immunity with this preparation in experimental models or in humans is not yet elucidated. Hence, C57BL/6 interleukin-12-deficient mice (IL-12p40<sup>-/-</sup>) and wild-type controls (wt) were infected with *L. amazonensis* and the course of infection, parasite burden and cytokine production were compared. IL-12p40<sup>-/-</sup> mice were more susceptible to *L. amazonensis* than wt: lesions and parasite burden were larger in IL-12p40<sup>-/-</sup> when compared to wt. Interestingly, IL-4 was not produced in the absence of IL-12 in response to infection with *L. amazonensis*. To evaluate the role of IL-12 in the vaccine-induced immunity against *L. amazonensis* infection, IL-12p40<sup>-/-</sup> wt mice were vaccinated in the base of the tail and subsequently challenged with *L. amazonensis* in the footpads. Surprisingly, vaccinated IL-12p40<sup>-/-</sup> mice developed smaller lesions and had fewer parasites in footpads than non-vaccinated controls. Lymph node and spleen cells from vaccinated IL-12p40<sup>-/-</sup> mice did not produce high levels of IFN- $\gamma$  in response to in vitro stimulus with antigen. Hence, partial protection against infection with *L. amazonensis* could be obtained in the absence of functional IL-12 and a typical Th1 response. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** *Leishmania*; Leishmaniasis; Protozoa; Parasite; Vaccine; Adjuvant; IL-12

### 1. Introduction

Leishmaniasis are parasitic infections of animals and humans caused by different species of a protozoan of the genus *Leishma-*

*nia*. The clinical manifestations of the diseases are determined by the species of *Leishmania* that infects the host and by the immune response of the host to parasite [1]. *Leishmania amazonensis*, a member of the *Leishmania mexicana* complex, has been isolated from patients with diverse clinical forms of the disease in South American countries, including cutaneous leishmaniasis, diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis [2].

A clear paradigm has been established for the role of cytokines in resistance and susceptibility during experimental

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infection with another parasite of the genus, *L. major*, in inbred mice: IL-4 production by BALB/c mice leads to susceptibility, while IL-12-dependent IFN- $\gamma$  production by most mouse strains leads to macrophage activation and control of parasite growth [3]. However, there is evidence that the host immune response to *L. amazonensis* infection is different from that directed to *L. major*. C3H, C57BL/6 and C57BL/10 mice, which are resistant to *L. major*, develop chronic lesions with persistent parasitism when infected with *L. amazonensis* [4–7]. The maintenance of chronic lesions is independent of the expression of IL-4 and a corresponding Th2 response [4,5,7]. *L. amazonensis* triggers early production of IL-12 and IFN- $\gamma$  in C57BL/6 mice similarly to *L. major* [8], but infection of C3H mice results in production of low levels of IL-12 and IFN- $\gamma$  by antigen-specific CD4<sup>+</sup> T cells [5]. In fact, chronic infection by *L. amazonensis* in C3H and C57BL/6 mice persists even after administration of exogenous IL-12 [5] or IFN- $\gamma$  [9]. The inability of IL-12 to drive an effective cell-mediated immune response during *L. amazonensis* infection suggests that the parasite can evoke a potent immunomodulatory mechanism to evade widespread parasite killing and promote a chronic infection. However, lesion development and parasite burden have been shown to be exacerbated by CD4<sup>+</sup> T cells [10,11], demonstrating that T cells are activated during *L. amazonensis* infection and that they contribute significantly to the immuno-pathology of the chronic disease [10]. In addition, sub-optimal IFN- $\gamma$  production seems to favor parasite growth [10]. However, the mechanisms responsible for susceptibility of mice to this New World parasite species remain obscure. The importance of understanding the factors involved in the maintenance of this chronic disease is highlighted by the fact that chronic cutaneous leishmaniasis in humans is often correlated with a poor T-cell-mediated immune response [12] or a mixed T-cell response [13].

Adoptive or vaccine-induced protection against *Leishmania* is largely dependent on cell-mediated immunity, Th1 lymphocytes and IFN- $\gamma$  production [14]. Different antigen preparations, including defined and recombinant antigens, have been demonstrated to induce this type of beneficial response in experimental models [15–18]. During the last 20 years, first generation vaccines composed of killed *Leishmania* promastigotes and manufactured according to Mayrink et al. [17] have been subjected to clinical trials in Brazil [15,17,19–22]. For the sake of standardization, a more simplified version of the vaccine was produced by a former licensed Brazilian biotechnology company, Biobrás (Montes Claros, MG), using only one *Leishmania* strain (*L. amazonensis* strain IFLA/BR/1967/PH8), selected among the five present in the former vaccine.

Since Leishvacin is protective against infection with *L. amazonensis* in association with *Corynebacterium parvum* in the murine model, we chose this as our model vaccine to evaluate the role of IL-12 in the vaccine-induced protection. C57BL/6 interleukin-12-deficient mice (IL-12p40<sup>-/-</sup>) and wild-type controls (wt) were used. Our results show that IL-12 p40 is important in the late control of lesions caused by *L. amazonensis* and that, in its absence, partial resistance to infection is obtained in vaccinated mice.

## 2. Materials and methods

### 2.1. Animals

Female C57BL/6 and BALB/c mice (4–6 weeks old) were obtained from CEBIO (Centro de Bioterismo do Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brazil). Matrices of mice deficient in the production of the p40 chain of IL-12 by homologous recombination (IL-12p40<sup>-/-</sup>) in the C57BL/6 mice were kindly provided by Dr. Luiz Vicente Rizzo, Department of Immunology, University of São Paulo (São Paulo, Brazil), and bred in the Gnotobiology and Immunology Laboratory of the Instituto de Ciências Biológicas. During the experiments mice were kept in an animal facility with controlled environmental conditions and environmental barriers. Animals were fed a commercial diet for rodents (Labina—Purina SP, Brazil) ad libitum.

### 2.2. Parasites and antigens

*L. amazonensis* (IFLA/BR/1967/PH8 strain) and *L. major* (WHO MHOM/IL/80/Friedlin) promastigotes were grown to stationary phase (5-day-old culture) at 27 °C in Grace's insect medium (GIBCO Laboratories, Grand Island, NY) with 20% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, Utah), 2 mM L-glutamine, 100 U of penicillin-G-potassium and 100  $\mu$ g of streptomycin sulfate per ml. *Leishmania* antigen was obtained from stationary-phase promastigotes washed four times in 0.1 M phosphate-buffered saline pH 7.3 (PBS) and adjusted to a concentration of 10<sup>8</sup> organisms/ml. Parasite suspensions were submitted to four cycles of freezing at -70 °C followed by thawing at 37 °C. Antigens were stored at -70 °C and thawed immediately before use in cell cultures.

### 2.3. Vaccine

Vaccine was produced and provided by Biobrás (Montes Claros, Brazil). The vaccine strain of *L. amazonensis* was the same used for infections (IFLA/BR/1967/PH8).

### 2.4. Vaccination and infection

C57BL/6 wild-type (wt) and IL12p40<sup>-/-</sup> C57BL/6 mice were vaccinated according to Costa et al. [16]. Vaccine was administered subcutaneously in 0.15 ml at the base of the tail. Each animal received two inoculations at an interval of 7 days (unless otherwise stated), each dose containing 100 mg of vaccine protein plus 250 mg of *Corynebacterium parvum* (Laboratório de Extratos Alergênicos Ltda, Rio de Janeiro, RJ, Brazil). Twenty-eight days after the second dose, animals received a further 10 mg of vaccine, without adjuvant. Control groups in this study were unvaccinated C57BL/6 and IL12p40<sup>-/-</sup> mice and mice injected with *C. parvum* and saline (no antigen). Seven days after the last booster, animals were challenged with 10<sup>4</sup> *L. amazonensis* purified metacyclic promastigotes [23] from stationary phase cultures (5 days of culture) in the hind footpad.

In one experiment, mice were challenged 21 days after the last booster. Lesion size was measured during the course of infection with a dial micrometer and expressed as the difference in size between the infected footpad and the contralateral uninfected footpad.

### 2.5. Cell culture and parasite quantification

Spleen and lymph node (popliteal and inguinal) single cell suspensions were obtained as previously described [8] and cultured at  $5 \times 10^6 \text{ ml}^{-1}$  in the presence or absence of antigen preparation for 72 h. Supernatants were collected and used for cytokine assays. Parasites were quantified by limiting dilution, as previously described [24]. The footpads were homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at  $150 \times g$  and cells were concentrated by centrifugation at  $2000 \times g$ . Pellets were resuspended in 500  $\mu\text{l}$  of Grace's supplemented culture medium (see above). 220  $\mu\text{l}$  were plated onto culture plates and diluted in log-fold serial dilutions in supplemented Grace's insect tissue culture medium starting with a 1:10 dilution. Each sample was plated in duplicates and read 15 days after the beginning of the culture. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (i.e., the dilution corresponding to the last positive well) adjusted per microgram of tissue.

### 2.6. Cytokine assays

IL-4 and IFN- $\gamma$  were assayed by two-site ELISA as described [8]. IFN- $\gamma$  in the supernatants from spleen or lymph node cell cultures was assayed by two site ELISA using rat anti-IFN- $\gamma$  monoclonal antibody (mAb) R46A2 and polyclonal rabbit serum specific for the cytokine. ELISA for IFN- $\gamma$  had a sensitivity of 16 pg/ml. The assay for IL-4 was performed using 11B11 mAb for coating and biotinylated BVD6 mAb as detection of bound IL-4. ELISA for IL-4 had a sensitivity of 15 pg/ml.

### 2.7. IgE and antigen-specific IgG1 and IgG2a ELISAs

Total serum immunoglobulin E (IgE) was quantitated by ELISA. The plates were coated with mouse anti-IgE (clone 2363, Southern Biotechnology, Birmingham, AL, USA) at a 1:500 dilution overnight at 4 °C. Sera were diluted 1:20. After 1 h incubation at 37 °C, plates were washed. After washing, wells were incubated with biotinylated murine anti-IgE antibody (clone R35-72, Southern Biotechnology) at a 1:500 dilution, washed as above, developed using horseradish peroxidase-streptavidin (Sigma, St. Louis, MO, USA). A positive control was performed using serum from BALB/c mice rendered allergic to ovalbumin (kindly provided by Janaina S. Saldanha and Dr. Denise Carmona, Departamento de Patologia/ICB-UFMG, Belo Horizonte). In order to detect antigen-specific IgG1 and IgG2a, ELISA for specific IgG1/IgG2 antibodies was optimized regarding antigen concentrations, sera and conjugate dilutions. *L. amazonensis* antigen preparation was derived from in vitro

promastigote cultures (10  $\mu\text{g}$  protein/ml) was diluted in 0.1 M sodium carbonate buffer (pH 9) and 100  $\mu\text{l}$  per well were used to coat flat bottom 96-well plates overnight at 4 °C. Plates were blocked for 1 h at 37 °C with PBS containing 1% bovine serum albumin (BSA, Sigma). Mouse sera were diluted 1:50 with PBS containing 1% BSA and serially diluted in the plate (1:3 dilutions) After 2 h incubation at 37 °C, plates were washed five times with PBS containing 0.05% Tween 20 (Sigma, St. Louis, USA). Wells were incubated with goat anti-mouse IgG1 or IgG2a (Southern Biotechnology) at a 1:4000 dilution washed and then incubated with rat anti-goat horseradish peroxidase-labeled antibody (Southern Biotechnology) at a 1:2000 dilution. Absorbance values were read at 492 nm in a Spectra Max Plus reader (Spectra Max Plus reader Molecular Devices Corporation, Sunnyvale, CA, USA).

### 2.8. ELISPOT assays

IL-4 and IFN- $\gamma$ -producing cells were determined by ELISPOT assay. MultiScreen-HA Cellulose Ester filtration 96-well plates (Millipore Corp., Bedford, MA, USA) were coated with 0.5  $\mu\text{g}/50 \mu\text{l}$ /well of anti-IL-4 (clone BVD4-1D11, PharMingen, San Diego, CA, USA) or 0.5  $\mu\text{g}/50 \mu\text{l}$ /well of anti-IFN- $\gamma$  (clone R4-6A2, PharMingen) in PBS overnight at 4 °C. The plates were washed twice with PBS and blocked using 5% FBS in DMEM (Dulbecco's minimal essential medium containing 2 mM L-glutamine, 100 U of penicillin 6-potassium/ml, 100  $\mu\text{g}$  of streptomycin/ml, 25 mM HEPES) for 2 h at 37 °C. The plates were washed with PBS. Single cell suspension of the popliteal lymph node and spleen cells the infected mice were obtained 5 and 8 weeks post-infection as indicated below. The cells were disassociated with a tissue homogenizer and were resuspended in complete tissue culture medium DMEM containing 10% FBS and were adjusted to a concentration of  $3 \times 10^5/200$  and  $1 \times 10^6/200 \mu\text{l}$ /well in 96-well plates. The plate was incubated with or without 100  $\mu\text{l}$  of *L. amazonensis* antigen (1 mg/ml) for 20 h at 37 °C in a humidified chamber containing 5% CO<sub>2</sub>. The wells were washed four times each with 0.01% Tween 20 in PBS and twice with PBS and overlaid with 0.025  $\mu\text{g}/50 \mu\text{l}$ /well of biotinylated anti-IL-4 (clone BVD6-24G2, PharMingen) or anti-IFN- $\gamma$  (clone XMG1.2, PharMingen) for 2 h at room temperature. Subsequently, the plates were washed, treated with 1:2000 dilution of Streptavidin-conjugated alkaline phosphatase (PharMingen) for 1 h at room temperature and washed six times with 0.01% Tween-PBS and twice with PBS. The IL-4 or IFN- $\gamma$  secreting cells was visualized by the addition of the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich, Chicago, IL, USA) diluted to 1 mg/ml in 0.1 M 2-amino-2-methyl-1 propanol buffer (Sigma-Aldrich) for 30 min. Colorimetric analysis was halted by washing with water. After the plates were dried, the resulting spots were counted on a computer-assisted Immunospot image analyzer (Cellular Technology Ltd., Cleveland, OH, USA). The computer-assisted image analyzer has 100% reproducibility when repeatedly counting the same well using single, defined criteria (data not shown). Results are presented as mean values of ELISPOTs detected in duplicate wells.

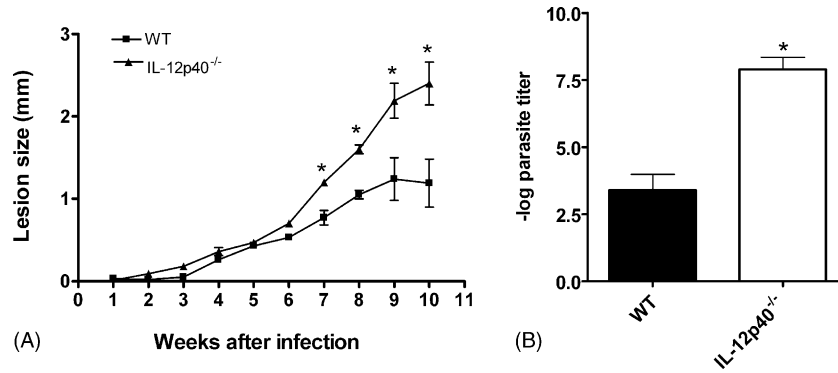


Fig. 1. Course of infection with *L. amazonensis* in C57BL/6 (wt) and IL-12p40<sup>-/-</sup> mice. Mice were injected in the left hind footpad with 10<sup>4</sup> stationary-phase promastigotes. (A) Lesion sizes were measured weekly. Each point represents the mean difference in size ± standard deviation of the mean between infected and uninfected footpads for five mice per group at each time point. (B) Parasite quantification in footpad lesions after 10 weeks of infection. Each bar represents mean ± standard deviation of the mean for five animals per group. The asterisk indicates a statistically significant difference ( $p \leq 0.05$ ) compared with the other group.

2.9. Histopathology

At the indicated time periods, foot tissues were collected, fixed in a formalin solution and embedded in paraffin. Sections were stained with hematoxylin and eosin. Sections were photographed using an Olympus photomicroscope equipped with an Olympus exposure control unit (Olympus Cop., New Hyde Park, NY).

2.10. Reproducibility and statistical analysis

Experiments were performed at least three times. Means were considered statistically different when  $p \leq 0.05$  by Student’s *t*-test.

3. Results

3.1. Course of infection with *L. amazonensis* in IL-12p40<sup>-/-</sup> and wt C57BL/6 mice

IL-12p40<sup>-/-</sup> and wt mice were infected with 10<sup>4</sup> *L. amazonensis* stationary-phase promastigotes, and lesion progres-

sion was monitored (Fig. 1A). Lesions in infected wt mice were at their peak size by 8 weeks and remained constant. IL-12p40<sup>-/-</sup> infected mice exhibited quite a distinct pattern of disease. Although lesion sizes could remain similar to the wt group for 6 weeks (Fig. 1A), or even up to 10 weeks (data not shown), at later time points IL-12p40<sup>-/-</sup> mice developed larger lesions, which progressively increased in size and did not show signs of healing for up to 14 weeks (one experiment performed, data not shown). Parasite burden in IL-12p40<sup>-/-</sup> mice at 10 weeks of infection was significantly larger (about 10<sup>4</sup> times) than parasite numbers in lesions from wt mice (Fig. 1B). These results show that IL-12p40<sup>-/-</sup> mice are highly susceptible to infection with *L. amazonensis*.

3.2. Cytokine production by C57BL/6 and IL-12p40<sup>-/-</sup> mice following infection with *L. amazonensis*

IL-12 is a crucial cytokine for the differentiation of the Th1 subset of helper cells and mice have been shown to default to a Th2 response in the absence of this cytokine [6,14,25,26]. In order to determine if the same default response would be found in *L. amazonensis*-infected mice in the absence of IL-12, we

Table 1

In vitro cytokine production by spleen and lymph node cells from naïve (c) and vaccinated (v) wt and IL12p40<sup>-/-</sup> mice infected for 10 weeks with *L. amazonensis*

		IFN-γ (ng/ml)		IL-4 (ng/ml)	
		Bkg <sup>a</sup>	LA <sup>b</sup>	Bkg	LA
Spleen	C57BL/6 c	6.1 ± 5.1	9.1 ± 7.5 <sup>c</sup>	0.02 ± 0.01	0.02 ± 0.01
	C57BL/6 v	20.3 ± 12.2	51.5 ± 22.7 <sup>d</sup>	0.04 ± 0.01	0.04 ± 0.01
	IL12p40 <sup>-/-</sup> c	0.05 ± 0.1	0.2 ± 0.2	0.01 ± 0.0	0.01 ± 0.0
	IL12p40 <sup>-/-</sup> v	0.4 ± 0.5	0.7 ± 0.7 <sup>e</sup>	0.01 ± 0.01	0.02 ± 0.01
Lymph node	C57BL/6 c	10.5 ± 7.6	43.2 ± 20 <sup>c</sup>	0.02 ± 0.01	0.01 ± 0.0
	C57BL/6 v	61.3 ± 18.5	140.2 ± 13 <sup>d</sup>	0.04 ± 0.02	n.d.
	IL12p40 <sup>-/-</sup> c	n.d. <sup>f</sup>	0.9 ± 1	0.1 ± 0.0	0.1 ± 0.0
	IL12p40 <sup>-/-</sup> v	1.8 ± 1.3	2.1 ± 1.6 <sup>e</sup>	0.1 ± 0.0	0.1 ± 0.0

<sup>a</sup> No antigen added to the culture.

<sup>b</sup> *L. amazonensis* freeze-thawed antigen added to the culture.

<sup>c</sup>  $P < 0.05$  wt c vs. IL12p40<sup>-/-</sup> c by Student’s *t*-test.

<sup>d</sup>  $P < 0.05$  wt v vs. wt c by Student’s *t*-test.

<sup>e</sup>  $P < 0.05$  wt v vs. IL12p40<sup>-/-</sup> v by Student’s *t*-test.

<sup>f</sup> Not detected. Limits of detection were: 0.016 ng/ml for IFN-γ and 0.015 ng/ml for IL-4.



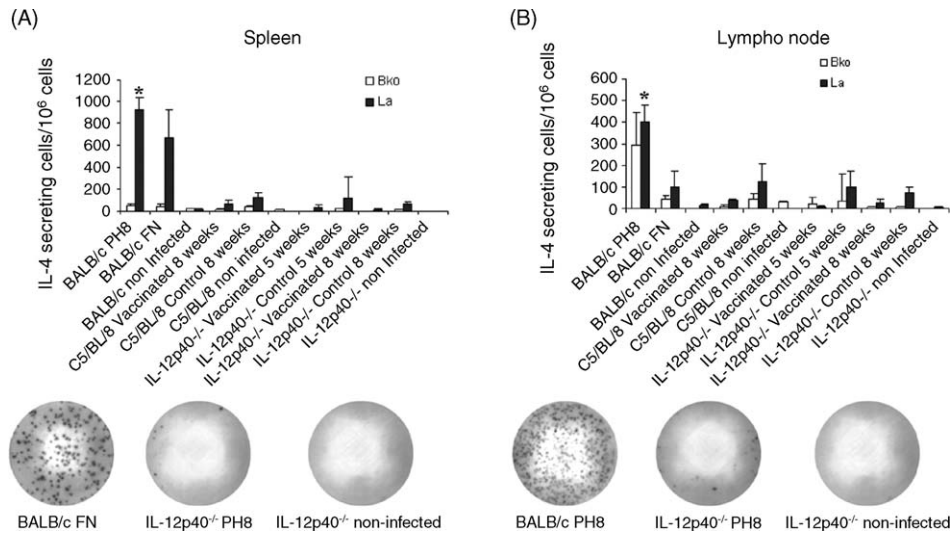


Fig. 2. IL-4-producing spleen and lymph node cells in control and vaccinated C57BL/6 and IL-12p40<sup>-/-</sup> mice after infection with *L. amazonensis* for 5 and 8 weeks. IL-4 secreting cells were measured by ELISPOT assay. BALB/c mice infected with *L. major* (BALB/c FN) and *L. amazonensis* (BALB/c PH8) were used as positive controls. Spleen cells and popliteal lymph node cells draining the site of infection were incubated over wells (seeded with  $5 \times 10^5$  and  $1 \times 10^6$  cells) that had been precoated with mAbs against IL-4, as described in Section 2. Pictures of IL-4 ELISPOT membranes from the ELISPOT assay are shown at the bottom. Each bar represents mean  $\pm$  standard deviation of the mean for five animals per group. The asterisks indicate a statistically significant difference ( $p \leq 0.05$ ) compared with the other group. Bkg, no antigen added to the culture, LA, *L. amazonensis* freeze-thawed antigen added to the culture.

determined the cytokine production by spleen and lymph node cells from wt and IL-12p40<sup>-/-</sup> mice in vitro. Results from a representative experiment are shown in Table 1. Cells from wt mice responded to *L. amazonensis* with IFN- $\gamma$  production, and, quite expectedly, cell cultures from IL-12p40<sup>-/-</sup> mice produced low levels of this cytokine both in the absence and in the presence of antigenic stimulus. These results were confirmed by ELISPOT: there were no detectable IFN- $\gamma$ -producing cells in spleens and lymph nodes of IL-12p40<sup>-/-</sup> mice infected with *L. amazonensis*, nor in BALB/c mice infected with *L. major* or *L.*

*amazonensis* (Table 2). Wild-type C57BL/6 mice showed significant numbers of IFN- $\gamma$  producing cells when infected with *L. amazonensis*. Surprisingly, however, IL-4 production was either undetectable or just above the detection limit of the assay in cell culture supernatants from both IL-12p40<sup>-/-</sup> and wt mice of mice (Table 1). These results were confirmed by ELISPOT assays. As shown in Fig. 2, IL-12p40<sup>-/-</sup> mice did not show an increase in the number of IL-4-secreting cells at 5 or 8 weeks after infection. In addition, we did not find a significant inversion of the IgG1/IgG2a ratio, nor a significant increase in the

Table 2

Mean IFN- $\gamma$  ELISPOT response by spleen and LN cells in vaccinated and control C57BL/6 and IL-12p40<sup>-/-</sup> mice after infection with *L. amazonensis*

	IFN- $\gamma$ secreting cells (cells/10 <sup>6</sup> ) <sup>a</sup>			
	Spleen		Lymph node	
	Bkg <sup>b</sup>	LA <sup>c</sup>	Bkg	LA
BALB/c PH8 <sup>d</sup>	0.66 $\pm$ 0.40	0.86 $\pm$ 0.50	8.2 $\pm$ 1.9	17.5 $\pm$ 7.5
BALB/c FN	0.66 $\pm$ 0.60	0 $\pm$ 0	7.0 $\pm$ 1.6	4.1 $\pm$ 1.6
BALB/c ni	1 $\pm$ 0.22	0 $\pm$ 0	0 $\pm$ 0	15 $\pm$ 2
C57BL/6 v 8 weeks	5.1 $\pm$ 0.82	34.8 $\pm$ 13.44	469 $\pm$ 23.12*	1028 $\pm$ 30.41*
C57BL/6 c 8 weeks	1.6 $\pm$ 0.3	3.8 $\pm$ 2.2	8.6 $\pm$ 2.89	17 $\pm$ 2
C57BL/6 ni	0 $\pm$ 0	4 $\pm$ 0.2	2 $\pm$ 1	2 $\pm$ 1
IL-12p40 <sup>-/-</sup> v 5 weeks	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
IL-12p40 <sup>-/-</sup> c 5 weeks	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
IL-12p40 <sup>-/-</sup> v 8 weeks	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
IL-12p40 <sup>-/-</sup> c 8 weeks	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
IL-12p40 <sup>-/-</sup> ni	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0

<sup>a</sup> Spleen cells and popliteal LN cells draining the site of infection were incubated over wells (seeded with  $5 \times 10^5$  and  $1 \times 10^6$  cells) that had been precoated with mAbs against IFN- $\gamma$ . After undisturbed incubation for 20 h, cells were washed and the captured cytokine developed using a second mAb and colorimetric reagent.

<sup>b</sup> No antigen added to the culture.

<sup>c</sup> *L. amazonensis* freeze-thawed antigen added to the culture.

<sup>d</sup> BALB/c PH8: BALB/c mice infected with *L. amazonensis* for 8 weeks; BALB/c FN: BALB/c mice infected with *L. major* for 8 weeks, ni: non-infected mice, v: vaccinated, c: infected non-vaccinated.

\*  $P < 0.05$  wt v vs. wt c, IL12p40<sup>-/-</sup> c and IL-12p40<sup>-/-</sup> v by Student's *t*-test.

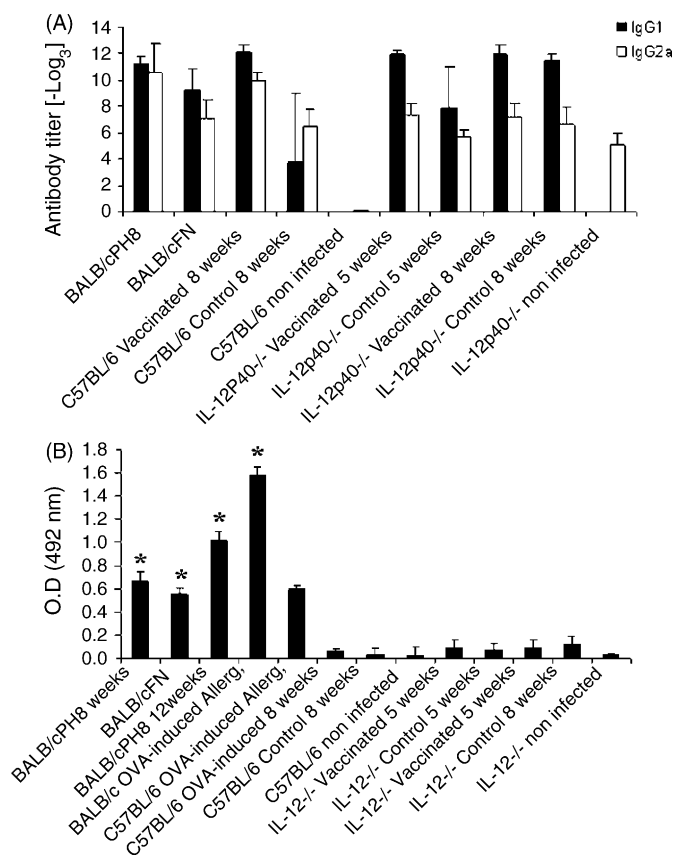


Fig. 3. Antibody isotypes in control and vaccinated C57BL/6 and IL-12p40<sup>-/-</sup> mice after infection with *L. amazonensis* for 5 and 8 weeks. Uninfected mice are shown for comparison. Results for BALB/c mice infected with *L. amazonensis* (BALB/c PH8) or *L. major* (BALB/c FN) for 8 weeks are also shown. (A) IgG1 and IgG2a were measured by incubating serially diluted sera (1:3) over wells precoated with *L. amazonensis* antigen, as described in Section 2. Results are expressed as the log<sub>3</sub> of the antibody titer. (B) Total serum IgE was quantitated using a sandwich ELISA, as described in Section 2. Results are expressed as mean of the optical density (OD). BALB/c OVA-induced allergy mice were used as a positive control. Each bar represents mean  $\pm$  standard deviation of the mean for five animals per group. The asterisks indicate a statistically significant difference ( $p \leq 0.05$ ) compared with the other group.

IgE titers in IL-12p40<sup>-/-</sup> mice (Fig. 3). BALB/c mice infected with *L. major* or rendered allergic to ovalbumin were used as positive controls of a typical Th2 response [3,27,28].

Hence, IL-12p40<sup>-/-</sup> mice did not default to a Th2 response when infected with *L. amazonensis*.

### 3.3. Vaccination of IL-12p40<sup>-/-</sup> mice against *L. amazonensis*

IL-12 has been shown to be an adjuvant for vaccination against *L. major* [14]. Hence, we decided to investigate if IL-12 was also essential for vaccination against *L. amazonensis*. A protocol that was previously shown to protect C57BL/6 mice against infection with this parasite was used [16] and, as can be seen in Fig. 4, vaccinated wt C57BL/6 mice showed smaller lesions and smaller parasite numbers than non-vaccinated controls (Fig. 4A and insert). In order to confirm that *Leishmania* antigens were important for the vaccination protocol, we performed infections

in mice in which *C. parvum* alone was injected along with saline. *C. parvum* alone did not confer protection in C57BL/6 mice (Fig. 4B), in accordance with previously published [29–31]. We then proceeded to investigate the importance of IL-12 in the immunization against *L. amazonensis*. IL-12p40<sup>-/-</sup> mice were vaccinated using the same protocol used for wt mice. Surprisingly, IL-12p40<sup>-/-</sup> were protected by the vaccination protocol for at least 4 weeks, when lesions started to grow, but were still smaller than lesions in non-vaccinated mice (Fig. 4C and D). Comparison of lesions from vaccinated and non-vaccinated IL12p40<sup>-/-</sup> mice showed a statistically significant difference from 4 to 8 weeks post-infection (Fig. 4C and D). Moreover, in the absence of functional IL-12, vaccinated mice were able to control parasite growth more efficiently: 10,000 times fewer parasites were found in their lesions (insert Fig. 4C). *C. parvum* alone did not protect IL-12p40<sup>-/-</sup> against infection with *L. amazonensis* (Fig. 4D). Moreover, IL-12p40<sup>-/-</sup> mice (as well as wt) challenged 21 days after the last boost of antigen were still protected, showing that, at least for this time period, the efficacy of our vaccination protocol was lasting (Fig. 4E). Hence, in the absence of IL-12, mice could be protected by immunization with *L. amazonensis* antigens in Leishvacin in association with *C. parvum*.

Histological analysis of the infected footpad from IL-12p40<sup>-/-</sup> non-vaccinated mice shows an extensive vacuolated area and parasitized macrophages (Fig. 5). These areas were smaller in vaccinated IL-12p40<sup>-/-</sup> mice (compare Fig. 5B and D).

### 3.4. Cytokine production in vaccinated mice

Lymph node and spleen cell cultures from wt C57BL/6 vaccinated mice presented higher levels of IFN- $\gamma$  when compared to non-vaccinated mice (Table 1) and higher numbers of IFN- $\gamma$ -producing cells (Table 2). Cells from IL-12p40<sup>-/-</sup> mice produced practically undetectable levels of IFN- $\gamma$  in vitro, regardless of vaccination. IL-4 was not detected in supernatants from lymph node or spleen cell cultures (Table 1). In addition, no significant numbers of IL-4-producing cells were found in vaccinated wt or IL-12p40<sup>-/-</sup> (Fig. 2). The absence of significant IL-4 production was further confirmed by IgG1 and IgG2a levels, as well as by IgE levels in sera from infected mice (Fig. 3).

## 4. Discussion

IL-12 is a heterodimeric pro-inflammatory cytokine that induces production of IFN- $\gamma$  by NK cells and T cells [32]. IL-12 is critical for promoting the differentiation of naive T cells into the Th1 subset and bridges innate and adaptive immunity [33–35]. The development of a Th1 response is crucial for the protection of the host against many pathogens, including *L. major* [36–40]. Mice that are genetically deficient for the expression of IL-12 (IL-12p40<sup>-/-</sup>) are susceptible to infection with *L. major* and default to a Th2 response [41]. Moreover, a clear requirement for IL-12 for the maintenance of a Th1 response directed to this parasite was found [42]. Thus, *L. major*-infected IL-12p40<sup>-/-</sup> mice required continuous treatment with IL-12 for

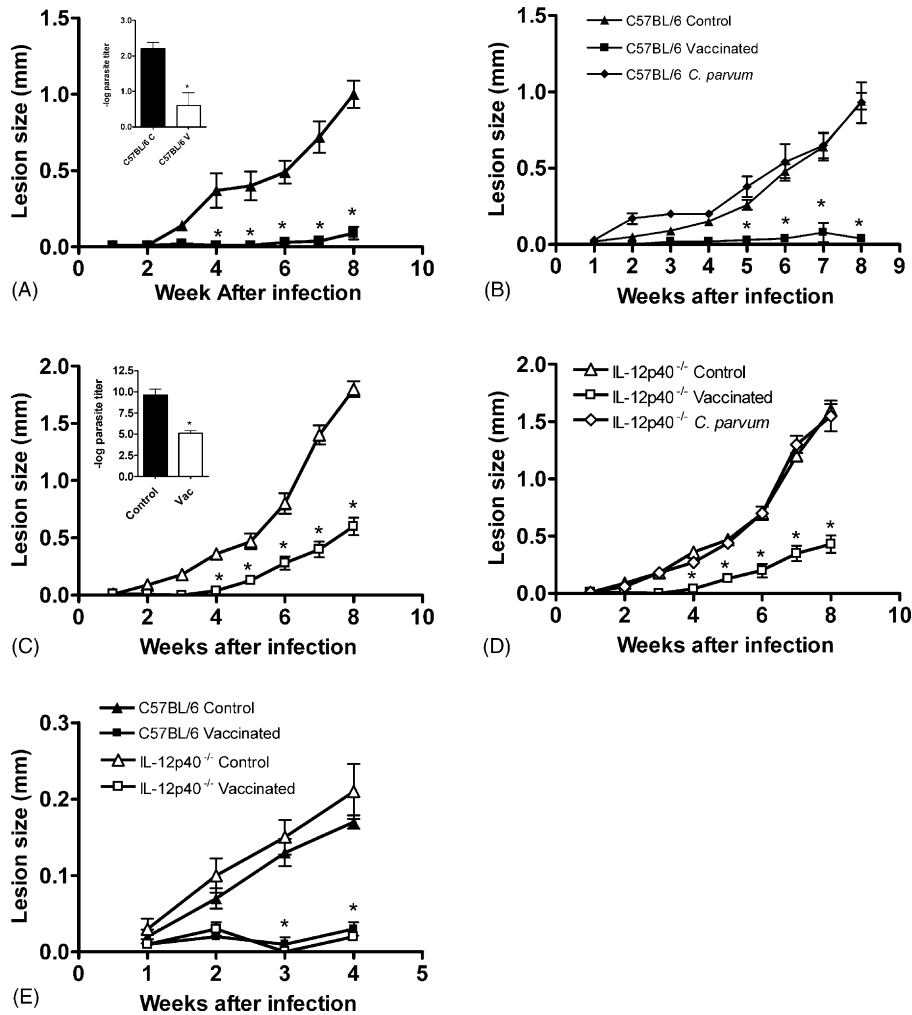


Fig. 4. Course of infection with *L. amazonensis* in vaccinated and control C57BL/6 and IL-12p40<sup>-/-</sup> mice. *C. parvum* was used as an adjuvant. Challenge was performed by injecting the left hind footpad with 10<sup>4</sup> stationary-phase promastigotes. Lesion sizes were measured weekly. Each point represents the mean difference in size  $\pm$  standard deviation of the mean between infected and uninfected footpads for five mice per group at each time point. (A and C) C57BL/6 mice vaccinated and non-vaccinated controls. (B and D) IL-12p40<sup>-/-</sup> mice vaccinated and non-vaccinated controls. Inserts: parasite quantitation in footpad lesions 8 weeks after infection. (E) C57BL/6 and IL-12p40<sup>-/-</sup> mice vaccinated and non-vaccinated controls, challenged 21 days after the last boost of antigen. Each bar represents mean  $\pm$  standard deviation of the mean for five animals per group. The asterisks indicate a statistically significant difference ( $p \leq 0.05$ ) compared with the other group.

the maintenance of a *Leishmania*-specific Th1 response. Susceptibility in these animals was associated with a loss of the Th1 response and the development of a Th2 response [42,43].

A less clear role for the development of a Th1 response in protection is found during infection with *Leishmania* belonging to the mexicana complex. In one report, infection of IL-12p40<sup>-/-</sup> mice with *L. mexicana*, had the same outcome as in the wild-type controls [44]. In another study, IL-12 was critical for long-term stabilization of lesions caused by *L. mexicana*, but insufficient to totally cure the infection, in contrast with *L. major* infection [26]. The early control of *L. mexicana* infection appears to be, thus, independent of IL-12 [26]. Here, we show that IL-12p40<sup>-/-</sup> mice are more susceptible to infection with *L. amazonensis*. Similar to the study by Torrentera et al. [26], IL-12 p40 seems to be more critical to long-term stabilization of lesions. However, contrary to what was found during *L. major* infection [41,42], IL-12p40<sup>-/-</sup> mice do not default to a Th2 response,

as demonstrated by IL-4 production in vitro, by the number of IL-4-producing cells detected ex vivo and by low IgE levels. In our study, IgG1 levels did not correlate with IL-4 production. Hence, in the absence of functional IL-12, the higher susceptibility to *L. amazonensis* seems to be due to a lack of an efficient Th1 response rather than a Th2 response. Indeed, this was previously suggested by Afonso and Scott [4], who found that susceptibility to *L. amazonensis* in the wt C57BL/10 mouse was not due to a Th2 response, but to a lack of an efficient Th1 response.

While infection with *L. amazonensis* induced IL-12 and IFN- $\gamma$  production in early stages of infection [8], infected mice fail to express a functional IL-12R [5]. This fact could explain the failure to produce sufficient IFN- $\gamma$  to control parasites to an extent that would allow lesion healing [4,5,7]. It is possible that another factor that is essential for control of parasite growth is missing in *L. amazonensis*-infected mice. In addition, it has been shown that IFN- $\gamma$  increases growth of *L. amazonensis* amastig-

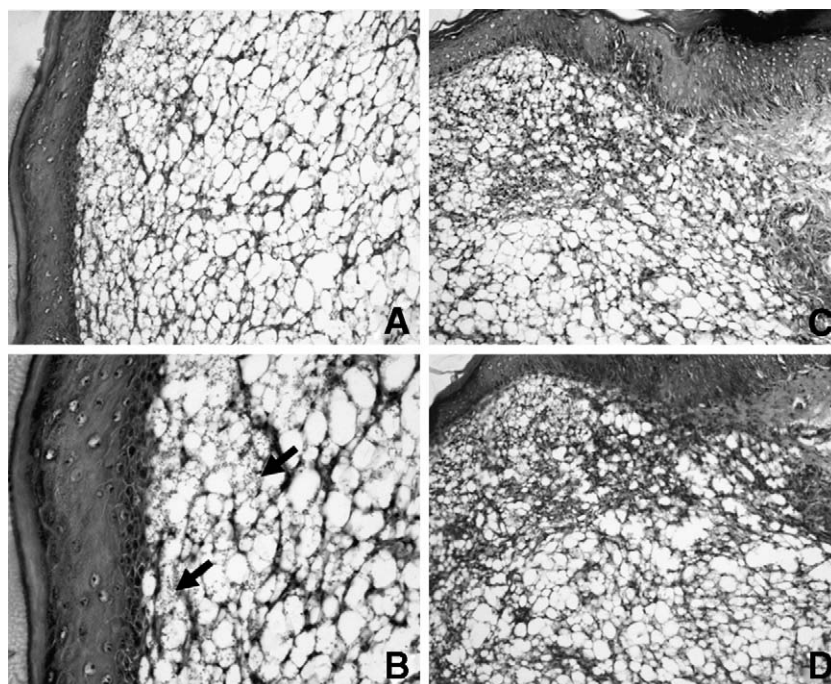


Fig. 5. Representative histology of foot tissues from control and vaccinated IL-12p40<sup>-/-</sup> mice infected with *L. amazonensis*. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin. (A) A section from control IL-12p40<sup>-/-</sup> mouse 8 weeks post-infection, showing a portion of an extensive vacuolated area (magnification 200 $\times$ ). (B) Numerous vacuolated and parasitized macrophages (arrows) (magnification 400 $\times$ ). (C) A section from IL-12p40<sup>-/-</sup> mice vaccinated at 8 weeks after infection, showing a portion of an extensive inflammatory area and the destruction of normal structures (magnification 200 $\times$ ). (D) A section from IL-12p40<sup>-/-</sup> mice vaccinated showing a limited vacuolated area compared with that in B (magnification 400 $\times$ ).

otes in macrophages [45]. According to this latter report, IFN- $\gamma$  would be, in fact, favoring the maintenance of *L. amazonensis* in the host at the later stages of infection. However, the protective vaccination protocol used in our study induced higher levels of IFN- $\gamma$  production in wt C57BL/6 mice after challenge with *L. amazonensis*, when compared to non-vaccinated mice. It is possible, also, that other factors, such as TNF- $\alpha$ , are being produced at higher levels in vaccinated mice. This report did not investigate these other possibilities.

The higher IFN- $\gamma$  production found in wt mice correlated with less severe disease: there was a significant decrease in the number of parasites and lesions were almost negligible. This suggests to us that the decrease in lesion size may be the result of lower parasite numbers as well as a decrease in the inflammatory response. Remarkably, histological aspects of lesions from vaccinated and non-vaccinated IL-12p40<sup>-/-</sup> mice were very similar, albeit lesions in vaccinated mice were considerably smaller than non-vaccinated mice.

IL-12 is an effective adjuvant for the initiation of protective cell-mediated immunity against *L. major*, since treatment with recombinant IL-12 and parasite antigens promoted the development of *Leishmania*-specific CD4<sup>+</sup> Th1 cells and the protection of a susceptible mouse strain [14,38,40]. Moreover, IL-12 was successfully used as an adjuvant for vaccination against *L. amazonensis* [46,47]. Surprisingly, the early control of *L. amazonensis* conferred by vaccination with Leishvacin<sup>®</sup> and *C. parvum* shown here is independent of IL-12. In addition, spleen and lymph node cells from mice that were protected against infection did not produce high levels of IFN- $\gamma$  in culture upon stimula-

tion with parasite antigens. IL-12 promotes the differentiation of CD4<sup>+</sup> T cells into the Th1, IFN- $\gamma$ -producing subtype. CD8<sup>+</sup> T cells are also able to synthesize IFN- $\gamma$  but, although some evidence for a protective role played by these cells has already been accumulated, this role has been far less characterized [48,49]. However, it is thought that CD8<sup>+</sup> T cells are also dependent on IL-12 for the production of IFN- $\gamma$  [50]. Another possible explanation for the results described here is that even in the absence of IL-12 enough *Leishmania*-specific Th1 cells developed in the vaccinated animals. Results from Jankovic et al. [51] demonstrate that repeated stimulation of IL-12 deficient mice induced low levels of IFN- $\gamma$ -producing- CD4<sup>+</sup> T cells. It is possible that the repeated inoculation of antigens in our vaccination protocol was sufficient to induce the development of a number of Th1 cells capable of partially controlling the infection. However, these cells were not detected in ELISPOT assays. Hence, the mechanism for resistance to infection after vaccination in IL-12p40<sup>-/-</sup> mice is still obscure, and is under investigation in our laboratory.

Here, we used vaccinated mice to study the mechanisms of resistance against *L. amazonensis*. Although in wild-type mice vaccination conferred protection, as measured by the parasite burden and the size of lesions, and this protection correlated with higher levels of IFN- $\gamma$ , the absence of IL-12 p40 did not prevent the vaccination protocol from protecting mice. Hence, although IL-12p40<sup>-/-</sup> mice were more susceptible to infection, protection was conferred by the vaccination protocol. The mechanisms involving this protection are, at present, unknown.



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