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Short-term protection conferred by Leishvacin® against experimental *Leishmania amazonensis* infection in C57BL/6 mice

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ABSTRACT

To date, there is no vaccine available against human leishmaniasis. Although some vaccination protocols can induce immunity in murine models, they fail to induce protection in humans. The reasons for that remain unclear. The aim of the present study was to characterize the changes in the pattern of the immune response during subcutaneous vaccination with Leishvacin® in mice. We also investigated whether IFN-γ and nitric oxide synthase are indispensable for the protection elicited by the vaccine. C57BL/6 WT vaccinated mice showed smaller lesions and fewer numbers of parasites in footpads until 8 weeks post-infection. Up to this time, they produced higher levels of IFN-γ, IL-2, IL-4, IL-17A and IL-10 and higher specific antibody response than control non-vaccinated mice. Moreover, we showed that IFN-γ, most likely by induction of iNOS expression, is essential for immunity. However, after 12 weeks of infection, we observed loss of difference in lesion size and parasite burden between the groups. Loss of resistance was associated with the disappearance of differences in cytokine patterns between vaccinated and control mice, but not of antibody response, which remained different until a later time of infection. The reversal of resistance to *L. amazonensis* could not be explained by upregulation of regulatory cytokines. Our data point to a subversion of the host immune response by *L. amazonensis* even when a protective response was previously induced.

1. Introduction

Leishmaniasis is a disease with a spectrum of clinical manifestations, depending on the species of *Leishmania* and the state of the host's immunity. Clinical manifestations include localized cutaneous leishmaniasis, mucocutaneous leishmaniasis, diffuse cutaneous leishmaniasis and the systemic form, visceral leishmaniasis. This disease is a relevant public health problem, affecting about 90 countries in the world. Statistical studies have estimated that there are 0.9–1.6 million new cases per year of cutaneous and visceral leishmaniasis [1,2]. To this day, there is no vaccine available against leishmaniasis and the traditional treatment is based on pentavalent antimonials, which have been associated with antimony-resistant strains of *Leishmania* and toxicity [3,4]. Also, prophylactic measures for cutaneous leishmaniasis are ineffective. Therefore, the development of a vaccine would be the most effective measure to eliminate this disease worldwide [5]. Mouse models, albeit useful, are, however, not a completely accurate reproduction of the human disease [6]. Nevertheless, experimental models, especially mouse models, have been the startpoint of choice to test for the efficacy of vaccines.

Resistance to *Leishmania major*, which causes cutaneous leishmaniasis in the Old World, is mediated by Th1 immune responses. IFN-γ and TNF-α are important mediators that induce NO production by macrophages, which are consequently able to kill the parasite, as evidenced in experimental models [7]. Therefore, protocols of vaccination against some species of *Leishmania* aim at inducing polarization to Th1 responses. Some studies have found a correlation between higher production of IFN-γ and protection induced by vaccination [8,9]. However, other studies have demonstrated that increased production of IFN-γ was not enough to induce immunity [10] or was not confirmed to be related to protection [11,12]. On the other hand, immune response to *Leishmania amazonensis* infection, which is responsible for different clinical manifestations such as localized cutaneous and diffuse cutaneous leishmaniasis in the New World [13,14], is different from that induced by *L. major*. Differently from *L. major* infection, susceptibility to

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L. amazonensis is more associated with a weak Th1 immune response than with a polarization to a Th2 immune response [15]. IFN-γ is, surprisingly, able to induce the proliferation of amastigote forms of the parasite *in vitro* [16]. Despite these differences, immunization protocols against *L. amazonensis* have the same aim: to induce the production of high levels of IFN-γ and polarization to a Th1 immune response. However, a few studies have shown that this kind of response in *L. amazonensis* infection does not lead to healing [17,18].

Leishvacin® is a vaccine composed of killed promastigote forms of *L. amazonensis* strain PH8 (IFLA/BR/67/PH8). This vaccine has been shown to induce the production of IFN-γ by murine splenic cells and the production of anti-*Leishmania* IgG and IgM antibodies, as well as to promote proliferation of murine lymphocytes and to confer protection to C57BL/10 and C57BL/6 mice [19–22]. Although this vaccine induces protection in mice, it fails to induce protective immunity against cutaneous leishmaniasis in humans [23], even with the administration of BCG as adjuvant [24].

The aim of this work was to characterize the immune responses induced by Leishvacin® during *L. amazonensis* infection in mice, through the identification of components of cellular and humoral immunity, and its persistence for several weeks after infection. We chose to use Leishvacin ® as part of an effort to elucidate the reasons for the contrast between its experimental success and the lack of protection in human trials. The use of adjuvant (*Corynebacterium parvum*) was necessary, since parasite antigens by themselves do not lead to protection in mice [25]. Furthermore, we analyzed the role of IFN-γ in this immunization protocol.

2. Materials and methods

2.1. Animals

Four- to 6-week-old male and female C57BL/6 mice were obtained from the Bioterism Center (CEBIO), Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. IFN-γ −/− (B6.129S7-Ifng^{tm1Ts}/J) and iNOS −/− (B6.129P2-Nos2^{tm1Lau}/J) were purchased from The Jackson Laboratory (Glesnsville, NJ, USA). Mice were kept in conventional conditions with barriers, controlled light cycle and controlled temperature. Animals were fed a commercial diet for rodents (Labina-Purina, SP, Brazil) *ad libitum*. This project was approved by the local ethical committee under the protocol CETEA 063/09. Animal care was in accordance with institutional guidelines and those are in accordance with international guidelines.

2.2. Parasites and antigen

L. amazonensis (IFLA/BR/67/PH8) were maintained in Grace's insect medium (GIBCO Laboratories, Grand Island, NY) supplemented with 20% heat-inactivated FBS (Cultilab, Campinas, SP, Brazil), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Antigens were prepared from log phase promastigotes that were washed in 0.1 M phosphate-buffered saline pH 7.3 (PBS) and submitted to seven freeze-thaw cycles.

2.3. Leishvacin®

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

2.4. Vaccination and infection

C57BL/6, IFN-γ −/− and iNOS −/− mice were immunized subcutaneously at the base of the tail using 0.12 mL of a mixture of 100 μg of vaccine protein plus 250 μg of *C. parvum* (Laboratório de Extratos Alergênicos Ltda, Rio de Janeiro, RJ, Brazil) [26]. Each animal received two inoculations at an interval of 7 days. Twenty-eight days after the second dose, animals received booster injections with 10 μg of vaccine, without adjuvant. Control groups in this study were unvaccinated C57BL/6, IFN-γ −/− and iNOS −/− mice. We have previously shown that mice vaccinated with *C. parvum* without antigen were not protected against infection [20]. Seven days after the last booster, animals were challenged with 10^5 *L. amazonensis* promastigotes from stationary phase cultures (4 days of culture) in the hind footpad.

2.5. Parasite quantification

Parasites were quantified by limiting dilution assay [20]. Results are expressed as the negative logarithm of the titer.

2.6. Cell culture and cytokine assays

Cells obtained from maceration of spleen and popliteal lymph node were cultured at 5×10^6 cells/mL in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Cultilab), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma). Cells were stimulated or not with 50 μg/mL of *L. amazonensis* antigen. Cell culture supernatants were collected after 24 h for detection of TNF-α, or after 72 h in order to evaluate IFN-γ, IL-4, IL-2, IL-17A, IL-10 and TGF-β production by ELISA. IFN-γ was assayed using monoclonal antibody R46A2, a polyclonal rabbit anti-mouse IFN-γ, and anti-rabbit IgG conjugated with peroxidase (Zymed Laboratories, Inc, San Francisco, CA, USA). ABTS (Sigma-Aldrich, Inc., St. Louis, MO, USA) and hydrogen peroxide were used as substrates for the peroxidase. The detection limit for this IFN-γ ELISA was 20 pg/mL. The ELISA assay for IL-4 was performed using 11B11 mAb for coating and biotinylated BVD6 mAb (kindly provided by Dr. Phill Scott, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA). The detection limit for this assay was 30 pg/mL. The other cytokines were assayed using commercially available kits: IL-17A (e-Bioscience, San Diego, CA, USA), TNF-α, IL-2 and IL-10 (BD Pharmingen, San Diego, CA, USA) and TGF-β (R&D Systems, Minneapolis, MN, USA). The detection limit for IL-17A was 10 pg/mL, 32 pg/mL for TNF-α, IL-2 and IL-10, and 30 pg/mL for TGF-β.

2.7. Antigen-specific IgG1 and IgG2a ELISA

L. amazonensis antigen derived from *in vitro* promastigote cultures (10 mg protein/mL) were diluted in 0.1 M sodium carbonate buffer (pH 9.6) and 100 μL per well were used to coat flat-bottomed 96-well plates overnight at 4 °C. Mouse sera were diluted 1:10 with PBS-5% FBS and incubated with goat anti-mouse IgG1 or IgG2a (Southern Biotechnology, Birmingham, AL, USA) at a 1:5000 and 1:10000 dilution. Absorbance values were read at 405 nm in a Spectra Max Plus reader (Spectra Max Plus reader Molecular Devices Corporation, Sunnyvale, CA, USA).

2.8. Histopathology

At the indicated time periods, foot tissues were collected. Tissue samples were fixed in 10% neutral buffered formalin, dehydrated, cleared, embedded in paraffin, cut into 4- to 5-μm-thick sections and stained by hematoxylin and eosin (H&E) to analyze inflammation. Sections were photographed using an Olympus photomicroscope equipped with an Olympus exposure control unit (Olympus Corp., New Hyde Park, NY, USA).

2.9. Statistical analysis

Experiments were performed at least three times. The comparison of two groups was performed by using Mann–Whitney test. For comparison of more than two groups, a two-way ANOVA with Bonferroni's *t*-test was carried out. Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Leishvacin® induces short-term protection against L. amazonensis

We followed the course of infection of vaccinated and control C57BL/6 mice challenged with 1×10^5 stationary phase promastigote forms of *L. amazonensis* in the hind footpad. Immunized mice showed smaller lesions when compared to control mice at 5 weeks of infection. Lesions continued to be smaller in immunized mice until 8 weeks post-infection, when the experiment was terminated (Fig. 1A). In addition to smaller lesions, vaccinated mice presented fewer parasites than the control group at the site of infection at 4 and 8 weeks post-infection (Fig. 1C). Thus, Leishvacin® protected mice against infection by *L. amazonensis* until 8 weeks post-infection, as evidenced by lesion size and parasite burdens.

Next, we were interested in verifying if Leishvacin® could induce long-term protection against *L. amazonensis* infection. We followed the development of the disease until 16 weeks after infection. Differences in lesion size between control and vaccinated groups were found until 12 weeks after challenge. However, after 10 weeks post-infection, the control group showed a partial reduction in lesion size, while the vaccinated group showed a slow but steady increase in lesion size. After 12 weeks of infection, there were no differences in lesion sizes till the end of the experiment at 16 weeks (Fig. 1B). Moreover, the vaccinated group did not show lower parasite burden at the site of infection at 12 and 16 weeks after challenge (Fig. 1C). Also, we followed the production of antigen-specific IgG2a and IgG1. Both immunoglobulins were higher in the vaccinated group when compared with the control group at 4, 8 and 16 weeks post-infection (Fig. 2). Taken together, our data showed a short-term protection induced by Leishvacin® in C57BL/6 mice.

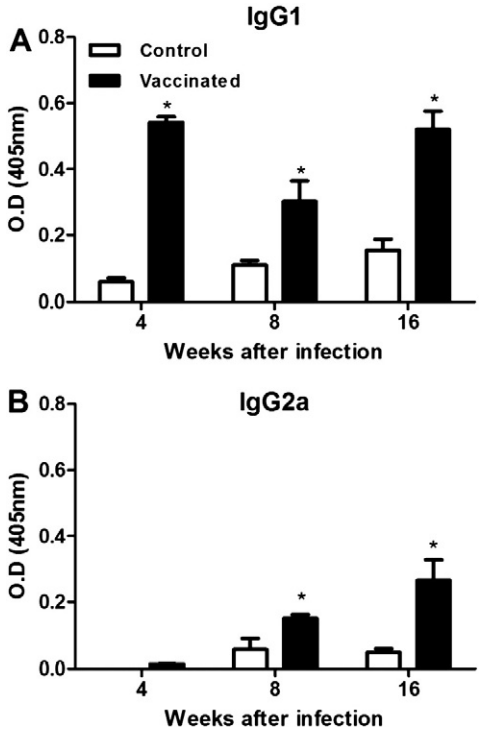

Fig. 2. IgG1 (A) and IgG2a (B) binding to *L. amazonensis* were measured by ELISA, as described in Materials and methods. Serum from control and vaccinated C57BL/6 mice were collected at 4, 8 and 16 weeks post-infection. Data are representative of three experiments. Results for week 16 are from a different experiment than the ones for weeks 4 and 8. $^*P < 0.05$.

3.2. Immunization by Leishvacin® induces temporary higher production of Th1-, Th2- and Th17- related cytokines

To determine the cytokine profile in vaccinated mice infected with *L. amazonensis*, we measured cytokine production by splenocytes and draining lymph node cells during *in vitro* re-stimulation with *L. amazonensis* antigen. To evaluate the induction of a Th1 response, we assessed the production of IFN-γ, IL-2 and TNF-α. Vaccination induced the production of increased levels of IFN-γ and IL-2 by lymph node cells and spleen cells until 8 weeks post-infection, a period when Leishvacin® induced protection against *L. amazonensis* infection

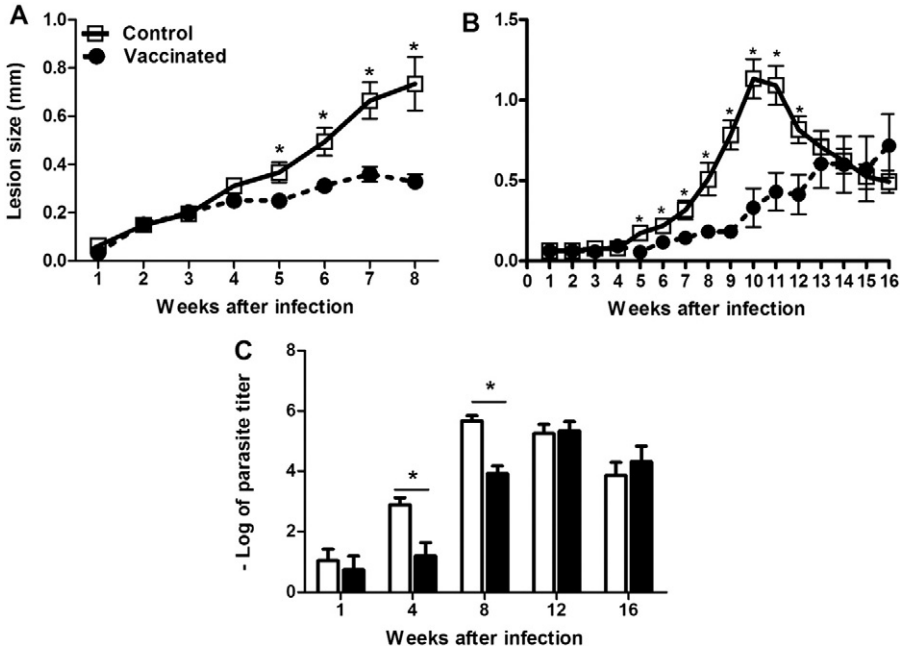

Fig. 1. Course of infection in vaccinated and control C57BL/6 mice infected with 10^5 *L. amazonensis* promastigotes in the right hind footpad (A,B). Lesion sizes were measured weekly and are expressed as the increase in thickness footpad. Each point represents the mean difference in size ± standard deviation between infected and uninfected footpads of five mice per group. (C) Parasite burdens in infected footpads at 1, 4, 8, 12 and 16 weeks post-infection. Data are representative of three or more experiments. Data for weeks 1–8 are from the experiment in (A), data for weeks 12 and 16 are from the experiment in (B). $^*P < 0.05$.

(Fig. 3A, B, F, G). Vaccination did not affect TNF-α production (data not shown). Th17 cells, which are known to produce IL-17, can be induced by vaccine protocols and is important for long-term protection against *L. major* [27]. Vaccination with Leishvacin® induced IL-17A production as early as 1 week post-challenge (Fig. 3C, H). Conversely, no production of IL-17 was detected in control mice on the first and fourth weeks post-infection. This difference in IL-17 production was found in both spleen and lymph node cells and was maintained at week 4, but not at week

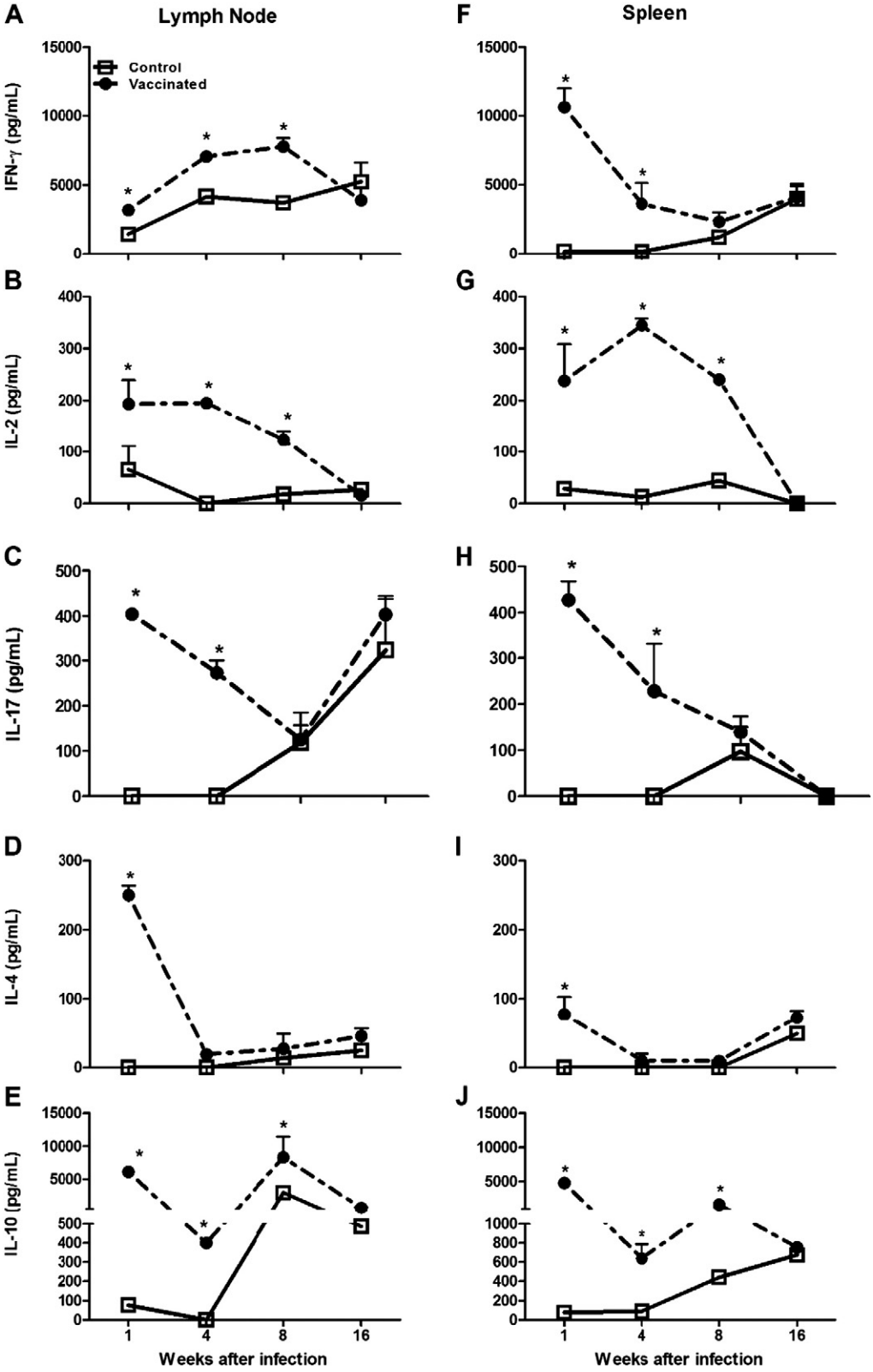

Fig. 3. IFN-γ, IL-2 , IL-17, IL-4 and IL-10 production by lymph node (A, B, C, D, E) and spleen (F, G, H, I, J) cells stimulated with parasite antigen in culture. Mice were infected with 10^5 *L. amazonensis* in the right hind footpad. The results are from one experiment that is representative of three performed, 5 mice per group at each time point. Results for week 16 are from a different experiment from those for weeks 1, 4 and 8. $^*P < 0.05$.

8 post-challenge, when similar levels of IL-17A were found in both groups. Regarding anti-inflammatory and Th2 cytokine production, no differences in TGF-β production between control and vaccinated groups were found (data not shown). However, immunization led to an increased production of IL-4 on the first week post-challenge (Fig. 3D, I) and induced higher production of IL-10 by lymph node and spleen cells until 8 weeks after challenge (Fig. 3E, J). Taken together, our data showed that vaccination with Leishvacin® induced an increase in Th1-related cytokines, IFN-γ and IL-2; in Th17-related cytokine, IL-17, but also promoted an increase in IL-4 and IL-10 production until 8 weeks post-challenge in immunized mice.

On the other hand, when we evaluated the cytokine production by lymph node and spleen cells 16 weeks post-infection, no differences on the production of IFN-γ, IL-2, IL-4, IL-10 and IL-17A (Fig. 3) were

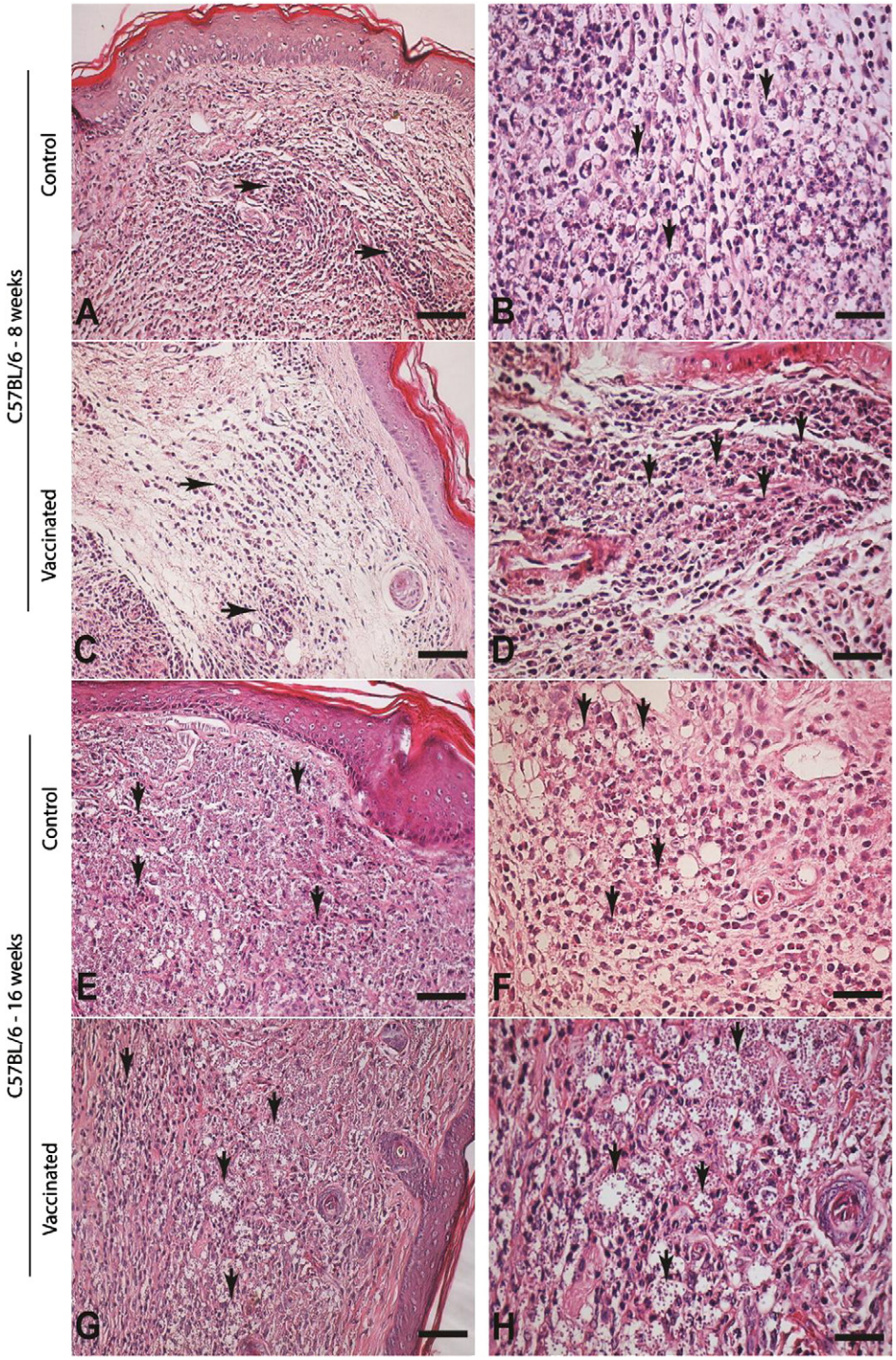

Fig. 4. (A and B) Histological representation of the footpad dermis of C57BL/6 control mice infected with *L. amazonensis* (8 weeks). (A) Presence of an intense chronic inflammatory exudate in the dermis (arrows). Bar = 32 μm. (B) High magnification showing mononuclear cells and numerous macrophages loaded with amastigote forms of *Leishmania* (arrows). Bar = 16 μm. (C and D) Histological representation of the footpad dermis of C57BL/6 vaccinated mice infected with *L. amazonensis* (8 weeks). (C) Moderate chronic inflammatory exudate in the deep dermis (arrows). Bar = 32 μm. (D) Some macrophages loaded with amastigote forms of *Leishmania* could be observed (arrows). Bar = 16 μm. Histological representation of the footpad dermis of C57BL/6 control (E) and vaccinated mice (G) infected with *L. amazonensis* (16 weeks). An intense chronic inflammatory exudate was observed in the dermis (arrows). Bar = 32 μm. (F) Higher magnification showing amastigote forms of *Leishmania* inside vacuolated macrophages in C57BL/6 control mice. Bar = 32 μm. (H) Higher magnification showing details of figure (G) where numerous amastigote forms of *Leishmania* could be easily observed inside vacuolated macrophages. Bar = 16 μm. All plates stained by hematoxylin and eosin.

found. Therefore, the loss of protection due to the vaccination protocol was paralleled by a loss of differences in cytokine production profiles, but not in antibody response.

In accordance with the data in Fig. 1A, histological analysis of the infected footpad from control C57BL/6 mice showed higher inflammatory exudate in the dermis than vaccinated mice, and an extensive vacuolated area 8 weeks post-infection (Fig. 4A, B, C and D). On the other hand, 16 weeks after the infection, both groups showed a chronic inflammation process (Fig. 4E, G), in accordance with the loss of difference in the lesion size between control and vaccinated groups. In addition, extensive vacuolated areas and high numbers of parasites were seen in tissues of control and vaccinated mice at this time point (Fig. 4F and H).

3.3. IFN-γ and iNOS are essential for Leishvacin®-induced protection until 12 weeks after challenge

The role of IFN-γ during *L. amazonensis* infection is controversial. In the presence of low levels of this cytokine, amastigote forms of the parasite can proliferate during *in vitro* infection of macrophages [16]. On the other hand, during *in vivo* infection, IFN-γ appears to be important for the control of parasitism at later times of infection [28]. In order to verify whether this cytokine is essential for the short-term protection induced by Leishvacin®, we attempted to immunize IFN-γ −/− mice. As shown in Fig. 5A and C, IFN-γ is essential for the protection induced by our vaccination protocol, since we did not find any difference during the course of infection or parasite burden until 16 weeks post-infection between control and vaccinated IFN-γ −/− mice. We also evaluated the participation of the enzyme iNOS, which can be induced by IFN-γ. Our data revealed that iNOS is also essential for the protection induced by Leishvacin® (Fig. 5B and D). Also, we measured the production of antigen-specific IgG1 and IgG2a in these mice, and we found higher production of IgG1 in vaccinated IFN-γ −/− and iNOS −/− mice (Fig. 5E) and higher production of IgG2a in vaccinated iNOS −/− mice (Fig. 5F). Interestingly, these results showed that although our vaccine protocol was able to change the pattern of immune response in these mice, as evidenced by the increase in serum antibody levels, these alterations did not influence lesion development or tissue parasitism. The experiments showed in Figs. 5A, B and 1B were performed simultaneously using the same parasite culture.

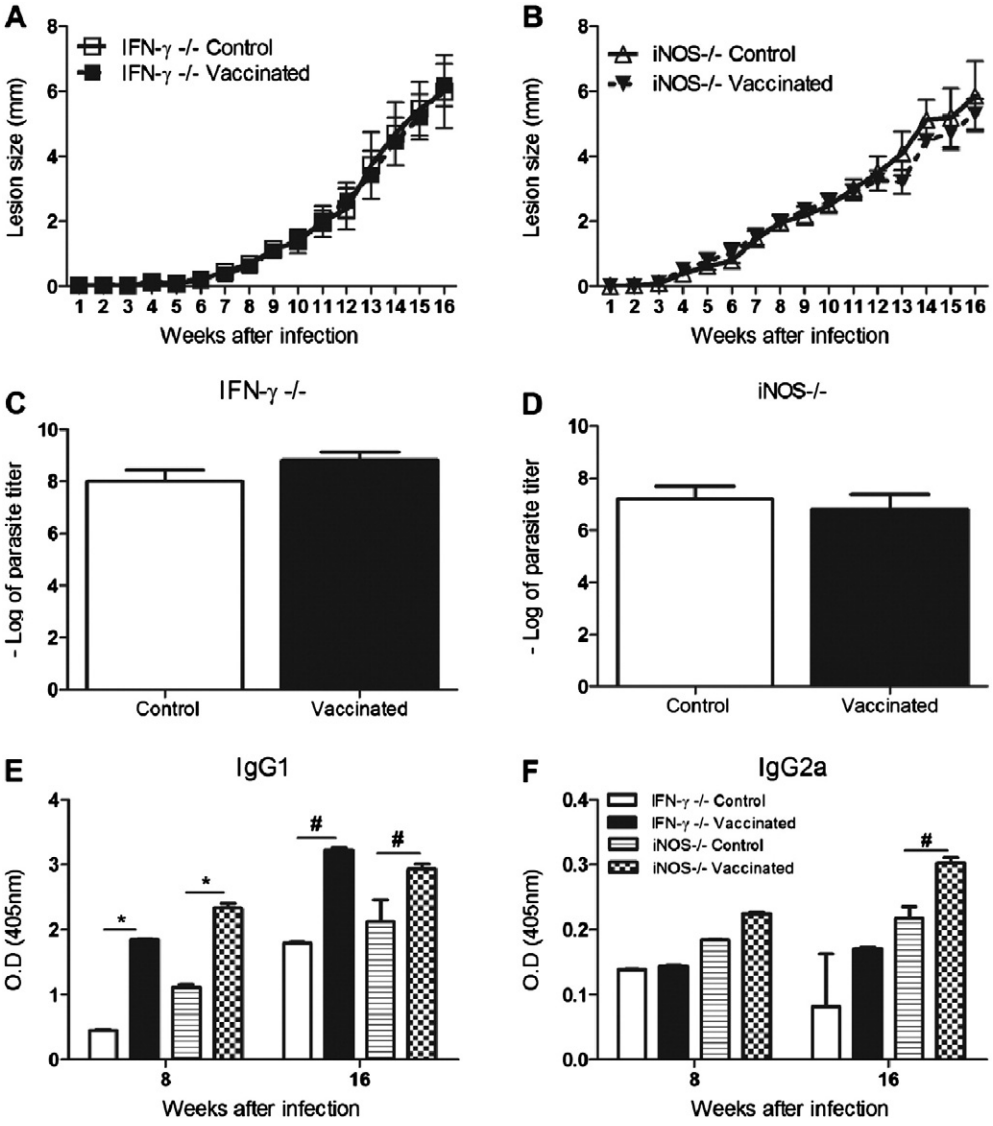

Fig. 5. Course of infection in vaccinated and control IFN-γ −/− (A) and iNOS −/− (C) mice infected with 10^5 *L. amazonensis* in the right hind footpad. Each point represents the difference in size ± standard deviation of the mean between infected and uninfected footpads for five mice per group. Parasite burdens were determined at 16 weeks post-infection at the site of infection in vaccinated and non-vaccinated IFN-γ −/− (B) and iNOS −/− (D) mice (5 mice per group). Levels of anti-*L. amazonensis* IgG1 (E) and IgG2a (F) were measured by ELISA, as described in Materials and Methods. Data are representative of three or more experiments. $^{*}P < 0.05$ indicates a difference between control and vaccinated IFN-γ −/− mice and $^{\#}P < 0.05$ indicates difference between control and vaccinated iNOS −/− mice.

4. Discussion

Protection induced by Leishvacin® in mice has been previously shown. Immunization induced increased production of IFN-γ, although a positive correlation between protection and this increase in C57BL/10 mice was not found [12]. Although our group has shown, in C57BL/6 mice, that Leishvacin® was able to confer protection against challenge with *L. amazonensis* for up to 10 weeks post-infection, the mechanisms of protection were not clear [20,21]. Immunization did not require $CD8^+$ T cells and could be partially achieved in the absence of IL-12 (which is important for protection against *L. major* [29,30]) and only a slight increase in IFN-γ production [20,21]. In the present study, we showed that Leishvacin® protected mice against infection with *L. amazonensis,* as shown by the smaller lesion size and the number of parasites at the site of infection. This protection was evident for up to 12 weeks post-infection and was associated with a change in the pattern of cytokine production and with *Leishmania*-specific antibodies. Moreover, we showed that IFN-γ, probably through iNOS induction, is essential for protection.

The use of *C. parvum* or other adjuvant is necessary for the effective vaccination of mice, since parasite antigens alone are not sufficient to confer protection [25]. This adjuvant has been shown to induce IL-12 production in macrophages [31,31]. In addition, *C. parvum* can be replaced by IL-12 in vaccination against *L. major* [29]. Therefore, the use of *C. parvum* as an adjuvant skews the immune response towards a Th1, IFN-γ-producing response.

IFN-γ is a key cytokine for protection against many infections caused by intracellular parasites [32–34]. Therefore, the induction of higher production of IFN-γ is a major aim of immunization protocols [9,35]. Surprisingly, the relevance of IFN-γ for *L. amazonensis* control *in vivo* occurs only in later times of infection [28]. In addition, *L. amazonensis* amastigotes are stimulated to proliferate in the presence of this cytokine in certain circumstances *in vitro* [16]. Our data showed that Leishvacin® induced the production of high levels of IFN-γ, by lymph node and spleen cells, until eight weeks post-infection. Despite the apparently contradictory roles of this cytokine during *L. amazonensis* infection, we showed that it was essential for protection in our vaccine protocol since we failed to induce protection in IFN-γ −/− mice. On the other hand, this induction was not enough to maintain the protection conferred by vaccination, as described before [6]. The mechanism by which IFN-γ acts against intracellular parasites is through the classical activation of macrophages, which express iNOS, leading to the production of NO and consequent death of the parasites [36]. Therefore, we tried to vaccinate iNOS −/− mice, and these mice were not able to control lesion development and parasite replication. Thus, it is most likely that the role of IFN-γ in protection is ultimately to induce iNOS production. In addition to IFN-γ production, induction of IL-2 and TNF-α are also markers of Th1 immune response [37,38]. The generation of multifunctional Th1 cells, instead of cells that produce only IFN-γ, is important in order to sustain the protection induced by vaccines. Hence, cells that produce IFN-γ, IL-2 and TNF-α simultaneously are important for the magnitude and quality of the induced immunity [39,40]. Thus, we investigated the production of IL-2 and TNF-α by vaccinated mice. We found higher levels of IL-2 produced by vaccinated animals. IL-2 may be contributing to the transient immunity these mice develop, since this cytokine mediates T-cell proliferation and induces effector functions in macrophages [41,42]. Regarding the production of TNF-α, we did not find differences between the groups in any time we investigated (data not shown), although higher levels were produced in response to *in vitro* stimulation of cells from both groups.

The role of IL-17 is not well established for *Leishmania* infection. Although a few studies have shown its protective effect during *L. braziliensis* and *L. donovani* infections [43,44], it seems to be a susceptibility factor during *L. major* infection in BALB/c mice [45]. Live *L. major* vaccine containing CpG motifs induces IL-17 production, which, in this case, has proven to be important for the generation of immunity [27]. We found higher production of this cytokine by vaccinated mice until four weeks post-infection, which may be associated with the protection. Interestingly, the difference in IL-17 production between groups was lost after 8 weeks post-infection. In addition, after 12 weeks of infection, when no differences in parasite burdens and lesion sizes were apparent, we found no differences in IL-17 between groups. In the first week post-infection, vaccinated mice produced higher levels of IL-4; however, this was not a very robust response. Consequently, it is difficult to evaluate what kind of effect this production had on counter-protective immunity. Also, vaccination produced only transient increase in IL-4 levels. After 4 weeks of infection, low levels of IL-4 were detected in both groups. Thus, it is possible that IL-4 does not play an important role in our vaccine protocol. IL-4 induces arginase activity in macrophages, and we did not find any difference in arginase activity in the footpads between control and vaccinated mice (data not shown). Furthermore, IL-4 is not considered a strong susceptibility factor for *L. amazonensis* infection in C57BL/10 mice and is only partially responsible for susceptibility in BALB/c mice [15]. It is curious that infection after Leishvacin® is able to induce concomitantly the production of Th1-type (IFN-γ, IL-2), Th2-type (IL-4) and Th17-type (IL-17) cytokines, especially at the beginning of infection. In addition, along with the induction of pro-inflammatory cytokines, we found that Leishvacin® induced higher production of IL-10. Increased IL-10 production has already been shown in other vaccine protocols that utilize *L. amazonensis* antigen [9]. This induction can also be associated with the fact that our vaccine is made of killed promastigotes, since inoculation of killed *L. major* promastigotes into healed mice causes loss of immunity by inducing higher production of IL-10 [46]. IL-10 inhibits TNF-α production by macrophages. This fact could explain why we did not find increased production of TNF-α after immunization [47].

The induction of *Leishmania*-specific IgG1 and IgG2a antibodies was found in vaccinated mice at all times post infection, even in late stages of infection. No correlation, however, was found between the increase of IgG1 or IgG2a and the cytokine profile of vaccinated mice. Furthermore, although antibody production during *L. amazonensis* infection has been associated with the pathogenesis of the disease (since mice that are deficient in functional B cells and antibodies are more resistant to the infection) [48], in our system, the induction of antibodies was a marker of immunization with parasite antigens and did not seem to alter tissue parasitism.

Even though vaccination was able to control lesion development and tissue parasitism during the first 8 to 10 weeks of infection, after 12 weeks of infection, lesions in control and vaccinated mice did not differ: lesions in control mice began to recede, whereas lesions in vaccinated mice became larger than at earlier time points. Also, parasite numbers at 16 weeks seemed smaller in control mice than at 10 weeks of infection (Fig. 1C). In contrast, parasite numbers in vaccinated mice increased at later time points. These similar levels of lesion development and tissue parasitism correlated with cytokine production (in particular IL-10 and IFN-γ) in control and vaccinated groups at 10 and 12 weeks of infection. Interestingly, the levels of IL-2 were considerably lower at later time points in vaccinated mice than at the first four weeks of infection. This suggests that at some point around 8 to 10 weeks of infection, the ability of the vaccination protocol to promote T-cell proliferation was overcome allowing increased parasitism during the following weeks even in the presence of IFN-γ levels, which were capable of controlling parasite proliferation at early time points. The histological aspect of lesions at 8 and 16 weeks in vaccinated mice is striking: infected macrophages are clearly loaded with parasites at the latter time point. The mechanism involved in this subversion of the protective response by the parasite is not clear.

It has been previously postulated that *L. amazonensis* causes a strong subversion of the host immune response: at the beginning of the infection, low levels of some inflammatory cytokines and chemokines were found. Maturation of dendritic cells is impaired, activation of macrophages by IFN-γ or type I IFNs favors the replication of parasites and

activation of T cells is pathogenic for the host [49]. Moreover, *L. amazonensis* antigen can suppress the proliferation of T cells, which could not be reverted by addition of IL-2, IL-4 or IFN-γ [50]. After the challenge with *L. amazonensis*, we found a slow but continuous increase in lesion size, in the inflammatory infiltrate and in the number of parasites in vaccinated mice. It is possible that one of the reasons for the loss of protection induced by vaccine is related with this strong manipulation of the immune system by the parasite. Given the facts that IL-10 production at 16 weeks of infection was lower than at early time points and that TGF-β levels were similar between groups, these cytokines do not seem to be implicated in the reversal of protection conferred by our vaccination protocol. Alternatively, vaccination merely reduced initial parasite numbers, and the course of infection was delayed in the vaccinated group.

The fact that we used a high-dose infection (10^5 promastigotes) in our model could influence the efficacy of vaccination since a low-dose infection of *L. amazonensis* induced a delay in the development of lesions in C57BL/6 mice [51]. It should be interesting to also perform these experiments using a low-dose infection (10^3 promastigotes), which is closer to what happens in natural infection, to address if in this scenario Leishvacin® could induce a long-term protection [52]. In addition, in our model, the vector saliva is absent, and it could modulate the host response [53].

In summary, our data showed that a transient resistance to *L. amazonensis* is obtained by our vaccination protocol. Resistance was associated with high levels of IFN-γ, IL-2 and IL-17. Immunized mice also produced higher levels of IL-10 at early time points. Our protocol induced lasting B-cell response. However, even though B-cell response persisted throughout our experiment, a drop of hallmark T-cell cytokines was observed in vaccinated mice at later time points. As infection progressed, the cytokine profile of vaccinated mice changed and became similar to that of control, unvaccinated *L. amazonensis*-infected mice. As a consequence, lesions became larger and parasite loads in vaccinated mice became similar to those of control mice. Hence, *L. amazonensis* overcame the partial protection conferred by the vaccine, demonstrating, once more, the overwhelming capacity of this parasite to interfere with the host immune system, even in the face of a pre-existing protective response.

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