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Original article

CD8$^+$T cells are not required for vaccine-induced immunity against *Leishmania amazonensis* in IL-12/23P40$^{-/-}$ C57BL/6 mice☆

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Abstract

Vaccine-induced protection against leishmaniasis is largely dependent on cell-mediated type 1 response and IL-12-driven IFN-γ production. Surprisingly, our previous data showed that IL-12/23p40$^{-/-}$ mice could be vaccinated against *L. amazonensis* and were able to produce limited amounts of IFN-γ. Since the role of CD8$^+$T in immunization against *L. amazonensis* is obscure, the aim of this study was to evaluate the effects of CD8$^+$ cells in protection against *L. amazonensis* in IL-12/23p40$^{-/-}$ mice. In order to deplete CD8$^+$ cells, one group of vaccinated animals was treated with anti-CD8 mAb. Infection was followed for 8 weeks. The vaccinated CD8$^+$-depleted group developed smaller lesions than the non-depleted group. CD8 depletion did not affect tissue parasitism or antibody response against the parasite, and treated animals displayed milder inflammation and better tissue integrity. IFN-γ production in spleen and draining lymph node was impaired in the depleted group, suggesting that CD8$^+$ cells produced this cytokine in IL-12-independent vaccination. Such results suggest that this T cell subset contributes to augmented pathology in IL12/23p40$^{-/-}$ mice vaccinated and challenged with *L. amazonensis*. Although these cells could produce some IFN-γ the in the absence of IL-12, they do not affect the parasite tissue load.

Keywords: Leishmania; Leishmaniasis; Protozoa; Parasite; Vaccine; Adjuvant; CD8$^+$

1. Introduction

Leishmaniasis is a parasitic infection of animals and humans caused by several species of protozoa from the genus *Leishmania*. 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, namely cutaneous leishmaniasis and diffuse cutaneous [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-γ production by most mouse strains leads to macrophage activation and control of parasite growth [3].

There is evidence that the host immune response to *L. amazonensis* infection is different from the response 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* regardless of the development of a Th2 response [4–6]. *L. amazonensis* triggers early production of IL-12 and IFN-γ in C57BL/6 mice similarly to *L. major* [7], but infection of C3H mice results in production of low levels of IL-12 and IFN-γ by antigen-specific CD4 T cells [5]. Chronic infection by *L. amazonensis* in C3H and C57BL/6 mice persists even after administration of exogenous IL-12 [5] or IFN-γ [8]. The inability of IL-12 in driving 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 $CD4^+$T cells [9,10], demonstrating that T cells are activated during *L. amazonensis* infection and that they contribute significantly to the pathology of the chronic disease [9].

Previous data from our laboratory [11] and from others [12] showed that vaccination of wild-type mice with a crude extract of *L. amazonensis*, in the presence of *Corynebacterium parvum* as an adjuvant, confers IFN-γ-dependent protection against infection, as measured by the parasite burden and the size of lesions. Surprisingly, our data also showed that the absence of IL-12 did not prevent the vaccination protocol from protecting mice, as determined in mice deficient in the p40 chain of IL-12 and IL-23 by homologous recombination (IL-12/23p40$^{-/-}$). Although these mice were more susceptible to infection, partial protection was conferred by the vaccination protocol. Very little IFN-γ production was found during *in vitro* antigen-stimulated lymph node and spleen cells, in accordance to the previously described crucial role of IL-12 in the induction of IFN-γ production by $CD4^+$T cells in the *L. major* model [13,14].

In the current study we hypothesized that $CD8^+$T cells might be involved, at least partially, in the vaccine-induced protection against *L. amazonensis* in IL-12/23p40$^{-/-}$ mice. It had been previously shown that resistance to infection with low doses of *L. major* in the dermis of mice was dependent on the presence of $CD8^+$T cells at the site [15]. Moreover, β2 microglobulin and perforin deficient mice primed with a leishmanial antigen were not able to control a challenge infection with *L. amazonensis* [16] suggesting a protective role for $CD8^+$T cells during immune response against *L. amazonensis* infection. Our model provided a unique opportunity to investigate the role of $CD8^+$T cells in the protection against *L. amazonensis* in the absence of IL-12 and the $CD4^+$T cell response induced by this cytokine. We found that CD8 depletion did not affect tissue parasitism or antibody response against the parasite. Nevertheless, $CD8^+$-depleted animals displayed milder inflammation and better tissue integrity.

2. Materials and methods

2.1. Animals

Female C57BL/6 and BALB/c mice (4–6 week 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-12/23 by homologous recombination in the C57BL/6 background (IL-12/23p40$^{-/-}$) 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. Matrices of mice deficient in the production of β2-microglobulin in the C57BL/6 background (β2-M$^{-/-}$) were originally purchased from Jackson Laboratories (Bar Arbour, ME, USA) and bred in the Gnotobiology and Immunology Laboratory. All animals were housed in ventilated racks with filters, controlled temperature and light cycles. 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, São Paulo, SP, Brazil) *ad libitum*.

2.2. Parasites and antigens

Leishmania amazonensis (IFLA/BR/1967/PH8 strain) promastigotes were grown to stationary phase at 25 °C in Grace's insect medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), 2 mM L-glutamine, 100 U of penicillin-G-potassium and 100 μg of streptomycin sulfate per ml (GIBCO). Leishmania antigen was obtained from stationary phase promastigotes washed four times in phosphate-buffered saline pH 7.4 (PBS). Parasite suspensions were submitted to seven cycles of freezing at −70 °C followed by thawing at 37 °C. Antigen suspension were adjusted to a concentration of 1 mg per ml. Antigens were stored at −70 °C and thawed immediately before use in cell cultures.

2.3. Vaccine

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

2.4. Vaccination and infection

Wild-type (wt) C57BL/6, β2-M$^{-/-}$ and IL-12/23p40$^{-/-}$ mice were vaccinated according to Costa et al. [17]. Vaccine was administered subcutaneously in 0.15 ml at the base of the tail. Each animal received two inoculations at seven day intervals, each dose containing 100 μg of vaccine protein plus 250 μg 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 μg of vaccine, without adjuvant. Seven days after the last

booster, animals were challenged with 10^4 *L. amazonensis* purified metacyclic promastigotes [18] from stationary phase cultures in the hind footpad. Control groups in this study were unvaccinated C57BL/6, IL-12/23p40$^{-/-}$ and β2-M$^{-/-}$ mice infected with metacyclic *L. amazonensis*. 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. In our hands, mice vaccinated with *C. parvum* without antigen were not protected against infection. In addition, mice could be challenged for up to 21 days after the last booster and were still protected against infection [11].

2.5. CD8$^+$ cell depletion

In order to deplete the CD8$^+$ T-cell subset, IL-12/23p40$^{-/-}$ vaccinated mice were inoculated i.p. with 250 μg anti-CD8 monoclonal antibody (clone YST169, kindly provided by Dr. Oscar Bruna-Romero, Department of Microbiology, Universidade Federal de Minas Gerais) in PBS or control antibody on days −6, −3, +4, +7 and once weekly after *L. amazonensis* infection. This schedule resulted in greater than 90% depletion of CD8$^+$ cells as assessed by FACS analysis. Infection was followed for 8 weeks. Injection with rat IgG was used as control for antibody depletion experiments.

2.6. Flow cytometry analysis

Depletion of CD8$^+$ cells was monitored in peripheral blood before infection by flow cytometry using monoclonal antibody Phitoeritrin (PE)-conjugated anti-CD8 (clone 53-6-7, BD Pharmingen, San Diego, CA, USA). All reactions were carried out on ice in PBS containing 0.5% BSA (Sigma Chemical Co, St. Louis, MO, USA) and 2 mM de NaN_3 (Sigma Chemical Co.) (FACS medium). Before surface labeling, samples of 10^5 cells from peripheral blood were pre-incubated with 0.5 μg of anti-mouse anti-CD8 monoclonal antibody (clone 53-6-7, BD Pharmingen). Cells were washed after 30 min incubation and resuspended in 500 μl of FACS medium. For each sample, between 10,000 and 20,000 cells were analyzed.

Mononuclear cells were prepared from spleens and lymph nodes as described [7]. To avoid the non-specific binding of antibodies to FcγR, the cells were preincubated with anti-mouse CD16/32 (clone 2.4G2, BD Pharmingen, San Diego, CA, USA) and PBS 0.5% BSA plus 10% inactivated normal serum mouse and 2 mM de NaN_3 before staining. Two-color immunofluorescent staining was performed to identify CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cells by use of anti-mouse mAbs: anti-CD4 fluorescein isothiocyanate (FITC)-conjugated (clone GK1.5, BD Pharmingen), anti-CD8 peridinin-chlorophyll-protein complex (PerCP)-conjugated (clone 53-6.7, BD Pharmingen) and anti-CD25 PE-conjugated (clone PC61, BD Pharmingen). Briefly, 10^5 cells per well were incubated for 30 min at 4 °C with 0.5 μg of fluorescent antibody. The cells were washed and fixed with 2% formaldehyde (Sigma). A total of 20,000 cells were acquired for each sample. The data were collected and analyzed using CellQuest software (BD Immunocytometry Systems) and a FACSCan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.7. Cell culture and parasite quantification by serial dilution

Spleen and lymph node (popliteal and inguinal) single cell suspensions were obtained as previously described [7] and cultured at 5×10^6/ml 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 [11]. The footpads were homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at 150 × *g* and cells were concentrated by centrifugation at 2000 × *g*. Pellets were resuspended in 400 μl of Grace's supplemented culture medium (see above). Samples were plated onto culture plates and serially diluted 1:10 in supplemented Grace's insect tissue culture medium. Each sample was plated in duplicates and read 5 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).

2.8. Cytokine assays

IFN-γ and IL-4 were assayed in the supernatants of spleen or lymph node cell cultures were assayed by two site ELISA as described [7]. Rat anti-IFN-γ monoclonal antibody R46A2 was used as capture (APABCAM, Associação Técnico Científica Paul Ehrlich, Rio de Janeiro, RJ, Brazil) and polyclonal rabbit serum specific for the cytokine was used as detection antibodies. Sensitivity was 16 pg/ml. The assay for IL-4 was performed using 11B11 mAb (APABCAM) for coating and biotinilated BVD6 mAb (kindly provided by Dr. Phill Scott, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA) as detection of bound IL-4. ELISA for IL-4 had a sensitivity of 15 pg/ml. IL-2 and IL-17 were assayed in the supernatants using a mouse IL-2 and IL-17 DuoSet ELISA (R&D Systems, INC Minneapolis, MN, USA). ELISA for IL-2 had a sensitivity of 9 pg/ml and for IL-17 of 4 pg/ml.

2.9. IgE and antigen-specific 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, wells were incubated with biotinylated murine anti-IgE antibody (clone R35–72, Southern Biotechnology) at a 1:500 dilution, washed, incubated with horseradish peroxidase-streptavidin (Sigma Chemical Co.) and developed using *ortho*-phenilenediamine and peroxide as substrates. A positive control was performed using serum from BALB/c mice rendered allergic to

ovalbumin (kindly provided by Drs. Janaína S. Saldanha and Denise Carmona Cara, Departamento de Patologia, ICB, UFMG). In order to detect antigen-specific IgG2a, ELISA for specific IgG2a antibodies was optimized regarding antigen concentrations, sera and conjugate dilutions [11]. *L. amazonensis* antigens derived from *in vitro* promastigote cultures (10 μg protein/ml) were diluted in 0.1 M sodium carbonate buffer (pH 9.6) and 100 μl 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 (Sigma Chemical Co.) and mouse sera were diluted 1:50 with PBS-1% BSA, and 100 μl per well were added to the plates. After 2 h incubation at 37 °C, plates were washed five times with PBS containing 0.05% Tween 20 (Sigma Chemical Co.). Wells were incubated with goat anti-mouse IgG2a (Southern Biotechnology) at a 1:4000 dilution, washed, incubated with rat anti-goat horseradish peroxidase-labeled antibody (Southern Biotechnology) at a 1:2000 dilution and developed as above. Absorbance values were read at 492 nm in a Spectra Max Plus reader (Spectra Max Plus reader Molecular Devices Corporation, Sunnyvale, CA, USA).

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.

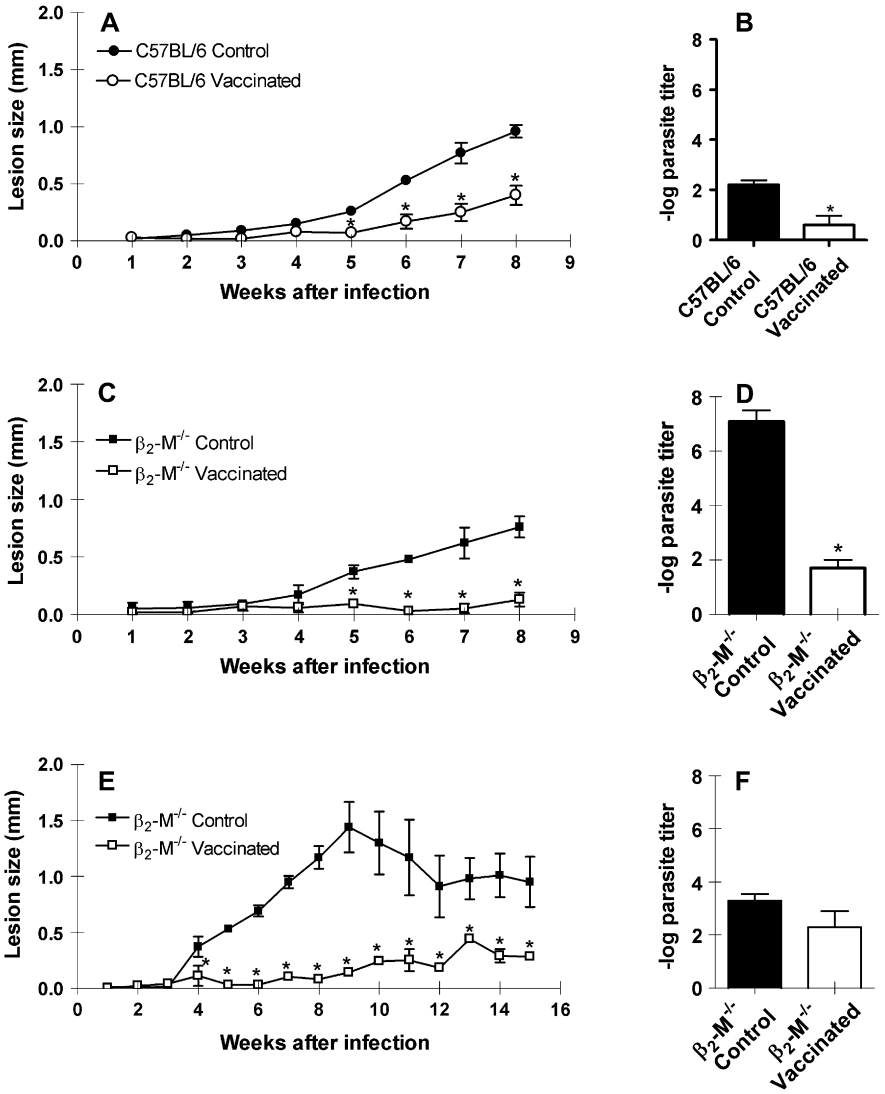

Fig. 1. Parasite burdens and course of infection with *L. amazonensis* in vaccinated and control wild type and β2-M$^{-/-}$ mice. Mice were vaccinated as described in Section 2 and challenged in the left hind footpad with 10^4 stationary-phase promastigotes. Control (non-vaccinated) mice were challenged in the same way. Lesion sizes were measured weakly in control and vaccinated wild type C57BL/6 (A) and β2-M$^{-/-}$ (C and E) mice. Each point in line graphs represents the mean difference in size ± standard deviation of the mean between infected and uninfected footpads. Parasite quantification in footpad lesions were performed at 8 (B and D) or 15 (F) weeks after infection of vaccinated and non-vaccinated wild type C57BL/6 (B) and β2-M$^{-/-}$ (D and F) or wild type C57BL/6 mice. Bars represent mean ± standard deviation of the mean for five animals per group; results are represented as the negative log of the titer, as described in Section 2. The asterisk indicates a statistically significant difference compared with the other group in the same experiment ($P \leq 0.05$).

3. Results

3.1. Effect of vaccination against L. amazonensis in β2-M$^{-/-}$ C57BL/6 mice

β2-M$^{-/-}$ mice and wt controls were vaccinated at the base of the tail. Lesion size was measured for 8 and 15 weeks after challenge with 10^4 *L. amazonensis* promastigotes. This protocol of vaccination protected C57BL/6 mice (wt) against infection: these mice showed smaller lesions and smaller parasite numbers than non-vaccinated controls (Fig. 1A,B). Lesion sizes in β2-M$^{-/-}$ mice were comparable to those of wt mice (Fig. 1A,C), but, as shown in Fig. 1D, the parasite burden in β2-M$^{-/-}$ mice at 8 weeks of infection was significantly larger (about 10^5 times, $P < 0.05$) than in lesions form wt mice. Comparison between vaccinated and non-vaccinated β2-M$^{-/-}$ mice showed a statistically significant difference in lesion sizes from 4 to 5 weeks post-infection (Fig. 1C,E). Again, parasite burdens at 8 weeks of infection were higher in lesions from β2-M$^{-/-}$ vaccinated mice than in lesions from the wt vaccinated group ($P < 0.05$). At 15 weeks of infection, control β2-M$^{-/-}$ mice presented smaller parasite burdens (Fig. 1F) than at 8 weeks of infection, equivalent to numbers found in vaccinated mice, albeit lesions were still larger in the control than in the vaccinated group (Fig. 1E).

Histological analysis of the infected footpad from non-vaccinated β2-M$^{-/-}$ mice shows an extensive vacuolated area and numerous parasitized macrophages (Fig. 2A,B), in accordance with the larger lesions and higher parasite numbers found in lesions. On the other hand, lesions from vaccinated β2-M$^{-/-}$ mice presented smaller vacuolated areas and less macrophages presenting parasites (Fig. 2C,D), also in accordance to the data in Fig. 1C,D.

3.2. Effect of CD8$^+$ cell depletion on vaccination against L. amazonensis in IL-12/23p40$^{-/-}$ mice

IL-12 has been shown to be essential for vaccination against *L. major* [14]. Previous data from our laboratory showed that, surprisingly, there was a partial control of *L. amazonensis* infection in vaccinated IL-12/23p40$^{-/-}$ mice. In addition, IFN-γ production, albeit small, was found up-regulated in the vaccinated IL-12/23p40$^{-/-}$ animals [11]. The main protective function of antigen-specific CD8$^+$T cells during infection with *L. major* seems to be to contribute to the release of IFN-γ in the parasite-loaded dermis [3]. Moreover, studies demonstrated that a specific and preferential activation of perforin and IFN-γ-producing CD8$^+$T cells occurs at the cutaneous site of infection of mice protectively immunized with P-8 antigen and challenged with *L. amazonensis* [16]. To evaluate

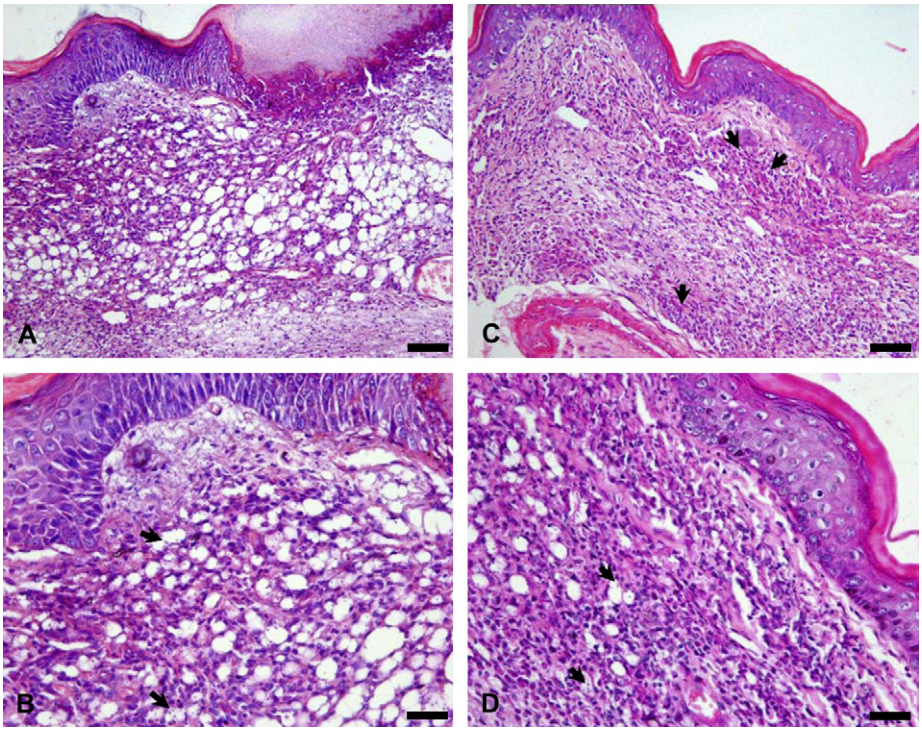

Fig. 2. Representative histopathology of lesions from vaccinated and non-vaccinated β2-M$^{-/-}$ mice. Animals were infected with *L. amazonensis* and tissues were collected at 8 weeks after infection, prepared and stained with hematoxilyn and eosin as described in Section 2. (A) Section from a lesions from infected β2-M$^{-/-}$ mice showing a portion of an extensive vacuolated area. (B) Section from a lesions from infected β2-M$^{-/-}$ mice showing numerous vacuolated and parasitized macrophages. Arrows indicate parasites. (C) Section from a lesion from vaccinated and infected β2-M$^{-/-}$ mouse, showing a portion of an extensive inflammatory area and the destruction of normal structures, indicated by arrows. (D) Section from a lesion from a vaccinated and infected β2-M$^{-/-}$ mouse, showing a limited vacuolated area (arrow) compared with that in B. Magnification in A and C: bars = 25 μm; in B and D: bar = 12.5 μm. Experiment was repeated with similar results.

the contribution of CD8$^+$T cells in protection against *L. amazonensis* in vaccinated IL-12/23p40$^{-/-}$ mice, we depleted CD8$^+$ cells in these animals. As can be seen in Fig. 3A,B, depletion of CD8$^+$ cells on days −6, −3, +4, +7 and once weekly after *L. amazonensis* infection reduced the CD8$^+$ cells by 90%. As previously [11], lesions from vaccinated and non-vaccinated IL-12/23p40$^{-/-}$ mice differed statistically from 4 to 8 weeks post infection (Fig. 3C). The vaccinated and CD8$^+$-depleted group developed smaller lesions when compared to the vaccinated non-depleted group (Fig. 3C). CD8$^+$ cell depletion did not, however, affect tissue parasitism (Fig. 3D). Histology of footpads from infected IL-12/23p40$^{-/-}$ mice showed an extensive vacuolated area and numerous vacuolated and parasitized macrophages (Fig. 4A,B), in accordance with higher numbers of parasites and large lesions. Sections from lesions from vaccinated and infected IL-12/23p40$^{-/-}$ mice showed an extensive inflammatory area and the destruction of normal structures, but a limited vacuolated area compared with that in B (Fig. 4C,D). Lesions from vaccinated, CD8$^+$-depleted and infected IL-12/23p40$^{-/-}$ mice displayed milder inflammation, in accordance with their smaller lesions and better tissue integrity (Fig. 4E,F). Interestingly, even though parasites are present in lesions from vaccinated CD8$^+$-depleted mice, macrophages were not vacuolated in these lesions (arrows in Fig. 4F).

After 8 weeks of infection, the levels of IFN-γ, IL-2, IL-4 and IL-17 in lymph node and spleen cell cultures from vaccinated, vaccinated and CD8$^+$ depleted and non-vaccinated IL-12/23p40$^{-/-}$ mice were analyzed. Although cells from IL-12/23p40$^{-/-}$ mice produced practically undetectable levels of IFN-γ *in vitro*, lymph node and spleen cell cultures from IL-12/23p40$^{-/-}$ vaccinated mice presented higher levels of IFN-γ when compared to CD8 depleted and non-vaccinated mice (Fig. 5A,B). The IL-2 levels were similar among the groups of IL-12/23p40$^{-/-}$ mice (Fig. 5C,D). IL-17 levels in lymph nodes similar in all groups. However, these levels were significantly higher in IL-12/23p40$^{-/-}$ vaccinated mice when compared with CD8-depleted vaccinated mice

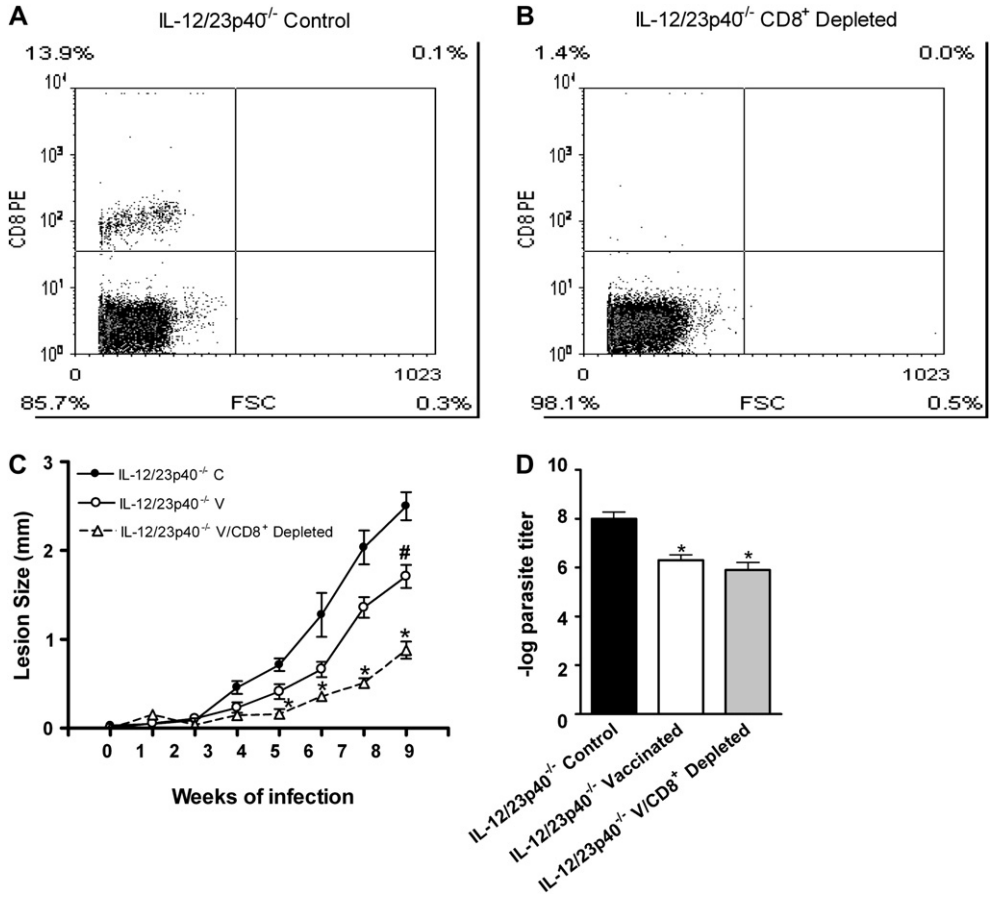

Fig. 3. Evaluation of the contribution of CD8$^+$T cells to the protection induced by vaccination in IL-12/23p40$^{-/-}$ mice. Vaccinated mice were treated with anti-CD8 antibody as described in Section 2. (A) Flow cytometry of cells from control IL-12/23p40$^{-/-}$. (B) Flow cytometry of cells from CD8$^+$-depleted IL-12/23p40$^{-/-}$ mice. Graphs represent FACS analyses of one representative mouse. The number in the top left represents the percentage of positive events in the quadrant. (C) Course of infection in vaccinated (V), vaccinated and CD8$^+$ depleted and non-vaccinated (C) IL-12/23p40$^{-/-}$ mice. 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. The asterisks indicate a statistically significant difference ($P \leq 0.05$) compared with the vaccinated non-depleted group. # indicates statistical difference between control and vaccinated IL-12/23p40$^{-/-}$ from week 5 of infection. (D) Parasite quantification in footpad lesions 8 weeks after infection. Each bar represents mean ± standard deviation of the mean for five animals per group. The asterisks indicate a statistically significant difference ($P \leq 0.05$) compared with the control group.

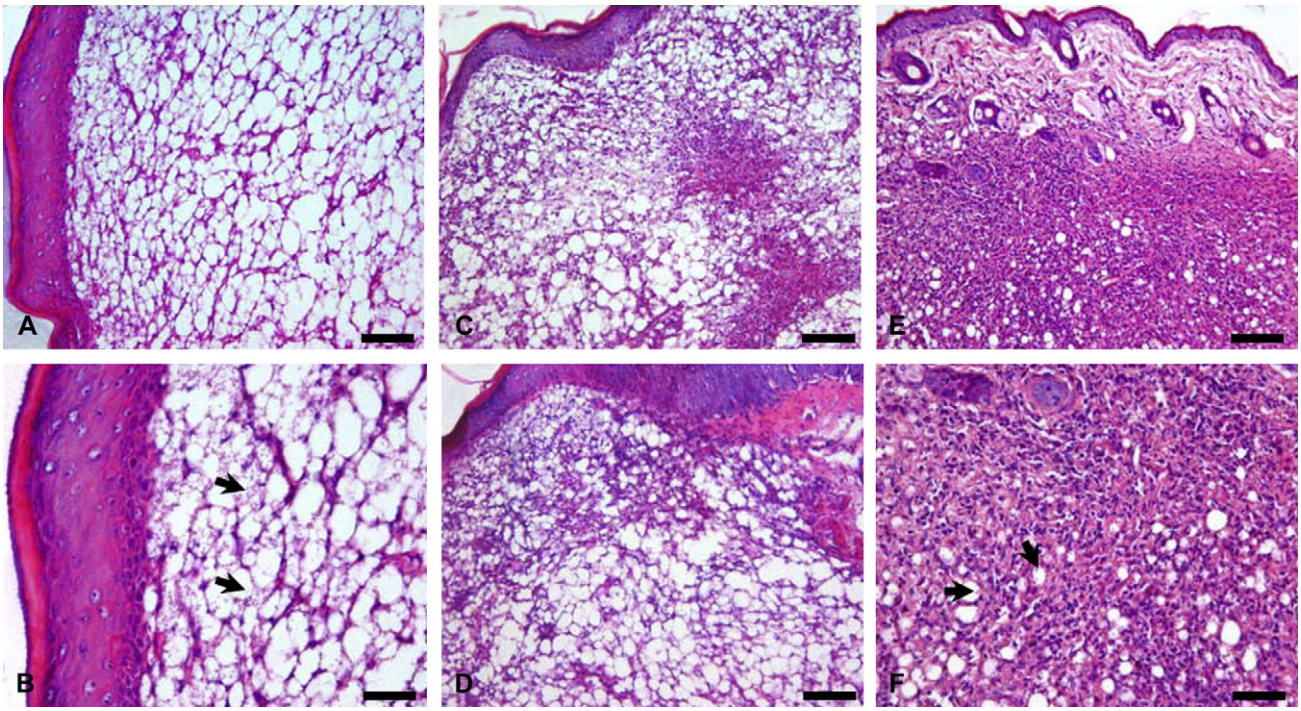

Fig. 4. Representative histopathology of lesions from vaccinated, vaccinated and $CD8^+$-depleted and non-vaccinated IL-12/23p40$^{-/-}$ mice. Mice were infected with *L. amazonensis* and tissues were collected 8 weeks after infection and prepared and stained with hematoxilyn and eosin as described in Section 2. (A) Section from lesions from infected IL-12/23p40$^{-/-}$ mice showing a portion of an extensive vacuolated area. (B) Detail from (A) showing numerous vacuolated and parasitized macrophages (arrows). (C) Section from a lesion from vaccinated and infected IL-12/23p40$^{-/-}$ mouse, showing a portion of an extensive inflammatory area and the destruction of normal structures. (D) Section from a lesion from a vaccinated and infected IL-12/23p40$^{-/-}$ mouse, showing a limited vacuolated area compared with that in B. (E) Section from a lesion from a vaccinated, $CD8^+$-depleted and infected IL-12/23p40$^{-/-}$ mouse showing a limited vacuolated area compared with that in A and B (F) Section from a lesion from a vaccinated, $CD8^+$-depleted and infected IL-12/23p40$^{-/-}$ mouse, showing a field with containing parasites (arrows). Magnification in A, C and E: bars = 25 μm; in B, D and F: bar = 12.5 μm. Experiment was repeated with similar results.

(Fig. 5E,F). IL-4 was not detected in supernatants from lymph node or spleen cell cultures, as previously described [11]. Furthermore, the absence of significant IL-4 production was further confirmed by IgG2a levels, as well as by IgE levels in sera from infected mice. IgG2a was lower in all groups when compared to vaccinated wt mice (Fig. 6A). No significant differences were detected in IgE levels, which were low in all groups (Fig. 6B). BALB/c mice rendered allergic to ovalbumin were used as positive controls of a typical Th2 response [19]. To evaluate the contribution of activated $CD4^+$ and $CD8^+$T cells in protection again *L. amazonensis* in our model, we analyzed the $CD4^+CD25^+$ and $CD8^+CD25^+$ population in the spleens and lymph nodes at 4 and 8 weeks after infection in IL-12/23p40$^{-/-}$ and wt mice. The percentage of $CD4^+CD25^+$T cells in draining lymph nodes was not different among groups of IL-12/23p40$^{-/-}$ mice after 4 and 8 week of infection. However, in spleens from IL-12/23p40$^{-/-}$ vaccinated mice after 8 week of infection, the percentage of $CD4^+CD25^+$T cells was significantly higher when compared with non-infected IL-12/23p40$^{-/-}$ mice. $CD8^+CD25^+$T cell percentages were not different among groups (Fig. 7).

4. Discussion

While infection with *L. major* leads to self healing lesions in most mouse models, mouse strains that are resistant to *L. major* generally develop a chronic lesion when infected with *L. amazonensis* or *L. mexicana* [20]. A partial resistance to infection may be obtained when mice are immunized with crude parasite antigens and *C. parvum* as adjuvant [11,17]. In this model of resistance, smaller lesions and parasite numbers correlate with higher IFN-γ production [11]. We have also shown that in the absence of functional IL-12 some protection was obtained after vaccination, which also correlated with an increase, albeit small, in IFN-γ production [11]. Hence, some protection against *L. amazonensis* could be obtained in the absence of IL-12, which would suggest a $CD4^+$T cell independent mechanism. $CD8^+$T cells are IFN-γ producers and may do so independently of IL-12 [21,22]. In this paper, we investigated the role of $CD8^+$T cells in the course of infection with *L. amazonensis* and in the resistance found in vaccinated IL-12/23p40 infected with *L. amazonensis*.

Several authors demonstrated the role of IL-12, $CD4^+$ and $CD8^+$T cells in resistance rendered by vaccination protocols to species belonging to the *Leishmania mexicana* complex [16,23], but contradictory data are found regarding the role of $CD8^+$T cells during primary infection with parasites of this complex. Overath and Harbecke [24] infected β2-M$^{-/-}$ mice with *L. major* or *L. mexicana* and found a course of infection similar to that in wt mice. On the other hand, Colmenares et al. [16] describe smaller lesions and parasite numbers in β2-M$^{-/-}$ mice infected with *L. amazonensis*. In the present

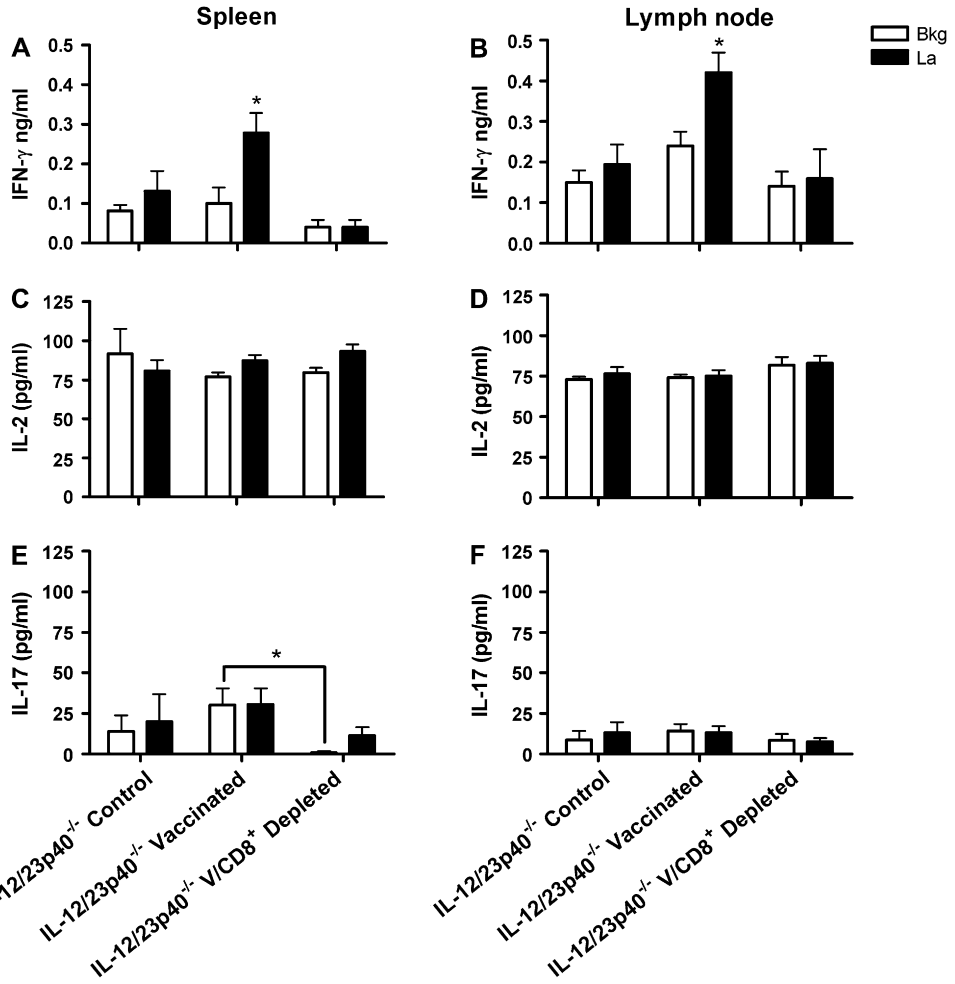

Fig. 5. *In vitro* IFN-γ, IL-2 and IL-17 production by spleen and lymph node cells from vaccinated (V), vaccinated and CD8 depleted and non-vaccinated IL-12/23p40$^{-/-}$ mice (control). Animals were infected for 8 weeks with *L. amazonensis*. Each bar represents mean ± standard deviation of the mean for five animals per group. The asterisks indicate a statistically significant difference ($P \leq 0.05$) from all other bars or from marked bars. Bkg: no antigen added to the culture. La: *L. amazonensis* freeze-thawed antigen added to the culture.

study, we investigated the role of CD8$^+$T cells in infection with *L. amazonensis* by two approaches: (1) we determined the course of infection with *L. amazonensis* in vaccinated and naïve β2-M$^{-/-}$ mice, which lack CD8$^+$T cells [25]; and (2) we depleted CD8$^+$T cells in IL-12/23p40$^{-/-}$ mice which would have no protection due to IL-12-induced CD4$^+$ Th1 cells [13,26].

Infection of β2-M$^{-/-}$ mice with *L. amazonensis* showed a course of lesion development that was very similar to that of wild-type C57BL/6 mice. These data are comparable to ones obtained by others [16,24,27,28], who found that C57BL/6 deficient in β2-microglobulin or CD8 showed similar courses of infection with *L. major*, *L. mexicana* or *L. amazonensis*. In our hands, parasite loads in wt mice infected with *L. amazonensis* is partially controlled over time [11]. Here, we found that this was also true for β2-M$^{-/-}$ mice infected with *L. amazonensis*, since the titers of parasites in lesions at 8 weeks of infection were several orders of magnitude higher than those found at 15 weeks of infection (Fig. 1D,F). However, β2-M$^{-/-}$ mice seemed to have impaired capacity to control parasites at the same rate as wt mice: at weeks 8 of infection the parasite titers in β2-M$^{-/-}$ were several orders of magnitude higher than wt at the same time point. Colmenares et al. [16] reported lower parasite loads (two orders of magnitude) in β2-M$^{-/-}$ mice when compared to wt at 10 weeks after infection with *L. amazonensis*. It is possible that there is a delayed control in β2-M$^{-/-}$ mice compared with wt as we reported here (five orders of magnitude), and a later similar parasite load. In our hands, parasite titers oscillate over time in *L. amazonensis*-infected mice at later time points (later than 6 weeks) by at least two orders of magnitude. Our data, together with the previously reported by others, show that CD8$^+$T cells are not an absolute requirement for the partial resistance to *L. amazonensis* found in wt C57BL/6 mice.

In contrast to data reported by Colmenares et al. [16], who showed that β2-M$^{-/-}$ mice could not be vaccinated by the amastigote antigen P-8 in combination with *C. parvum*, β2-M$^{-/-}$ mice were successfully vaccinated by our crude antigen preparation and *C. parvum*. It is possible that the pool of

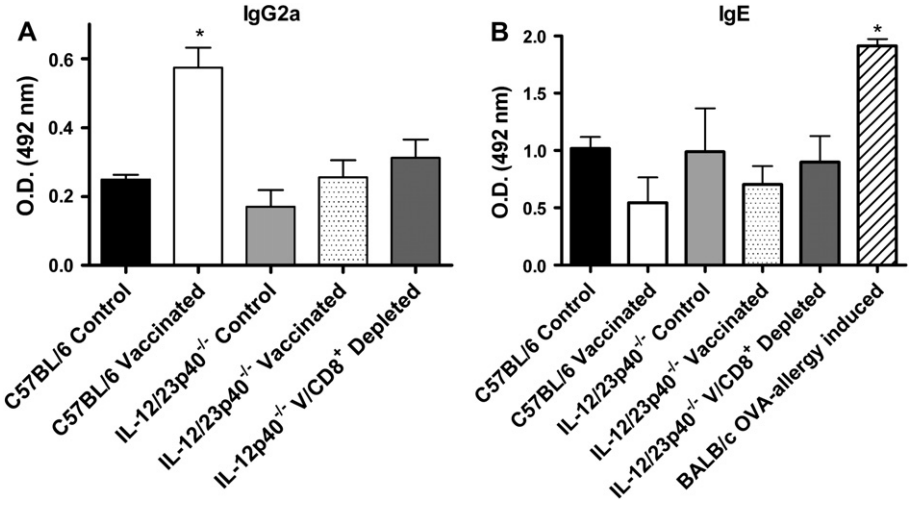

Fig. 6. Antibody isotypes in sera from vaccinated, vaccinated and CD8 depleted and non-vaccinated IL-12/23p40$^{-/-}$ mice challenged with *L. amazonensis*. Data from vaccinated and non-vaccinated wild type C57BL/6 mice infected with *L. amazonensis* are also shown for comparison. Mice were infected with *L. amazonensis* for 8 weeks. (A) IgG2a was measured by incubating sera over wells pre-coated with *L. amazonensis* antigen, washing, and quantitating the captured antibody using an isotype-specific mAb and colorimetric development. (B) Total IgE was quantitated using sandwich ELISA. Serum from BALB/c OVA-induced allergic mice was used as positive control for IgE. Results are expressed as mean of the optical density units (OD). Each bar represents mean ± standard deviation of the mean for five animals per group. The asterisk indicates a statistically significant difference compared with the other group in the same experiment ($P \leq 0.05$).

antigens offered in our immunization protocol overcomes the necessity for CD8$^+$T cells reported by those authors. We found both smaller lesions and parasite burdens in β2-M$^{-/-}$ mice, albeit parasite numbers were slightly higher than in vaccinated wt mice. Remarkably, histological aspects of lesions from vaccinated and non-vaccinated β2-M$^{-/-}$ mice were quite different: cellular infiltrates in vaccinated mice were considerably smaller than non-vaccinated mice and less parasitized cells were found, supporting the protection conferred by our immunization protocol.

Hence, in the absence of β2 microglobulin (and CD8$^+$T cells) the course of infection and immunization by antigens and adjuvant were similar to those found in mice expressing β2-microglobulin. However, would these cells play a role when CD4$^+$ IFN-γ-producing T cells were not present due to the absence IL-12? In order to answer this question, we infected IL-12/23p40$^{-/-}$ mice and depleted them with an anti-CD8 monoclonal antibody. We found that depletion of CD8$^+$ cells did not change the parasite burden in lesions, but the lesions were somewhat smaller when these cells were absent.

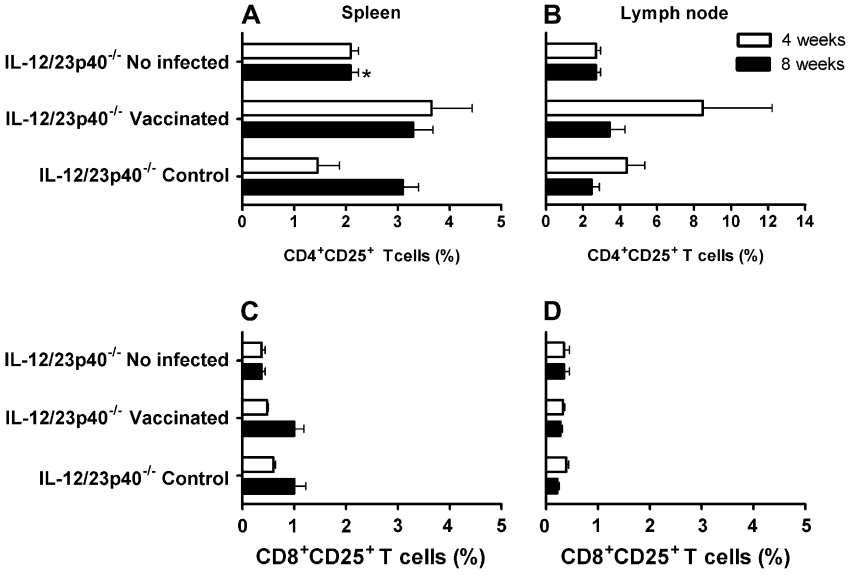

Fig. 7. Analysis by flow cytometry of CD4$^+$CD25$^+$ (A and B) and CD8$^+$CD25$^+$ (C and D) cell populations in IL-12/23p40$^{-/-}$ mice infected with *L. amazonensis* (control), vaccinated and infected or non-infected IL-12/23p40$^{-/-}$ mice. Cells from spleens (A and C) or lymph nodes (B and C) were harvested from mice 4 or 8 weeks after infection and stained as described in Section 2. Each bar represents the mean ± standard deviation of the mean for five animals per group. The asterisk indicates a statistically significant difference ($P \leq 0.05$) from the other groups at the same time point.

Examination of tissues showed that lesions were smaller due to a less vacuolated area and smaller cellular infiltrate to the site of infection. Thus, lesions could be smaller because $CD8^+$T cells could influence in the leukocyte recruitment to infected tissues through the production of pro-inflammatory chemokines such as MIP-1α and RANTES [29,30].

It was previously shown that migration of T cells to the site of infection is crucial for the development of pathogenesis [9], our data is consistent with this observation. Another interesting observation was the lack of vacuolization in the $CD8^+$-depleted lesions, the reason for which is unknown. Moreover, the small amounts of IFN-γ that were seen in culture supernatants of lymph nodes and spleens of IL-12/23p40$^{-/-}$ mice were abrogated when $CD8^+$T cells were depleted. On the other hand, He et al. [31] demonstrated a new mechanism for T cell-mediated delayed-type immune response, which is mediated by CD8 IL-17-producing T cells. Here, IL-17 levels were significantly higher in IL-12/23p40$^{-/-}$ vaccinated mice compared with CD8-depleted and vaccinated mice, which explain the decrease in the lesion size found in this latter group. Although several studies indicate that IL-17 production is mediated by IL-23 [32–34], Wozniak et al. found that IL-23 is not essential for the development of IL-17-secreting T cells [35]. In our model, in the absence of IL-12 and IL-23, IL-17 production was low is present. In addition, the percentage of activated $CD8^+$T cells was not different between IL-12/23p40$^{-/-}$ vaccinated groups and control mice infected with *L. amazonensis*.

The smaller IFN-γ found in IL-12/23p40$^{-/-}$ $CD8^+$-depleted mice was not reflected by a smaller IgG2a production. Higher IgG2a production was only found in wt vaccinated mice, which produce much higher levels of IFN-γ than IL-12/23p40$^{-/-}$ vaccinated animals [11]. No default to a Th2 response was found in any IL-12/23p40$^{-/-}$ mice regardless of the depletion of $CD8^+$ cells, as depicted by our inability to detect IL-4 and by low IgE levels. The IL-2 levels were not different among groups of IL-12/23p40$^{-/-}$ mice, suggesting that in this model $CD4^+$T cells are not significant contributors to immunity. However, the percentage of $CD4^+CD25^+$T cells in the spleens from IL-12/23p40$^{-/-}$ vaccinated mice after 8 weeks the infection were significantly higher compared with non-infected IL-12/23p40$^{-/-}$ mice.

In conclusion, we found that $CD8^+$T cells play a minor role in parasite control, as shown by higher parasite numbers in β2-M$^{-/-}$ mice. However, using a crude preparation we could immunize mice that lack $CD8^+$T cells. We also showed that the partial resistance conferred by immunization with crude extracts of *L. amazonensis* and adjuvant in the absence of functional IL-12 is not dependent on $CD8^+$ cells.

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