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Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania* confers protection against murine cutaneous leishmaniosis

Salvador Iborra, Manuel Soto, Javier Carrión, Carlos Alonso, Jose M. Requena*

Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, Campus de Cantoblanco, Madrid E-28049, Spain

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Abstract

Leishmania histones are relevant immunogens for the host immune system during both *Leishmania* infection and disease. In the present paper we have evaluated the prophylactic value of the four *Leishmania infantum* histones forming the nucleosomal core in the murine model of cutaneous leishmaniasis. In a first stage, the immune response elicited by the intramuscular injection of a mixture of four plasmid DNAs, encoding the *L. infantum* histones H2A, H2B, H3 and H4, was determined in BALB/c mice. It was found that the immunized animals developed a specific Th1 immune response, which was associated with an antigen-specific production of interferon (IFN-γ) and a limited humoral response against histones (dominated by antibodies of the IgG2a isotype). According to the pure Th1-type immune response elicited by the DNA vaccination with *Leishmania* histones, vaccinated mice showed a solid immunity that efficiently controlled the *Leishmania major* infection. The protection in mice vaccinated with histone-DNAs was associated with a low humoral response against leishmanial antigens, an enhanced IFN-γ production and little, if any, IL-4 production. The relative contribution of both $CD8^+$ and $CD4^+$ T cells to the IFN-γ production, and the IL-12 dependence were also evaluated. All these data indicated that DNA vaccination with *Leishmania* histones genes results in a specific Th1-like response during *L. major* infection, and that both $CD4^+$ and $CD8^+$ T cells contribute to the resistance of vaccinated mice to cutaneous leishmaniasis.

Keywords: Histones; DNA vaccines; Th1/Th2 immune responses

1. Introduction

Leishmania protozoa are obligate intracellular parasites that infect cells of the mononuclear phagocyte lineage of their vertebrate hosts. These parasites are the etiological agents of leishmaniasis, a group of diseases affecting an estimated 12 million people worldwide, there are 2 million new cases each year and another 350 million people are at risk of infection (World Health Organization Leishmaniasis page: http://www.who.int/emc/diseases/leish/leisdis1.html). The different clinical manifestations of leishmaniasis, ranging from self-healing cutaneous ulcers to potentially fatal visceral spread, are determined by the species of *Leishmania* that infects the host and by the response of the host to the parasite [1,2]. Current control measures rely on chemotherapy, but the chemotherapeutic arsenal is limited and far from satisfactory, it includes pentavalent antimonials as first-line drugs and amphotericin B and pentamidine as second-line drugs [1,3]. There is consensus that vaccines ought to become a major tool in the control of this group of diseases. However, to date, there are no vaccines against any form of clinical leishmaniasis [2,4].

Murine models of leishmaniasis have been extensively used to explore the requirements for effective vaccination. *Leishmania major* infection in mice causes a cutaneous form of the disease that resembles many aspects of the human disease, including a range of susceptibility states depending on the strain of mouse. Thus, resistance to *L. major* infection in mice correlates with the induction of an interleukin-12 (IL-12)-driven, interferon (IFN-γ)-dominated Th1 response. In contrast, susceptibility correlates with the dominance of an IL-4-driven Th2 response [5]. Genetic vaccination has recently provided a promising new approach to vaccination, and protective responses with DNA vaccines against

* Corresponding author. Tel.: +34 91 4978454; fax: +34 91 4974799.
E-mail address: jmrequena@cbm.uam.es (J.M. Requena).

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several pathogens have been demonstrated [6,7]. DNA vaccination has the ability to induce immune responses of the Th1-type and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses. Following this approach, several DNA vaccines containing *Leishmania* genes have been assayed in the *L. major* mouse model, showing different degree of efficacy: Gp63 [8], LACK [9], PSA [10], meta1 [11], and P0 [12]. Recent reports have shown that vaccination is improved when several distinct antigens are co-administered. Thus, a cocktail of plasmids expressing the *Leishmania* antigens LACK, LmST11 and TSA conferred complete protection against *L. major* infection [13]. Similarly, co-injection of two *L. major* genes expressing different cysteine proteinases induces a long lasting protective response, whereas the separate injection of cysteine proteases genes is not protective [14]. These results, therefore, are suggesting that effective vaccination against a complex parasitic infection such as leishmaniasis would require a multivalent vaccine containing a large number of candidate genes. In agreement with this idea is the finding that mice immunized with genomic expression libraries of *L. major* develop significant protective immunity against a challenge infection [15].

Several lines of evidence suggest that *Leishmania* histones are immunologically relevant molecules during leishmaniasis. Thus, Melby et al. [16] showed that a significant reduction in parasite burden was induced after immunization of BALB/c mice with a cDNA expression library from *Leishmania donovani*. Following a reductionist approach, they identified two groups of cDNAs that afforded protection; one group was composed by five cDNAs that encoded *L. donovani* histone proteins. In an independent study, searching for *Leishmania* antigens that are recognized by $CD4^+$ T cell lines from human donors recovered from the infection, the *Leishmania* histone H2B was identified as the most frequently recognized antigen [17]. Also, de Carvalho et al. [18] found that *Leishmania infantum* H2A and H3 stimulated PBMC, from either cutaneous leishmaniasis patients or asymptomatic *L. chagasi*-infected patients, to produce IFN-γ. On the other hand, antibodies reacting with the four core histones of *L. infantum* (H2A, H2B, H3 and H4) are frequently observed in the sera from humans and dogs with visceral leishmaniasis [19–22], indicating that they are strong immunogens during *Leishmania* infections. Altogether, these data prompted us to analyze the potential of *L. infantum* histones as vaccine candidates for leishmaniasis.

As described in this work, the sequences for histones in *L. infantum* and *L. major* are essentially the same. On the basis of this finding, we explored the immunoprophylactic value of a "DNA-cocktail" of eukaryotic expression plasmids containing the genes for the *L. infantum* histones (H2A, H2B, H3 and H4) in the prototypical mouse model of *L. major* infection. Interestingly, vaccinated mice developed a Th1 response against the four antigens and were protected against an *L. major* challenge.

2. Materials and methods

2.1. Mice and parasites

Female 6–8-week-old BALB/c mice were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). *L. major* (WHOM/IR/-173) was a kind gift of Dr. R.M. Gonzalo (Centro Nacional de Biotecnología, Madrid, Spain). Promastigotes were cultured at 26 °C in Schneider's medium (Gibco, BRL) supplemented with 20% fetal calf serum (FCS). Parasites were kept in a virulent state by passage in BALB/c mice. *L. major* amastigotes were obtained from popliteal lymph nodes, and after transformation to the promastigote form, parasites were grown until stationary phase and then harvested for inoculation in the left hind footpad of mice.

2.2. Plasmid constructs and DNA preparation

The cDNAs coding for the *L. infantum* histones were isolated by immunoscreening of a λgt11 expression library: H2A (clone cL72; [23]), H2B (clone LiH2B; [21]), H3 (clone LiB6; [24]) and H4 (clone LiH4-1; [21]). After *Eco*RI digestion, the cDNAs were ligated into the *Eco*RI site of the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA). Accordingly, the resulting clones were named pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 or pcDNA3-LiH4. DNAs from these pcDNA3-derived plasmids were purified by the endotoxin-free Giga-preparation Kit (Qiagen, Hilden, Germany).

2.3. Expression of Leishmania histones in mammalian cells

COS7 cells were transfected with 20 μg of either pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 or pcDNA3-LiH4 using the Lipofectin® Reagent (Gibco, BRL) according to the manufacturer's protocol. Briefly, 3×10^6 cells were seeded on 100 mm plates in Dulbecco's modified Eagle's medium plus 5% FCS and transfected when they reached 50–75% confluence. Seventy-two hours post-transfection, the cells were harvested, washed two times with ice-cold PBS and immediately lysed by addition of Laemmli's buffer. Protein derived from equivalent numbers of cells were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham, Aylesbury, UK). The blots were probed with either a serum from a rabbit immunized with *L. infantum* H2A recombinant protein [25] or affinity-purified antibodies against *L. infantum* histones (H2B, H3 or H4) from the serum of a dog suffering of visceral leishmaniasis.

2.4. Expression of the L infantum histones as recombinant proteins

ORFs coding for each one of the four core histones of *L. infantum* were PCR-amplified using the

following oligonucleotides as primers: H2A (forward, 5′-CGGGATCCAT GGCTACTCCT CGCAGC-3′; reverse, 5′-CCCAAGCTTA CGCGCTCGGT GTCGCCC-3′), H2B (forward, 5′-CGGGATCCAT GGCCTCTTCT CGCTCT-GC-3′; reverse, 5′-CCCAAGCTTC AAGCCGACGC GCTCGACAC-3′), H3 (forward, 5′-CGGGATCCAT GTC-CCGCACC AAGGAGAC-3′; reverse, 5′-CCCAAGCTTC TAGTGGCGCT CACCGCGCA-3′), and H4 (forward, 5′-CGGGATCCAT GGCCAAGGGC AAGCGTTC-3′; reverse, 5′-CCCAAGCTTA CGCGTAGCCG TACAGGA-3′). Underlined are the *Bam*HI and *Hin*dIII restriction sites included for direct cloning in the pQE30 prokaryotic expression vector (Qiagen, Hilden, Germany). The resulting clones were named pQE-H2A, pQE-H2B, pQE-H3 and pQE-H4. Expression and purification of the His-tagged recombinant proteins were performed in *Escherichia coli* M15 following standard procedures (Qiagen). After induction, bacteria were harvested, and lysed by sonication under denaturing conditions (8 M urea, 0.5 M NaCl, 20 mM Tris–HCl). After binding to a Ni-NTA agarose column (Qiagen), recombinants proteins were gradually refolded on the affinity column as described [26]. Afterwards, recombinant proteins were eluted with 0.3 M imidazol, and dialysed against PBS. Finally, proteins were passed through a polymyxin-agarose column (Sigma, St Louis, MO) in order to eliminate endotoxins. Residual endotoxin was measured with Quantitative Chromogenic *Limulus* Amebocyte assay (QCL-1000, BioWhittaker, Walkersville, MD), showing that recombinant histone preparations were essentially endotoxin-free (less than 30 ng/mg of recombinant protein). Fig. 1A shows a Coomassie blue-stained SDS-PAGE gel of the purified recombinant histones.

2.5. Soluble Leishmania antigen (SLA) preparation

L. major SLA was prepared from promastigotes harvested from culture and washed with sterile phosphate-buffered saline (PBS). Parasites were resuspended in PBS, freeze-

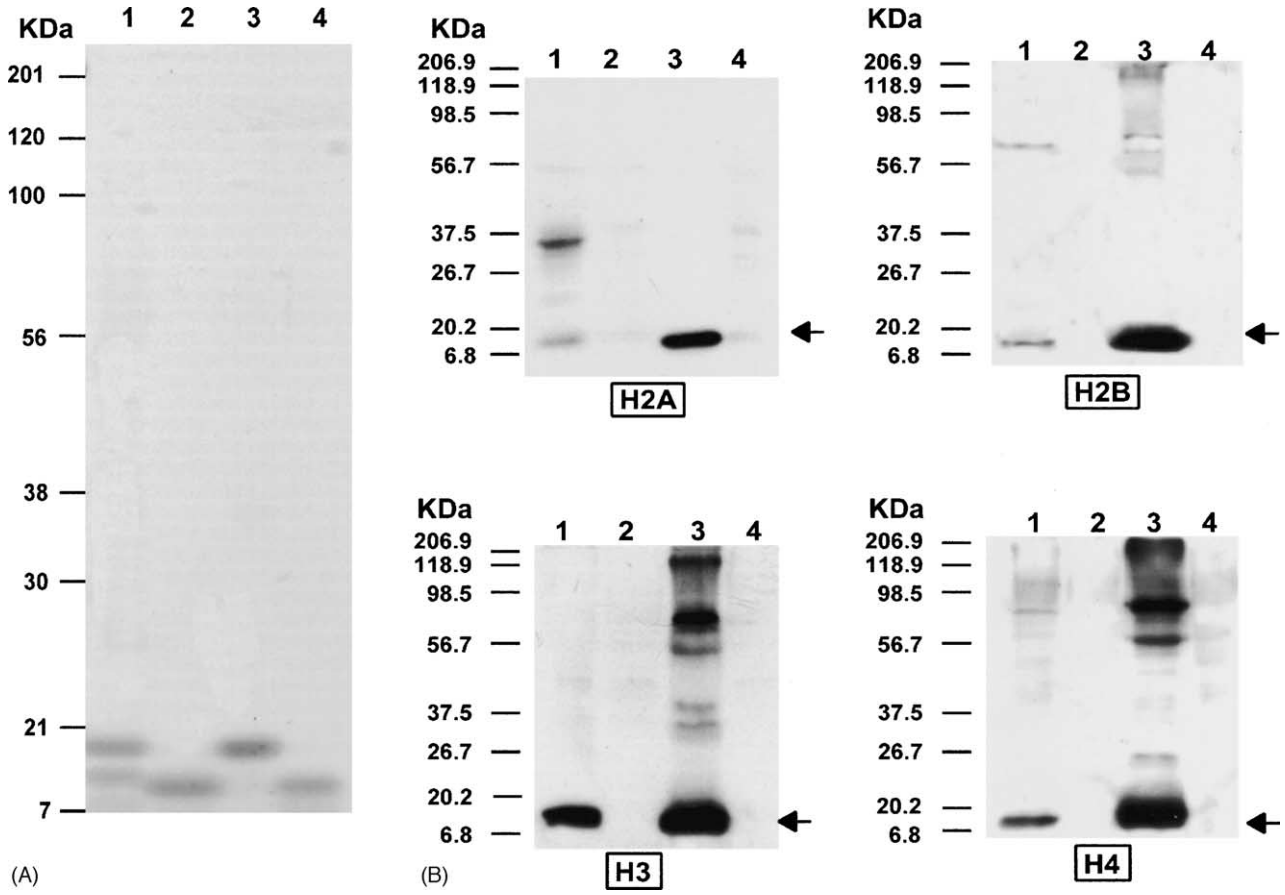

Fig. 1. (A) Purification of *L. infantum* histones after expressing in *E. coli*. Five microgram of histones H2A (lane 1), H2B (lane 2), H3 (lane 3) and H4 (lane 4) were electrophoresed on linear 10–14% gradient SDS-PAGE gel. The Coomassie-blue staining of the gel is shown. (B) Analysis of the expression in COS7 cells of the *L. infantum* genes coding for the four core histones. Separated cultures of COS7 cells were transiently transfected with DNA plasmids pcDNA3-LiH2A (panel H2A), pcDNA3-LiH2B (panel H2B), pcDNA3-LiH3 (panel H3) or pcDNA3-LiH4 (panel H4). At 72 h post-transfection, cells were harvested, lysed and separated on a 13.5% SDS-PAGE gel; afterwards, the expression of the protein was detected by Western blotting using specific antiserum against *Leishmania* histones H2A, H2B, H3, or H4. In addition to the cells transfected with the corresponding constructs (lanes 1), the following controls were included: COS7 cells transfected with pcDNA3 (lanes 2), nuclear extracts from *L. major* promastigotes (lanes 3), and untransfected COS7 cells (lanes 4). Arrows point to the antibody-reactive bands of the expected size.

thawed three times in liquid N_2 and the supernatant was taken after centrifugation in a micro-centrifuge (15,000 rpm) for 10 min.

2.6. Immunizations and parasite challenge

Immunization experiments were carried out in groups of ten mice. Mice were inoculated three times at 2-week intervals intramuscularly (i.m.) in both hind leg quadriceps with 50 μg (25 μg per leg) of each plasmid DNA (pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 and pcDNA3-LiH4) in a total volume of 100 μl of PBS. Control mice were inoculated following the same schedule using 200 μg of pcDNA3 in each inoculation or PBS alone. Fourteen days after each inoculation, mice were bled by orbital plexus puncture. Four weeks after the final inoculation, spleens and lymph nodes from four immunized mice were collected and groups of six immunized mice were challenged with 5×10^4 stationary phase promastigotes injected in the left hind footpad. The footpad swelling was measured every week and is given as the difference between the thicknesses of the infected footpad versus contralateral uninfected footpad.

2.7. Determination of antibody titres and isotypes

Serum samples were analyzed for specific antibodies against either *Leishmania* histones or *Leishmania* total proteins. Briefly, standard ELISA plates were coated overnight at room temperature with 100 μl of rLiH2A, rLiH2B, rLiH3 or rLiH4 (1 μg/ml in PBS), or SLA (2 μg/ml in PBS). A serial dilution of the sera was carried out in order to determine the titre, which is defined as the inverse of the highest serum dilution factor giving an absorbance > 0.2. The isotype-specific analyses were done with the following horseradish peroxidase-conjugated anti-mouse immunoglobulins (Nordic Immunological Laboratories, Tilburg, The Netherlands): anti-IgG1 (1:1000) and anti-IgG2a (1:500). Ortophenylenediamine dihydrochloride—OPD—(Dako, A/S, Glostrup, Denmark) was used as peroxidase substrate. After 15 min, the reaction was stopped with the addition of 100 μl of H_2SO_4 1 M, and the absorbance was read at 450 nm.

2.8. Measurement of cytokines in supernatants

Spleens and lymph nodes from BALB/c mice were removed aseptically after cervical dislocation. Splenocyte and lymph node cell (LNC) suspensions were prepared in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 10 μM 2-mercaptoethanol). In a final volume of 0.6 ml, 3×10^6 cells were seeded in 48-well plates during 72 h at 37 °C in the presence of rLiH2A, rLiH2B, rLiH3 or rLiH4 (12 μg/ml), histones from calf thymus (48 μg/ml; Sigma), or SLA (24 μg/ml). The release of IFN-γ and IL-4 was measured in the supernatants of splenocytes and LNC cultures, and was determined by commercial ELISA kits (Diaclone, Besançon, France).In parallel, splenocytes stimulated with the recombinant histones or SLA were incubated in the presence of 10 μg/ml of monoclonal antibody (mAb) against either mouse CD4 (GK1.5), mouse IL-12 (C17.8) or mouse CD8 (53-6.7). Appropriate isotype matched controls were also analyzed in this assay. These antibodies (no azide/low endotoxin™) were purchased from Becton Dickinson (PharMingen, San Diego, CA).

2.9. FACS analyses

For analysis of the frequency of T cell producing IFN-γ by intracellular staining, pooled LNCs (3 ml at 1×10^7 cells/ml) were seeded in a 6-well tissue plate. Subsequently, cultures were stimulated with PMA (5 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma), immediately the GolgiStop™ reagent was added and cells were cultured for 6 h at 37 °C in 5% CO_2. Afterwards, cells were harvested, washed twice in PBS with 1% FCS and stained with either PE-conjugated rat anti-mouse CD4 mAb (GK1.5) or PE-conjugated rat anti-mouse CD8 mAb (53-6.7) for 30 min on ice. Cells were then washed twice and fixed for 20 min in Cytofix/Cytoperm buffer. Next, cells were washed twice and incubated with FITC-conjugated rat anti-mouse IFN-γ mAb (XGM.1.2) for 30 min at 4 °C. Finally, cells were washed twice and analyzed on a FACSCalibur flow cytometer. The specificity of the anti-cytokine mAb was tested by both negative staining of non-permeabilized cells and a FITC-conjugated isotype-matched control (R3-34). Reagents and conjugate monoclonal antibodies were purchased from Becton Dickinson (PharMingen, San Diego, CA).

2.10. Determination of parasite burden

The number of parasites was determined in the popliteal lymph node from infected leg and spleen by quantitative limiting dilution [27]. Tissues were homogenized and serially diluted in a 96-well flat-bottomed microtiter plate containing Schneider's medium with 20% FCS. The number of viable parasites was determined from the highest dilution at which promastigotes grown after 7 days of incubation at 26 °C.

2.11. Statistical analysis

Statistical analysis was performed by a Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Immunogenicity of L. infantum histones administered as a DNA-vaccine

Each one of the genes coding for the *L. infantum* core histones (H2A, H2B, H3 and H4) was separately cloned

into the eukaryotic expression vector pcDNA3. Because expression of recombinant proteins by mammalian cells transfected with plasmid DNA is a critical condition for the stimulation of the immune system, the expression of the four *L. infantum* histones was initially assessed in COS7 cells transfected with the constructs. Transfected COS7 cells were cultured during 3 days and the expression of the *Leishmania* histones was analyzed by Western blotting (Fig. 1B). Cells transfected with the different constructs produced high levels of the corresponding histone that could be clearly detected by the specific antibodies (Fig. 1B). The additional bands present in lysates of pcDNA3-LiH2A (lane1, panel H2A), pcDNA3-LiH2B (lane 1, panel H2B), or pcDNA3-LiH4 (lane 1, panel H4) transfected cells must be representing interactions of the histones among them or with other cellular proteins since they are of higher molecular size than expected. In addition, it is unlikely that these bands are cross-reacting proteins because they were not detected by the antiserum in lysates of either pcDNA3-transfected cells (lanes 2) or untransfected cells (lanes 4). Furthermore, Fig. 1B illustrates that histones from *L. major* and *L. infantum* are antigenically cross-reactive: antisera against *L. infantum* histones strongly reacted with protein bands of the predicted size present in nuclear extracts from *L. major* promastigotes (lanes 3). In fact, a search in the ongoing project of sequencing *L. major* genome (http://www.genedb.org/genedb/leish/index.jsp) evidenced that the predicted sequences for *L. major* histones and the sequences of *L. infantum* histone used in this study are highly conserved. Thus, *L. infantum* H2A shows a 95% of sequence identity with the deduced amino acid sequence of LmjF29.1720 gene; similarly, sequences of *L. infantum* histones H2B, H3 and H4 are 94, 95 and 99% identical to the deduced amino acid sequences of genes LmjF9.1340, Lmj10.0990 and LmjF02.0020, respectively.

A DNA-mixture of the four plasmids encoding each one of the *Leishmania* core histones (50 μg each) was used to i.m. immunize BALB/c mice three times, 2 weeks apart. No anti-histone antibodies were detected in the sera of mice 2 weeks after the second immunization (data not shown), indicating that *Leishmania* histones, inoculated as "naked-DNA", are poor inductors of humoral responses in mice. Two weeks after the third immunization, it was detected the presence of antibodies against the *Leishmania* histones in those mice (Fig. 2), even though with low titres. The predominant isotype of antibodies against the four *Leishmania* histones was IgG2a, whereas IgG1-specific antibodies were almost undetectable (Fig. 2). Interestingly, no reactivity against calf thymus histones was observed in sera from immunized mice (data not shown), excluding the possibility that anti-histone autoantibodies were induced by the immunization process.

The cellular response induced in mice by immunization with the plasmid constructs was also measured. For that purpose, splenic mononuclear cells and lymph node cells were obtained 4 weeks after the last DNA immunization

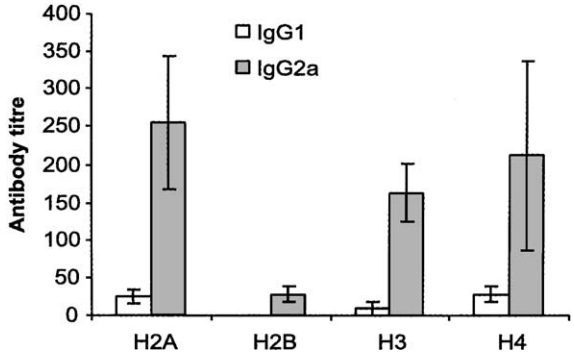

Fig. 2. Analysis of the specific humoral response induced in BALB/c mice immunized with a DNA cocktail containing four *Leishmania* histone genes. Fourteen days after the third inoculation, mice (n = 10) were bled and sera were tested by ELISA for specific anti-H2A, -H2B, -H3 and -H4 antibody responses of both IgG1 and IgG2a isotypes. Each sample was assayed separately, and the titre was determined as indicated in Section 2. None of the preimmune sera showed reactivity against these recombinant proteins.

and were stimulated in vitro with the recombinant histone proteins. After 3 days of incubation, the supernatants were harvested and assayed for both IFN-γ and IL-4. The results, depicted in Fig. 3, indicated that recombinant *Leishmania* histones stimulate the production of large quantities of IFN-γ by both splenocytes (Fig. 3A) and LNCs (Fig. 3B) from immunized mice. The IFN-γ production was found to be induced specifically by *Leishmania* histones, since stimulation of cell cultures with histones from calf thymus resulted in a very low production of this cytokine. In contrast, no histone-specific production of IL-4 was observed in the supernatants of any of the cultures (Fig. 3C and D). These results were in synchrony with the predominance of the IgG2a antibodies and indicated that DNA immunization with histone genes preferentially elicits a Th1-like immune response.

3.2. Protection induced by vaccination with a mixture of DNAs encoding for Leishmania histones

Since the immunogenicity experiments revealed that DNA immunization with *Leishmania* histone genes triggers the induction of a Th1-type immune response, we next assessed whether this vaccine formulation could provide protection against *L. major* infection of mice. Interestingly, mice vaccinated with a mixture of the four plasmid DNAs encoding *L. infantum* histones and challenged 4 weeks later with 5×10^4 *L. major* stationary promastigotes in the left footpad were almost completely protected against the development of lesions (Fig. 4A). The footpad swelling in the vaccinated mice was reduced (mean value of 1 mm at the eighth week) when compared with that of controls (mean value = 5 mm). Indeed, no lesion at all was observed in four out of the six vaccinated mice along the period of study (10 weeks post-infection). In contrast, the mice vaccinated with control DNA (empty plasmid) developed lesions

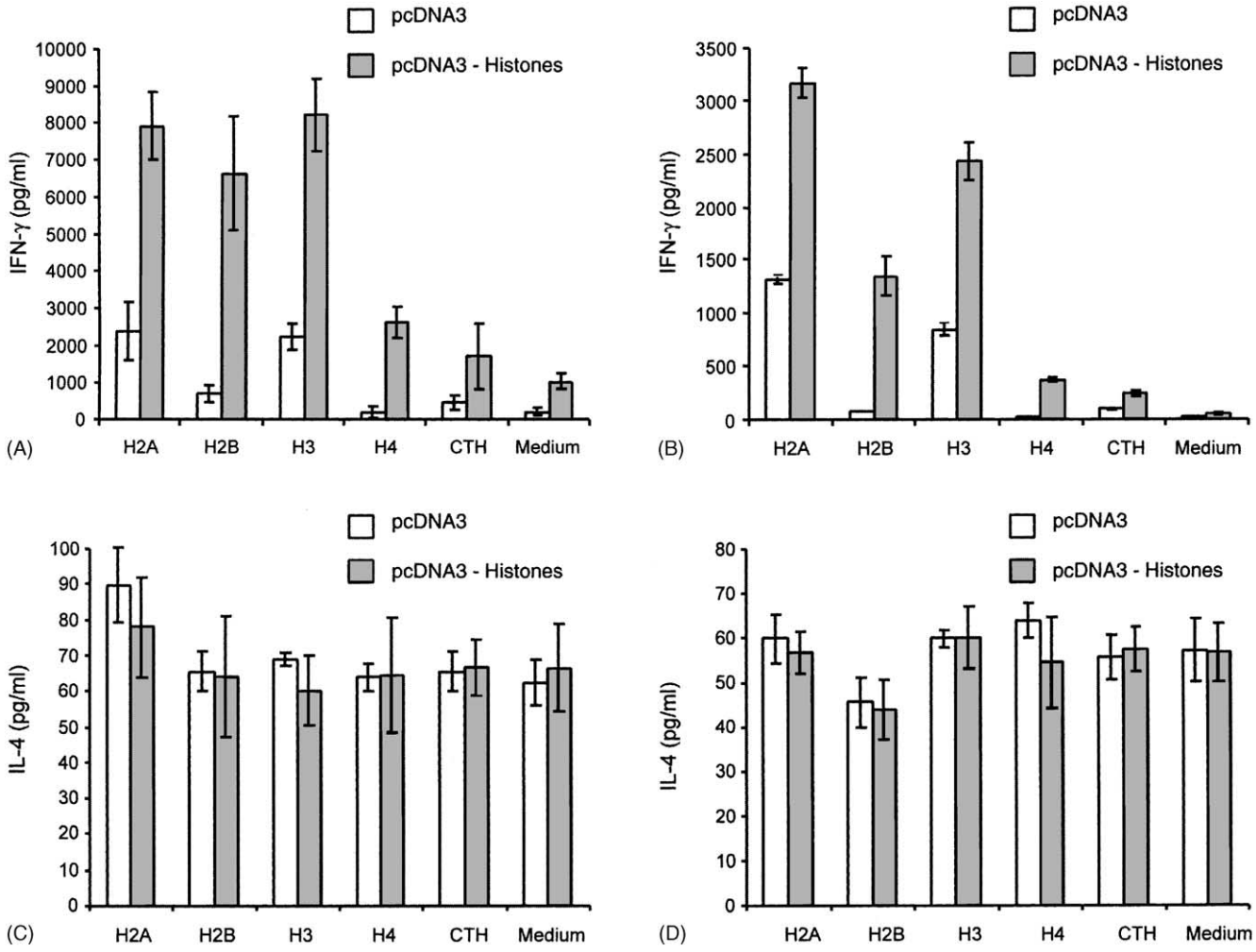

Fig. 3. Cytokine production by splenocytes and LNCs of DNA-vaccinated mice. Mice (four per group) were immunized three times i.m. with either pcDNA3 or a cocktail of the four pcDNA3-histone constructs. One month after the last immunization, the animals were sacrificed, and their splenocytes (panels A and C) and LNCs (panels B and D) were obtained and cultured in vitro for 3 days in the presence of recombinant histones H2A, H2B, H3, H4, calf thymus histones (CTH), or medium alone. The supernatants were harvested and assayed for both IFN-γ (panels A and B) and IL-4 (panels C and D). Data correspond to one representative experiment of three independent experiments with similar results.

similar in size to the unvaccinated mice (inoculated with PBS).

It is known that *L. major* promastigotes disseminate rapidly to the viscera in BALB/c [5]. Thus, we considered of interest to determine the parasite burden in popliteal lymph nodes and spleens as an additional parameter to evaluate the degree of protection induced by the genetic vaccination. *L. major* parasites were quantitated in spleens and popliteal lymph nodes from vaccinated mice (euthanized at week 10th post-infection) and controls (euthanized at week 8th post-infection). Vaccinated mice had a ≈2-log reduction in parasite burden (in the popliteal lymph node of the infected foot) compared with mice inoculated with control DNA (Fig. 4B). Also, we analyzed the visceralization of *L. major* by determining the parasite burden in the spleens from vaccinated and control mice. The parasite burden in spleens of vaccinated mice was ≈4-log lower than that of control mice (Fig. 4B); in fact, no parasites were found in two out of the six vaccinated mice. Altogether, these data were taken as an indication that vaccinated mice are controlling the infection by *L. major*.

3.3. Histone DNA vaccination modulates the immune response elicited in mice by L. major infection

Further experiments were designed to determine immunological parameters associated with protection against cutaneous leishmaniosis in vaccinated mice. First, we determined the humoral response, induced by *L. major* infection, against each one of the four recombinant *Leishmania* histones in both vaccinated and control mice (Fig. 5). Remarkably, the anti-histone humoral response induced by *L. major* infection of vaccinated mice was lower than that observed in control mice. In addition, whereas the anti-histone

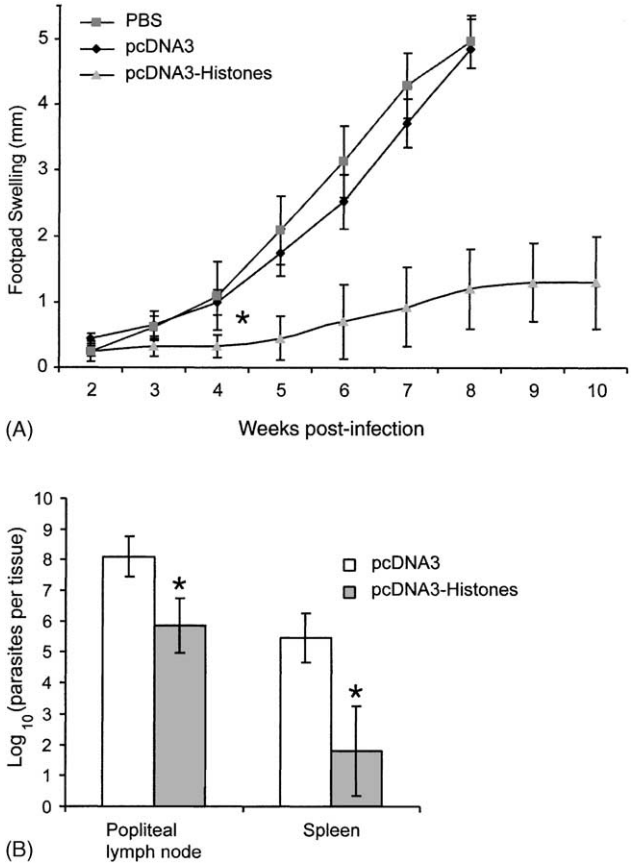

Fig. 4. Course of *L. major* infection in vaccinated mice. (A) BALB/c mice (six per group) were immunized three times i.m. with either PBS, pcDNA3 or a DNA-cocktail of the four pcDNA3-histone constructs. One month after the last immunization, the animals were infected in the left hind footpad with 5×10^4 *L. major* promastigotes. Footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. At fourth week after challenge, differences between controls and vaccinated mice were found to be significant ($^*P < 0.001$). This is one representative experiment of three separate experiments with virtually the same results. (B) At 8 weeks after infection for pcDNA3-immunized mice or at 10 weeks after infection for pcDNA3-histone immunized mice, the total numbers of viable parasites present in the popliteal lymph node of the infected leg and spleen were determined by limiting dilution. Results represents the mean and standard deviation for mice from two independent experiments ($^*P < 0.001$).

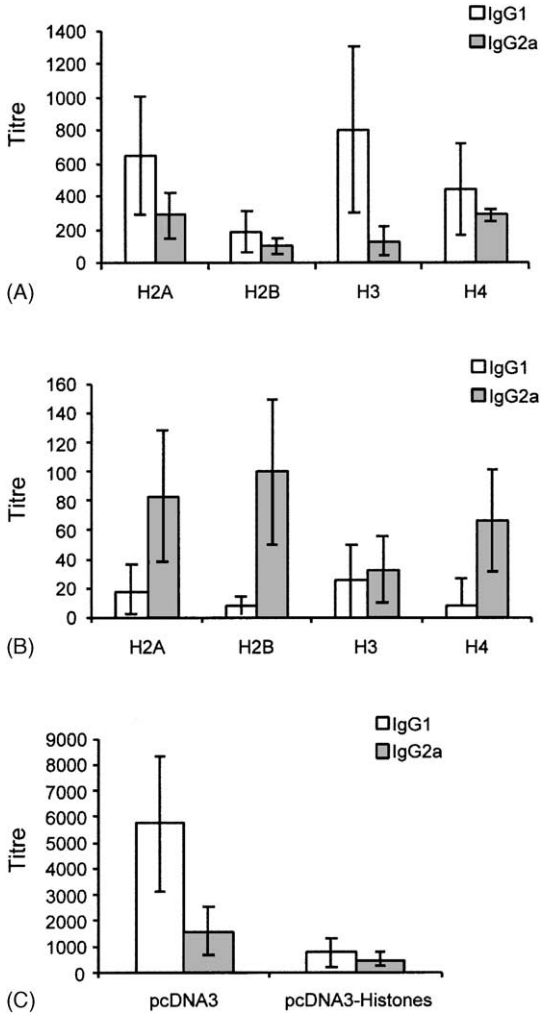

Fig. 5. Humoral response induced by *L. major* challenge in vaccinated and control mice. Eight weeks after infection in control mice (immunized with pcDNA3) and 10 weeks after infection in vaccinated mice (immunized with a mixture of the four pcDNA3-histone constructs), serum samples were obtained and tested by ELISA for specific anti-histone antibodies (panels A and B, respectively) or anti-SLA antibodies (C). Titres were determined for IgG1 and IgG2a isotypes. Results represent the mean and standard deviation for mice from two independent experiments.

antibodies found in control mice were predominantly of the IgG1 isotype (Fig. 5A), the anti-histone antibodies in vaccinated mice were mainly of the IgG2a isotype (Fig. 5B). Interestingly, vaccination with DNA encoding *Leishmania* histones also conditioned the global anti-*Leishmania* humoral response induced by *L. major* infection (Fig. 5C). Thus, vaccinated mice developed a limited humoral response against *Leishmania* antigens, while control mice showed substantially higher levels of anti-*Leishmania* antibodies compared with vaccinated mice. Also, as expected for a nonhealing phenotype, in control mice the predominant IgG isotype was the IgG1 while vaccinated mice exhibited a similar level of both IgG1 and IgG2a isotypes (Fig. 5C).

On the other hand, we assessed the production of IFN-γ and IL-4 by splenocytes, after in vitro stimulation with *Leishmania* histones, from vaccinated and control mice after *L. major* infection (Fig. 6). Splenocytes from vaccinated mice produced significantly more histone-specific IFN-γ than those from control mice. Remarkably, splenocytes from vaccinated mice also made higher levels of IFN-γ than controls after in vitro stimulation with SLA (Fig. 6A). In contrast, production of histone-specific IL-4 was not stimulated in splenocytes from vaccinated and control mice, even though splenocytes from control mice spontaneously liberated more IL-4 than splenocytes from vaccinated mice (Fig. 6B). Finally, it should be noted that there was an increase in the production of IL-4 by splenocytes from control

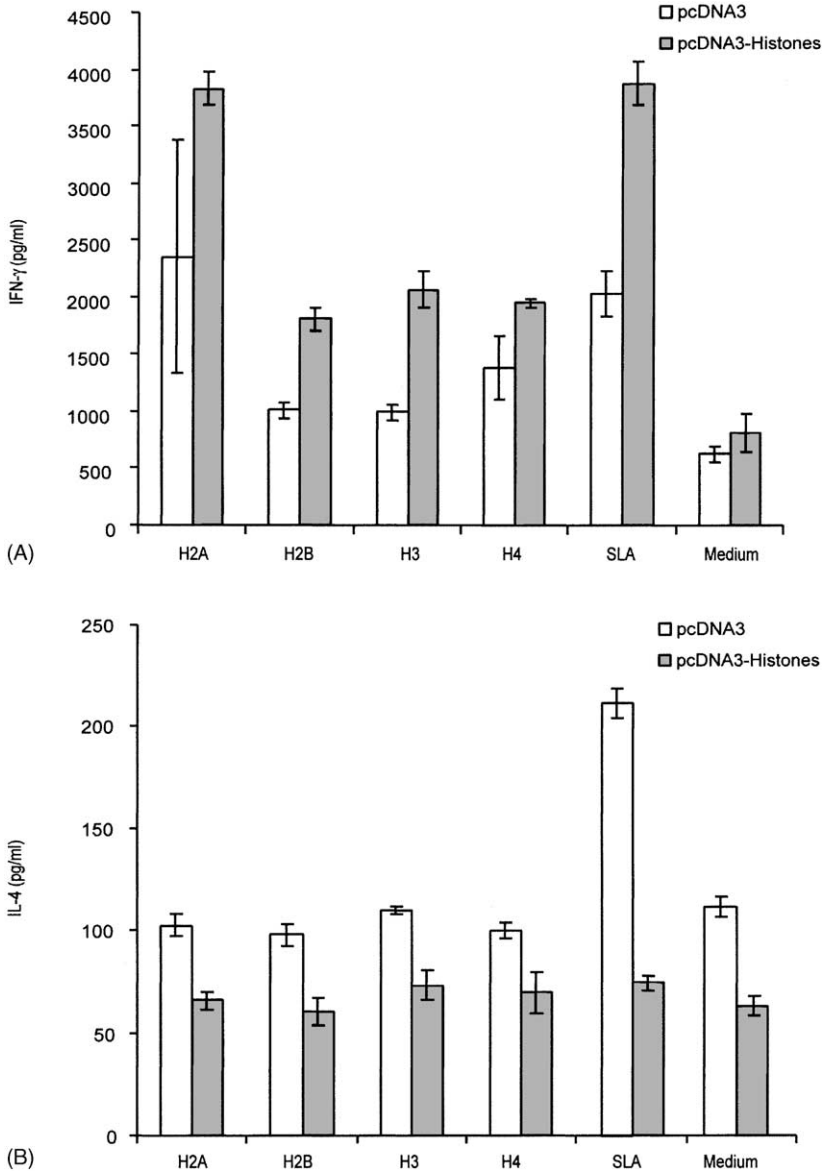

Fig. 6. Cytokine production by splenocytes from vaccinated mice after *L. major* challenge. BALB/c mice (six per group) were immunized with either pcDNA3 (controls) or a cocktail of the four pcDNA3-histone constructs and, afterwards, infected with *L. major* promastigotes (see legend to Fig. 4 for additional details). Eight weeks after infection in controls, or 10 weeks after infection in vaccinated mice, spleens were removed and the splenocytes were stimulated in vitro with recombinant *Leishmania* histones H2A, H2B, H3, H4, SLA, or medium alone. The production of IFN-γ (A) and IL-4 (B) was assessed by ELISA in supernatants harvested after 72 h of in vitro stimulation. This experiment was repeated two times with similar results.

mice, but not by splenocytes from vaccinated mice, when stimulated in vitro with SLA.

3.4. Analysis of the IL-12 dependence, and the involvement of $CD4^+$ and $CD8^+$ T cells in the production of IFN-γ

In order to characterize in more detail the immune response elicited in mice by DNA vaccination with the *Leishmania* histone genes and its evolution after challenging with *L. major*, we assessed the IL-12 dependence, and cellular contributions to the antigen-specific production of IFN-γ by splenocytes from vaccinated mice (Fig. 7). As shown in Fig. 7A, 25 days after *L. major* infection, splenocytes from vaccinated mice continued to produce more histone-specific IFN-γ than splenocytes from control mice. Interestingly, the production of SLA-mediated IFN-γ was two-fold higher in splenocytes from vaccinated mice as compared with that from controls. No IL-12 could be detected by ELISA assays in the supernatants of splenocyte cultures from any of the groups (data not shown). As IL-12-dependent production of IFN-γ is the main mechanism associated with the control of *L. major* infection [5], we assessed whether addition of anti-IL-12 monoclonal antibody to cultures substantially inhibited production of IFN-γ in splenocytes from vaccinated

mice (Fig. 7B). Since inhibition of IFN-γ production, by the presence of the anti-IL-12 antibody, was found to be higher than 80% for the four different stimuli (*L. infantum* histones H2A, H2B, H3 and H4), it can be concluded that histone-specific IFN-γ production is IL-12-dependent.

Also, we were interested to know the relative contributions of $CD4^+$ and $CD8^+$ T cells to the production of IFN-γ. The histone-mediated stimulation of IFN-γ production from splenocytes of all groups was substantially inhibited by addition of anti-CD4 monoclonal antibodies to cultures (Fig. 7C). However, addition of anti-CD8 monoclonal antibodies to the cultures diminished the production of IFN-γ only for the splenocytes in vitro stimulated with either histone H2A or H3 from vaccinated mice. Thus, DNA vaccination with genes encoding *Leishmania* histones H2A and H3 seems to be more potent activators of IFN-γ-producing $CD8^+$ cells than the other two histone genes (H2B and H4).

Finally, in order to provide further insights about the immune status associated with the protection elicited in mice by DNA vaccination with genes coding for the *L. infantum* histones, the frequency of $CD4^+$ and $CD8^+$ T cells producing IFN-γ in the LNCs was assessed by intracellular cytokine staining at the end of the protection studies (8th week post-infection for controls, and 10th week post-infection for vaccinated mice). As shown in Fig. 8, the frequency of both $CD4^+$ and $CD8^+$ cells producing IFN-γ was higher in vaccinated mice than in controls. The differences were accentuated after in vitro stimulation with PMA/ionomycin (Fig. 8). Thus, these data are a direct support that protection against *L. major* infection, achieved by DNA vaccination with *Leishmania* histones, is correlated with an enhanced frequency of T cells ($CD4^+$ and $CD8^+$) producing IFN-γ in mice.

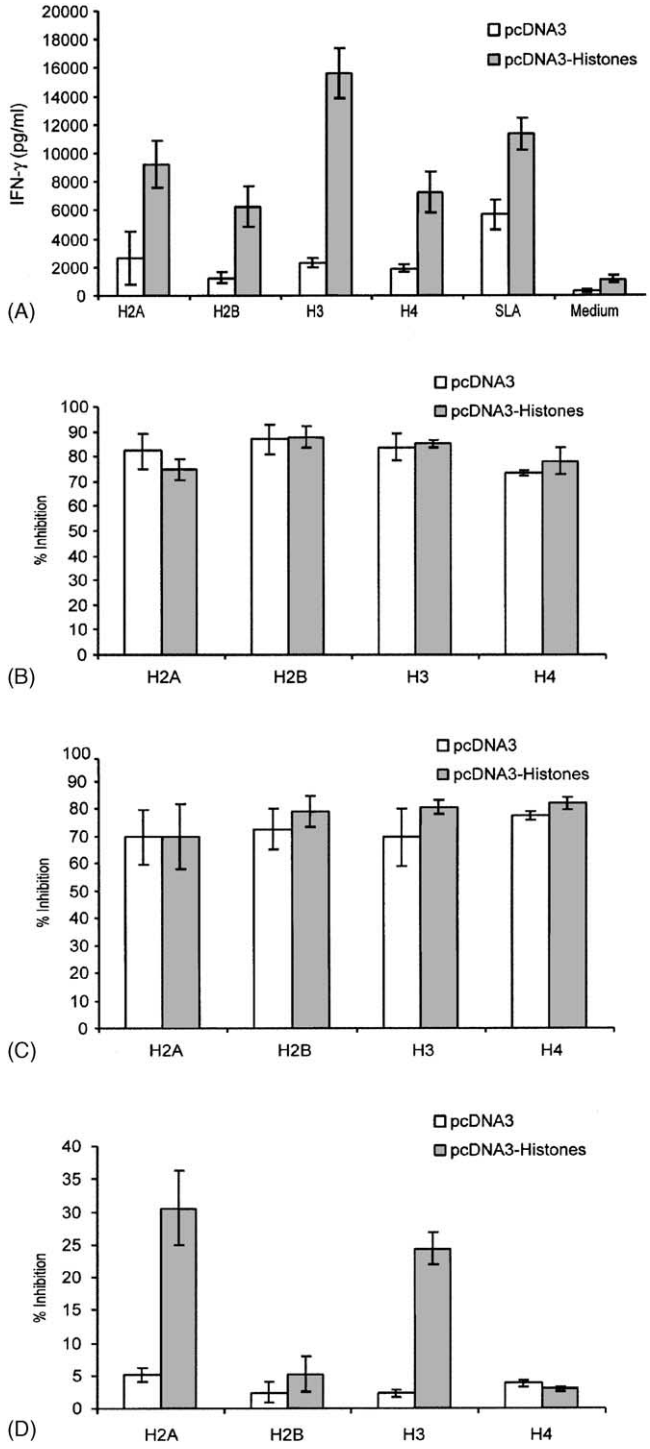

Fig. 7. Analysis of the involvement of IL-12 and T cells in the production of IFN-γ associated with the protection conferred by vaccination with *Leishmania* histone genes. BALB/c mice (four per group) were immunized three times i.m. with either pcDNA3 or a DNA-cocktail of the four pcDNA3-histone constructs. One month after the last immunization, the animals were infected in the left hind footpad with 5×10^4 *L. major* promastigotes. Twenty-five days after infection, spleens were removed and the splenocytes were stimulated in vitro with recombinant *Leishmania* histones H2A, H2B, H3, H4, or medium alone. After 72 h of stimulation, the production of IFN-γ was determined by ELISA (A). In parallel, splenocyte cultures were stimulated with recombinant *Leishmania* histones in the presence of either anti-IL-12 (B), anti-CD4 (C), or anti-CD8 (D) monoclonal antibodies. The production of IFN-γ was assessed by ELISA after 72 h of incubation and the results were expressed as percentage of inhibition (={[(histone-specific production of IFN-γ) − (histone-specific production of IFN-γ in the presence of a monoclonal antibody)]/histone-specific production of IFN-γ} × 100).

4. Discussion

The four *Leishmania* histones forming the nucleosomal core were selected for the development of vaccine against leishmaniasis based on previous studies showing that these proteins possess interesting immunogenic properties [16–22] (see also the Introduction section). In addition, we chose to use a DNA vaccine approach, because DNA vaccination has been probed as the more efficient method to attain effective protection for diseases requiring cellular immunity [28]. The data presented here show that immunization of BALB/c mice with a cocktail DNA vaccine encoding the *L. infantum* histones H2A, H2B, H3 and H4 resulted in a

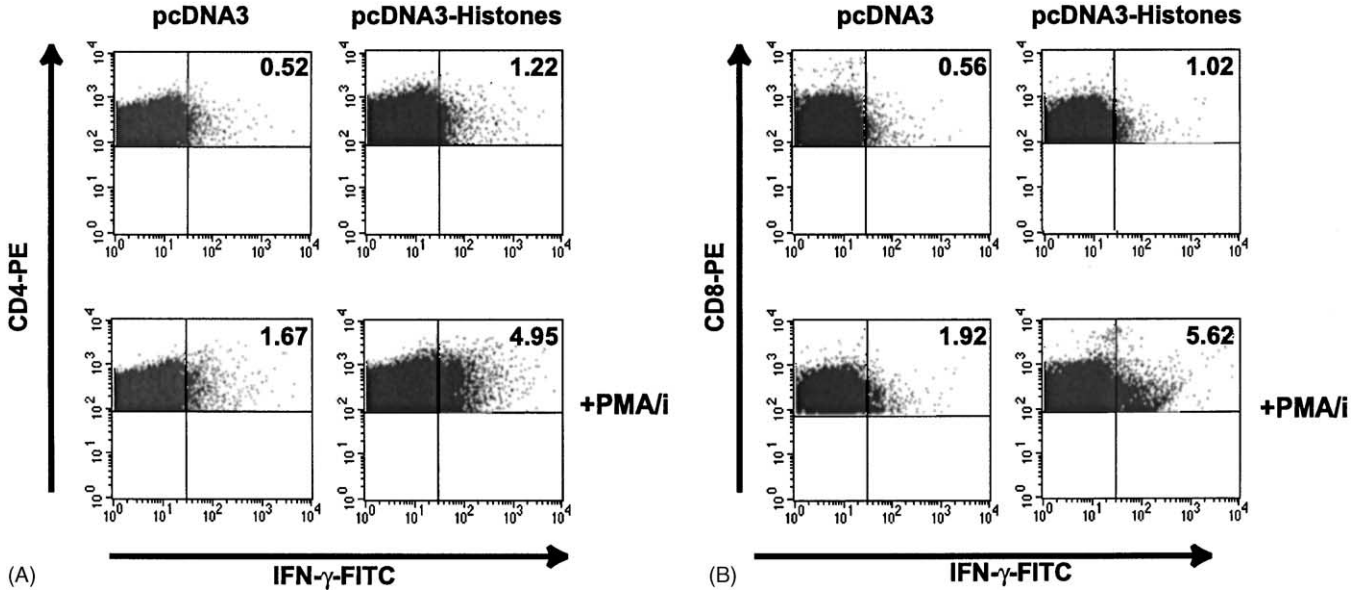

Fig. 8. Frequencies of $CD4^+$ and $CD8^+$ T cells producing IFN-γ in LNCs from vaccinated and control mice after challenge with *L. major*. BALB/c mice (six per group) were immunized with either pcDNA3 (controls) or a cocktail of the four pcDNA3-histone constructs and, afterwards, infected with *L. major* promastigotes (see legend to Fig. 4 for additional details). The percentages of T cell populations producing IFN-γ were determined in pooled LNCs. After 6 h of incubation in the presence of Golgi Stop reagent, cells were harvested and stained for intracellular IFN-γ staining and for the presence of either $CD4^+$ (A) or $CD8^+$ (B) surface markers. In parallel, T cell populations producing IFN-γ in LNCs were assessed after stimulation with PMA/ionoycin (bottom panels).

robust protection against cutaneous leishmaniasis due to *L. major*. Analysis of the immune response developed in mice after genetic vaccination with *Leishmania* histone genes was found to be exclusively of the Th1 type based on two features. First, immunized mice developed low anti-histone antibody responses with a predominance of antibodies of the IgG2a isotype (Fig. 2). Second, lymphocytes from vaccinated mice produced high amounts of IFN-γ, but not IL-4, after in vitro stimulation with recombinant *Leishmania* histones (Fig. 3). Although most of the DNA vaccines tested in murine models resulted in the development of a Th1-type response, an exception to this rule has been recently reported. Vaccination of mice with *Leishmania* meta 1 gene induced a immune response of the Th2-type that was associated with a lack of protection against infection [11], suggesting that some leishmanial antigens have an intrinsic potency to elicit Th2 responses that cannot be subverted by genetic vaccination. This is not the case for any of the *Leishmania* histones, since after DNA vaccination the immune responses, assessed individually against each one of the four histones, were exclusively of the Th1 type.

However, the natural response of BALB/c mice against *Leishmania* histones after challenging with *L. major* seems to be of the Th2 type. This is based on the fact that the anti-histone antibody response of controls (mice immunized with pcDNA3 plasmid and challenged with *L. major*) was predominantly of the IgG1 isotype (Fig. 5A). In addition, the anti-histone antibody titres were higher in sera from controls than in sera from vaccinated mice (Fig. 5B). The fact that a positive correlation between the titers of anti-*Leishmania* antibodies and disease progression exists in leishmaniasis has lead to the suggestion that uncontrolled humoral response against "pathoantigens" (histones are included within this category) are direct responsible for several of the pathological alterations observed in patients [29,30]. Remarkably, we have found that the Th2 natural response to *Leishmania* histones during *L. major* infection can be reversed to a Th1-type response after immunizing mice with a cocktail DNA vaccine containing the *L. infantum* genes coding for the four core histones (Fig. 5A). In addition, it is noticeable that immunization of mice with this histone-DNA vaccine also has a clear effect on the global humoral response elicited in mice by the *L. major* infection (Fig. 5C). Thus, the infection of vaccinated mice induces a limited anti-*Leishmania* humoral response, whereas the humoral response induced in control mice was high and with a predominance of antibodies of the Th2 type (i.e., IgG1 isotype). Therefore, these data favor the idea that DNA vaccination with *Leishmania* histones has direct influences on early decisions of the immune system at the time of *Leishmania* infection.

There is a precedent in the scientific literature showing that another *Leishmania* histone, i.e. histone H1 of *L. major*, has protective capacity against experimental murine cutaneous leishmaniasis [31]. Thus, inoculations of either perchloric acid extracted-histone H1, recombinant protein or synthetic peptides, emulsioned in IFA, were able to partially protect BALB/c mice against infection with *L. major* infective promastigotes. Furthermore, in a recent report

it has been described that vaccination with *Leishmania* histone H1 develops protection against cutaneous leishmaniasis in outbred vervet monkeys [32]. These data, together with the results described in this article, demonstrate that nuclear proteins of *Leishmania* have outstanding properties to induce protective immunity against leishmaniasis. In this way, it is remarkable that evolutionarily conserved, intracellular proteins, as histones are, show such immunoprotective properties. In fact, after an analysis of recent articles describing potential vaccine candidates against leishmaniasis, it is found that most of the promising immunoprotective antigens belong to this category of proteins; examples are: LACK [9], LeIF [33], and cysteine proteinases [14]. A potential drawback associated with the use of this class of proteins as vaccines could be the induction of autoimmune responses against the host molecules. However, this was not observed in this study: the anti-histone antibodies elicited in mice after genetic vaccination with *Leishmania* histone genes did not recognize histones from a mammalian origin. This finding is not unexpected because the histones of *Leishmania*, an organism located at the root of the eukaryotic evolutionary tree, have highly divergent domains where map the antigenic determinants recognized by sera from individuals with visceral leishmaniasis [19–21].

Acknowledgements

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