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PLoS NEGLECTED TROPICAL DISEASES

Therapeutic DNA Vaccine Encoding Peptide P10 against Experimental Paracoccidioidomycosis

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Abstract

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides brasiliensis*, is the most prevalent invasive fungal disease in South America. Systemic mycoses are the 10th most common cause of death among infectious diseases in Brazil and PCM is responsible for more than 50% of deaths due to fungal infections. PCM is typically treated with sulfonamides, amphotericin B or azoles, although complete eradication of the fungus may not occur and relapsing disease is frequently reported. A 15-mer peptide from the major diagnostic antigen gp43, named P10, can induce a strong T-CD4+ helper-1 immune response in mice. The TEPITOPE algorithm and experimental data have confirmed that most HLA-DR molecules can present P10, which suggests that P10 is a candidate antigen for a PCM vaccine. In the current work, the therapeutic efficacy of plasmid immunization with P10 and/or IL-12 inserts was tested in murine models of PCM. When given prior to or after infection with *P. brasiliensis* virulent Pb 18 isolate, plasmid-vaccination with P10 and/or IL-12 inserts successfully reduced the fungal burden in lungs of infected mice. In fact, intramuscular administration of a combination of plasmids expressing P10 and IL-12 given weekly for one month, followed by single injections every month for 3 months restored normal lung architecture and eradicated the fungus in mice that were infected one month prior to treatment. The data indicate that immunization with these plasmids is a powerful procedure for prevention and treatment of experimental PCM, with the perspective of being also effective in human patients.

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Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungus that causes a systemic granulomatous disease known as paracoccidioidomycosis (PCM). PCM is widespread in Latin America, mainly affecting rural workers, and its incidence has increased in recently deforested areas associated with soil churning [1]. Acquisition of *P. brasiliensis* may arise from inhalation of aerosolized conidia.

Recently we reviewed the death rates by systemic mycoses in Brazil [2]. PCM was the principal cause of death identified for 3,583 patients in the 1996–2006 decade and representing 51.2% of total deaths due to systemic mycoses. It ranks as the 10th most common cause of death among infectious diseases in Brazil [2].

There are distinct forms of PCM. The acute and sub-acute forms affect both genders with primary involvement of the reticuloendothelial/lymphatic system. The chronic form affects mainly adult males and predominantly causes pulmonary and/or mucocutaneous disease [3]. Antifungal chemotherapy is required for treatment, though treatment may not assure complete eradication of the fungus, with frequent relapses. Treatment with itraconazole usually takes 6–9 months in the low and 12–18 months in the moderately severe cases. Frequently, a combination of trimethoprim and sulfamethoxazole (TMP/SMZ) is used, held for 12 months in the low severity forms and 18–24 months in the moderately severe forms. Patients with severe PCM forms require endovenous treatment with anfothericin B or the TMP/SMZ association for long periods, monitored by clinical, radiological and serological tests [4].

The 43 kDa glycoprotein was characterized as the major diagnostic antigen of *P. brasiliensis* [5,6,7]. Immunization with gp43 elicited delayed hypersensitivity reactions in guinea pigs [8] and humans [9], implying the presence of T-CD4+ reacting epitopes. Based on the sequence of gp43 [6], which encodes a polypeptide of 416 amino acids with a single high mannose *N*-glycosylated chain [10], the T-cell epitope was mapped to a 15-mer peptide called P10 [11]. The hexapeptide HTLAIR comprises the essential core of P10 that induces proliferation of lymph node cells from mice sensitized to gp43 or infected with *P. brasiliensis* [11]. Type 1-T helper lymphocytes producing IL-2 and IFN-γ are induced by P10 [11,12,13]. Intratracheally infected mice previously immunized with P10 in the presence of complete Freund's adjuvant (CFA) had >200-fold reduction of lung *P. brasiliensis* colony-forming units (CFUs). In many cases the immunization rendered preserved lung

Author Summary

Paracoccidioidomycosis (PCM) is the predominant systemic mycosis in Latin America causing half of the total deaths among systemic fungal infectious diseases in Brazil. Chemotherapy is the standard treatment, but the long time required, severe cases of immunosuppression and frequent relapses indicate that additional methods should be introduced such as immunotherapy combined with antifungal drugs. Previously, the protective activity of P10, a peptide derived from the major diagnostic antigen gp43, was demonstrated, alone or combined with chemotherapy. P10 elicited a vigorous IFN-γ mediated Th-1 immune response. Presently, the reduction of fungal load, and even sterilization, was attempted using a specific DNA vaccine encoding P10. Plasmid pcDNA3 expression vector with P10 insert was tested as a vaccine in intratracheally infected BALB/c and B10.A mice. Our results showed that vaccination with pP10 induced a significant reduction of the fungal burden in the lung. Co-vaccination of pP10 with a plasmid encoding mouse IL-12 proved to be even more effective in the elimination of the fungus with virtual sterilization in a long term infection and treatment assay system. The data suggest that immunization with these plasmids, without the need of an adjuvant, could be used in the prevention and treatment of PCM in human patients.

architecture with few or no yeasts, whereas the infected, unimmunized mice displayed dense pulmonary inflammation characterized by epithelioid granulomas with numerous yeast cells [11,12].

The immunoprotection by P10 depends on the IFN-γ-producing Th-1 response since mice deficient in IFN-γ, IFN-γ-R or IRF-1, but not IFN-α-R/IFN-β-R, were not protected by P10 immunization [12] The essential role of IFN-γ in organizing granulomas that contain *P. brasiliensis* yeasts has also been recognized by other investigators [13,14,15].

Several experimental avenues have been pursued to validate P10 as a vaccine candidate. These studies have included: a) the presentation of P10 by MHC molecules from different murine haplotypes [11]; b) its conservation in nature, confirmed by examining gp43 molecules from different isolates [16]; c) its immunogenicity and effective immunoprotection in formulations that do not require complete Freund's adjuvant [17]; d) its presentation by most human HLA-DR molecules as well as that of neighbor peptides to P10, based on the sequence of gp43 [18]; and e) the effectiveness of P10 as an adjuvant to chemotherapy in normal [19] and anergic [20] mice challenged intratracheally with virulent *P. brasiliensis*.

The immunoprotective properties of P10 emulsified in Freund's adjuvant have been well documented in an established murine model of PCM [11]. Since CFA is not allowed in human vaccines and a tetramer of truncated P10 although immunogenic, involves laborious chemical methods [17], we have explored alternative approaches for P10 delivery. In the present work we have investigated the effectiveness of plasmid immunization with P10 and/or IL-12 inserts given prior to or after challenge with a virulent Pb18 isolate of *P. brasiliensis* using a murine pulmonary PCM disease model. Our results demonstrate that plasmid immunization with P10 with or without IL-12 inserts is highly therapeutic in mice intratracheally infected with this fungus. Most importantly, immunization was effective either prior to, or after infection suggesting that these plasmids are candidates for use in human PCM.

Materials and Methods

Plasmid constructions

Yeast cells of *P. brasiliensis* isolate 18 (Pb18) were grown in Sabouraud Dextrose Broth (BD, MD, USA) at 37°C for 7 days. Cells were washed and frozen in liquid nitrogen then disrupted by grinding on a mortar. Total RNA was isolated with Trizol according to manufacturer's instruction (Invitrogen, CA, USA). Complementary DNA was synthesized from 1 μg of total RNA in the presence of oligo$(dT)_{18}$ (Fermentas, MD, USA) and Revertaid M-MuLV(Fermentas, MD, USA).

The P10 nucleotide sequence was obtained using the sense PCR primer derived from the gp43 [6] 5′ nucleotide sequence: [5′-AAT AAG CTT CAA ACC CTG ATC GCC-3′], and the antisense primer derived from the 3′ end of the gp43 gene: [5′-AAT GAA TTC ATT GGC GTA ACG GAT TGC-3′]. A HINDIII site and an EcoRI site were added to the sense and antisense primers, respectively, for cloning into plasmid pcDNA3 (Invitrogen, CA, USA). PCR reactions (50 μl) were carried out following the protocol provided by Fermentas, using 100 ng of cDNA and 100 ng of each primer. The P10 PCR reaction started with one cycle at 94°C (2 min), followed by 40 cycles at 94°C (30 sec), 55°C (1 min) and 72°C (1 min), and a final 7-min extension at 72°C. PCR products were purified using Wisard SV gel and PCR Clean-UP system (Promega, Brazil) and each PCR product was digested with the appropriate restriction enzyme (Fermentas) and cloned into the pcDNA3 by directional insertion in the HINDIII/EcoRI sites. The resulting plasmid was called pP10.

Plasmid pORF-mIL-12 was acquired from InvivoGen (CA, USA). The confirmation of the insert was done using the primers: sense [5′-CGG GTT TGC CGC CAG AAC ACA-3′] and antisense [5′-GGC CAC CAG CAT GCC CTT GT-3′]. The IL-12 PCR started with one cycle at 94°C (2 min), followed by 40 cycles at 94°C (1 min), 45°C (1 min) and 72°C (2 min), and a final 7-min extension at 72°C.

Preparation of plasmid DNA

To prepare plasmid DNA for immunization, *Escherichia coli* XL1Blue and DH5α cells were transformed by electroporation using Celljet Duo according to the manufacturer's directions (Hybaid, Middx, UK) with the DNA constructs or the vector plasmid alone and then cultured at 37°C in Luria broth supplemented with ampicillin (50 μg/ml).

The positive clones were confirmed by automatic sequencing carried out following the protocol provided by Applied Biosystems (CA, USA) and analyzed by BioEdit and Blast. The parental vectors, pcDNA3 and pORF were used as negative controls. DNA for immunization was purified using the EndoFree Giga Kit (Qiagen, CA, USA) and was diluted in TE buffer to the final concentration of 1 μg/μl.

Plasmid gene expression in mammalian cells

For the expression of pORF-mIL-12 in mammalian cells, a transient-transfection assay was performed using Lipofectin (Invitrogen) and 1 or 2 μg plasmid transfected into HeLa cells (2×10^5 cells/well). The cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) (Cultilab, SP, Brazil). After 24 h incubation, the cells were harvested, and total RNA was isolated with Trizol for reverse transcription (RT)-PCR. IL-12 PCR was used as described above. IL-12p70 was detected (80 ng/ml) by ELISA, in the supernatant of transfected HeLa cells.

P10 expression from vector pcDNA3, followed by IFN-γ production

DNA immunization was performed by injecting groups of 5 six-week-old male BALB/c mice intramuscularly in both quadriceps with three doses of 100 μg of plasmid encoding P10 (pP10), 50 μg of either pP10 and pcDNA3 vector alone, or 50 μg of the pcDNA3 vector alone, each in 50 μl of TE buffer. A total of three immunizations were given at weekly intervals in alternating sites on the left and right hind legs. The mice were euthanized one week after the last immunization, their spleens were isolated and single-cell suspensions were prepared by gentle homogenization in RPMI medium supplemented with 1% FCS. Cells were suspended and treated with isotonic ammonium chloride to lyse erythrocytes. The splenocytes were washed by centrifugation, suspended in RPMI containing 10% FCS, and dispensed into wells on a microtitering plate (5×10^5 mononuclear cells per well). The cultures were stimulated with 20 μg/ml of synthetic P10. After 24 and 48-h incubation at 37°C with 5% CO_2, supernatants were collected and IFN-γ was assayed by a sandwich enzyme-linked immunoassay (ELISA) (BD Pharmingen, CA, USA). Splenocytes from animals immunized with pP10 and stimulated with synthetic P10 produced 10 and 15 ng/ml IFN-γ after 24 and 48 h,

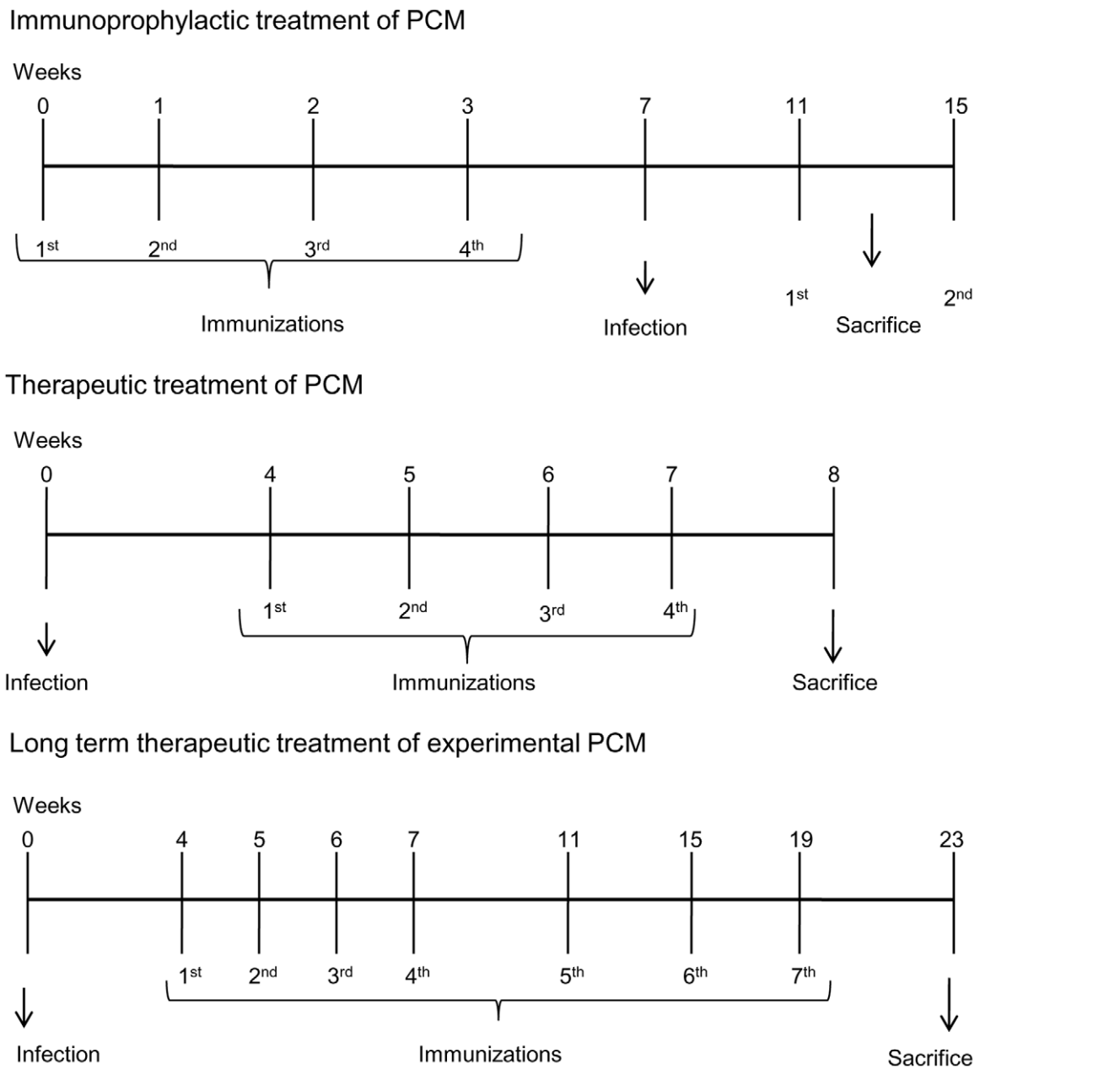

Figure 1. Summary of the treatment protocols used. First protocol: BALB/c mice received 4 weekly injections. Animals were infected and sacrificed 30 or 60 days later. **Second protocol**: BALB/c and B10.A mice were infected intratracheally. Mice received 4 weekly vaccine doses and animals were sacrificed 1 week after the last injection. **Third protocol**: B10.A mice were infected i.t. and one month after infection, they were immunized with the DNA vaccine. The animals were sacrificed one month after the last injection, six months after infection.
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respectively. When 50 μg of pcDNA3 was used for immunization, 9 and 11 ng/ml IFN-γ was released by splenocytes at the two examined times.

Ethics statement

This study was carried out as recommended by the Brazilian college of animal experimentation (COBEA). The protocol has been approved by the Ethical Committee on Animal Experimentation of University of São Paulo (Permit number: 039).

Animals

BALB/c and B10.A mice were bred at the Institute of Biomedical Science of University of São Paulo, Department of Immunology animal facility under specific-pathogen-free conditions.

Fungal strain

Yeast cells of the virulent isolate Pb 18 of *P. brasiliensis* were maintained by weekly subculturing on Sabouraud Dextrose Agar and incubation at 37°C. Before experimental infection, 7–10 day-old cells were inoculated into Sabouraud Dextrose Broth and incubated at 37°C for 5–7days with rotary shaking. Fungal cells were washed three times in phosphate-buffered saline pH 7.2 (PBS) and counted in a haemocytometer. The viability of fungal cells in the inoculum was determined by staining with Janus B (Merck, Darmstadt, Germany) and was greater than 90%.

Intratracheal infection of BALB/c and B10.A mice

BALB/c and B10.A mice (6- to 8-week-old males) were inoculated intratracheally (IT) with 50 μl suspension of 3×10^5 Pb18 yeast cells in sterile saline (0.85% NaCl). Mice were anesthetized i.p. with 200 μL of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 10 min, their necks were extended to expose the trachea at the thyroid level and cell suspensions were injected with a 26-gauge needle. The incisions were sutured with 5-0 silk.

Immunization of mice with plasmid DNA

Three different protocols were used (**Fig. 1**). Injections of 50 μg plasmid were given in the quadriceps muscle. **First protocol**: Groups of 10 BALB/c mice were injected with PBS (control), pcDNA3 (50 μg; control), pORF (50 ug; control), plasmid encoding P10 (pP10, 50 μg), plasmid encoding IL-12 (pIL-12, 50 μg) or with both pP10 and pIL-12 (50 ug each). A total of 4 injections were given weekly on alternating sites, on the left and right hind legs. One week after the last injection, mice were infected intratracheally then sacrificed 30 or 60 days later. **Second protocol**: BALB/c and B10.A mice (10 mice per group) were infected intratracheally. One month after infection, the mice received 4 weekly injections of either PBS (control), pcDNA3 (50 μg; control), pP10 (50 μg), pIL-12 (50 μg) or both pP10 and pPIL-12 (50 ug each). Mice were sacrificed 1 week after the last

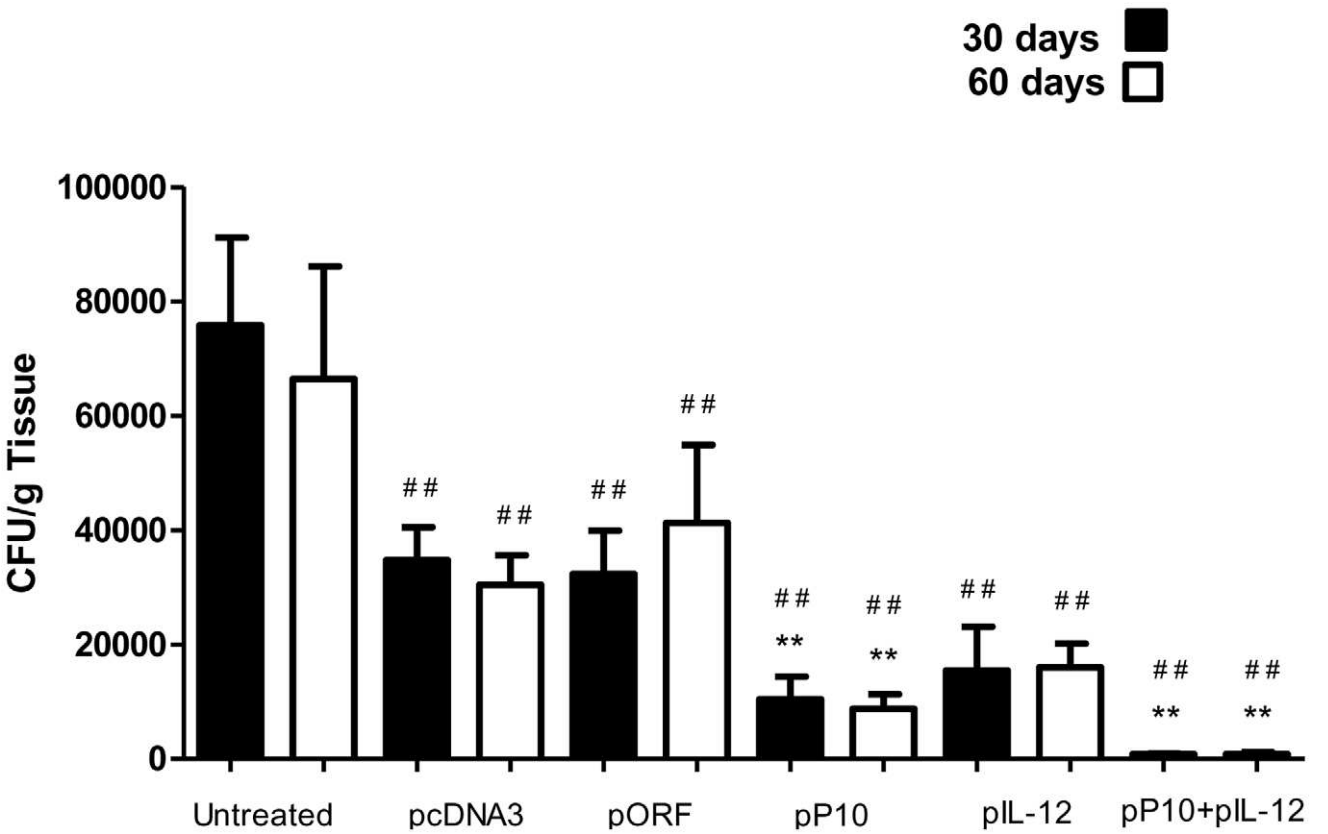

Figure 2. Immunoprophylactic treatment of PCM. Gene immunization was initiated 30 days before fungal challenge. CFUs are from lungs of BALB/c mice infected intratracheally with 3×10^5 yeast cells and subjected to immunization with vectors containing P10 (pP10) and/or IL-12 (pIL-12) DNA insert. Control mice were inoculated with PBS or with vectors without insert. Mice were sacrificed 30 (■) and 60 (□) days after infection. Each bar represents the average counts and standard deviations of CFUs in lungs from 10 animals in each group. Experiments were carried out in triplicate with similar results. **$p\leq0.0001$, comparing vector with and without insert; $^{\#\#}$ $p\leq0.0001$, .comparing untreated and other groups.
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injection. **Third protocol**: B10.A mice (10 mice per group) were infected intratracheally. One month after infection, they were treated with 4 weekly injections followed by a monthly booster for 3 additional months (total of 7 injections). The injections were with either PBS (control), pcDNA3, pP10, pIL-12 or both pP10 and pPIL-12. The mice were sacrificed one month after the last injection, six months after infection.

Fungal burden in organs of infected mice

Mice were sacrificed and the lungs, liver and spleen were removed. Weighed tissue sections were homogenized and then washed 3 times with PBS and suspended in 1 ml PBS. Suspensions (100 µl) were inoculated on brain-heart infusion (BHI) agar medium supplemented with 4% FCS and 5% spent culture medium of *P. brasiliensis* (strain-192), streptomycin/penicillin 10 IU/ml (Cultilab) and cycloheximide 500 mg/ml (Sigma, MO, USA). Colonies were counted after 10 days of incubation at 37°C.

Histopathology

Lung sections from sacrificed mice were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Four-micra sections were stained with haematoxylin-eosin (HE) or silver nitrate (Gomori) and examined microscopically (Optiphot-2; Nikon, Tokyo, Japan).

Cytokine detection

Sections of excised lungs were homogenized in 2 ml of PBS in the presence of protease inhibitors: benzamidine HCl (4 mM), EDTA disodium salt (1 mM), N-ethylmaleimide (1 mM) and Pepstatin (1.5 mM) (Sigma, St Louis, MO). The supernatants were assayed for IL-4, IL-10, IL-12, and IFN-γ using ELISA kits (BD OpTeia, San Diego, CA). The detection limits of the assays were as follows: 7.8 pg/ml for IL-4, 31.3 pg/ml for IFN-γ and IL-10, 62.5 pg/ml for IL-12, as previously determined by the manufacturer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism5 software. The results are expressed as means and standard deviations (SD). The nonparametric Kruskall-Wallis honestly significant difference test was used. *p* values are shown in the Figure legends.

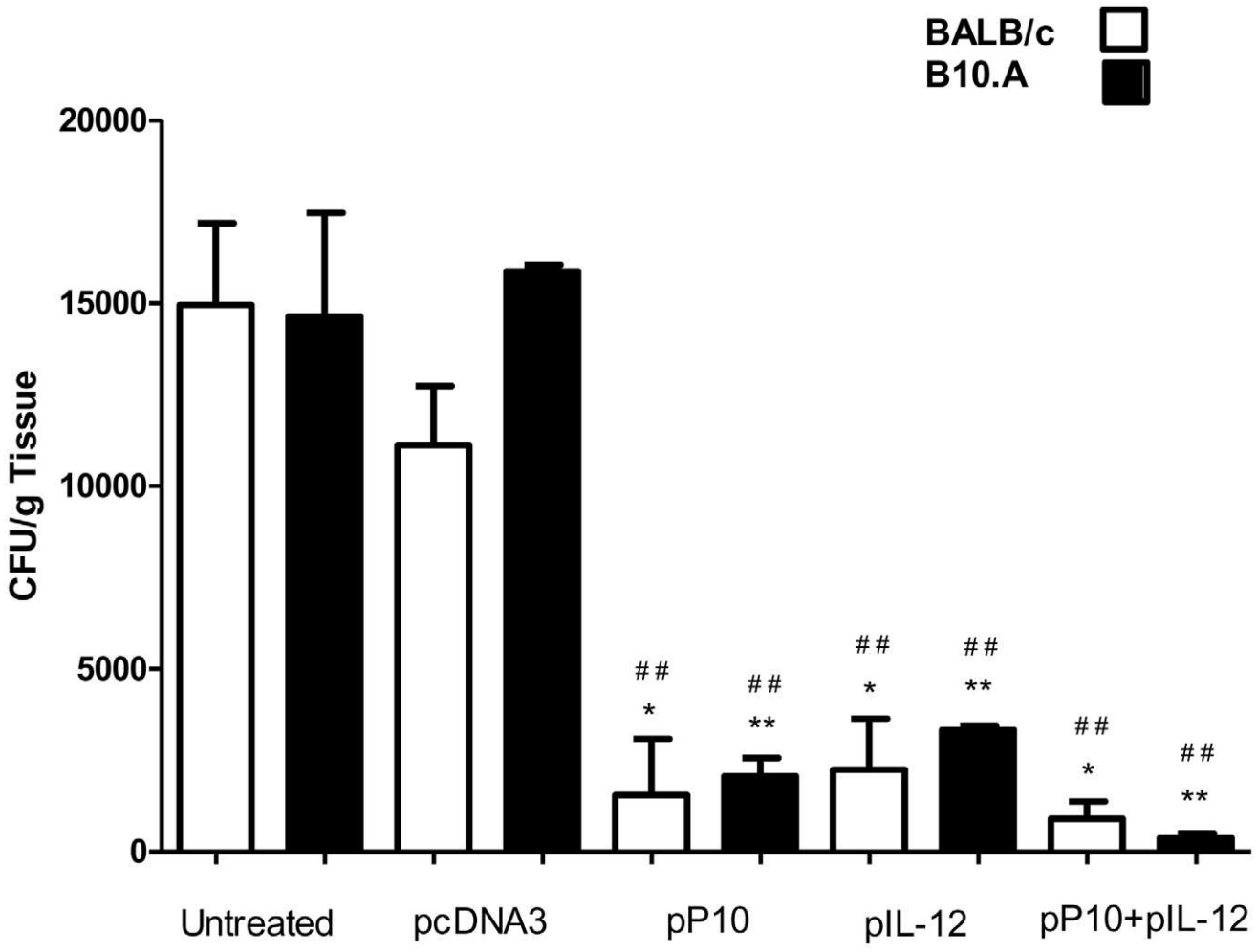

Figure 3. Therapeutic treatment of PCM. Gene immunization started 30 days after infection. CFUs are from lungs of BALB/c (□) and B10.A(■) mice infected intratracheally with 3×10^5 yeast cells and subjected to immunization with vectors containing P10 (pP10) or IL-12 (pIL-12) DNA inserts. Control mice were inoculated with PBS or with vectors without insert. Mice were sacrificed 60 days after infection. Each bar represents the average counts and standard deviations of CFU in lungs from 10 animals in each group. Experiments were performed three times and similar results were achieved. *$p\leq0.05$, **$p\leq0.005$, comparing vector with and without insert; $^{\#\#}$ $p\leq0.005$, comparing untreated and other groups.
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Results

Colony-forming units (CFU) in mice immunized prior to infection (prophylactic immunization). First protocol

To explore the effects of the plasmid with the P10 insert (pP10) with or without the murine IL-12 gene insert (pIL-12), BALB/c mice were immunized and then infected intratracheally with virulent *P. brasiliensis* Pb 18. Animals were sacrificed after 30 or 60 days, and the fungal burden in the lungs, spleens and livers was determined. The number of lung CFU per gram of tissue was significantly reduced in animals immunized with pP10 and/or pIL-12 compared to controls at both time intervals (**Fig. 2**). Notably, we observed that the empty plasmids (pcDNA3 and pORF) also induced a significant reduction in CFU relative to mice that received PBS alone, which is presumably a result of dendritic cell activation through Toll-like receptor 9 binding of plasmid unmethylated CpG motifs. Immunostimulation by DNA from *P. brasiliensis* also attributed to CpG motifs showed protective effects in susceptible mice [21,22]. Nevertheless, the fungal load measured in CFUs in mice receiving pP10 and/or pIL-12 was significantly lower than that in mice treated with control pcDNA3 and pORF. Livers and spleens from all animals had no detectable fungal cells.

Organ CFUs in mice immunized 1-month after infection (therapeutic immunization). Second protocol

The therapeutic protocol attempts to reproduce the clinical reality of patients presenting to medical attention after developing symptomatic PCM. We studied two mouse strains with different susceptibilities to PCM, BALB/c (susceptible) and B10.A (highly susceptible) [23]. The data showed that immunization with pP10 and/or pIL-12 was therapeutic in mice infected with *P. brasiliensis* for 1 month prior to receiving plasmid immunizations (**Fig. 3**). CFU reductions were significant in infected mice receiving pP10 and/or pIL-12 compared to mice injected with PBS or pcDNA3. In contrast to the first protocol, injection of pcDNA3 after installing PCM was not sufficient to reduce the fungal burden. The most significant reduction in the lung CFUs from B10.A mice was achieved when pP10 and pIL-12 were combined. The CFUs from the livers and spleens were barely detectable in all groups.

Organ CFUs in a long-term infection model of B10.A mice submitted to gene immunization. Third protocol

This protocol allowed us to analyze the efficacy of therapeutic plasmid treatment during long-term infection (six months) of the highly susceptible mouse strain, B10.A. Treatment of mice with PCM using pP10 and/or pIL-12 significantly reduced lung CFUs (**Fig. 4**). However, the impact of pIL-12 alone was not as dramatic as either pP10 alone or pP10 with pIL-12. Notably, treatment with the combination of pP10 and pIL-12 virtually eradicated the infection in all organs examined.

Lung histopathology

The lungs of control animals in each experimental protocol group showed intense inflammation and large numbers of yeast cells, whereas mice receiving pP10 with or without pIL-12 had significantly reduced inflammation, and lower or undetectable fungal cells. Analysis of the lungs of animals from the third protocol (6 months infection) that received control plasmids revealed dense infiltration of inflammatory cells, mainly of macrophages, lymphocytes and epithelioid cells, and numerous fungal cells (**Fig. 5A**). Around the foci of epithelioid granulomas, giant cells were observed. In contrast, there were large areas of normal lung architecture in pP10-immunized mice and a global reduction in the number of granulomas with few yeast cells (**Fig. 5B**). Treatment with pIL-12 resulted in histological findings that were more similar to controls than to pP10-immunized mice (**Fig. 5C**). Importantly, the lungs of mice treated with the

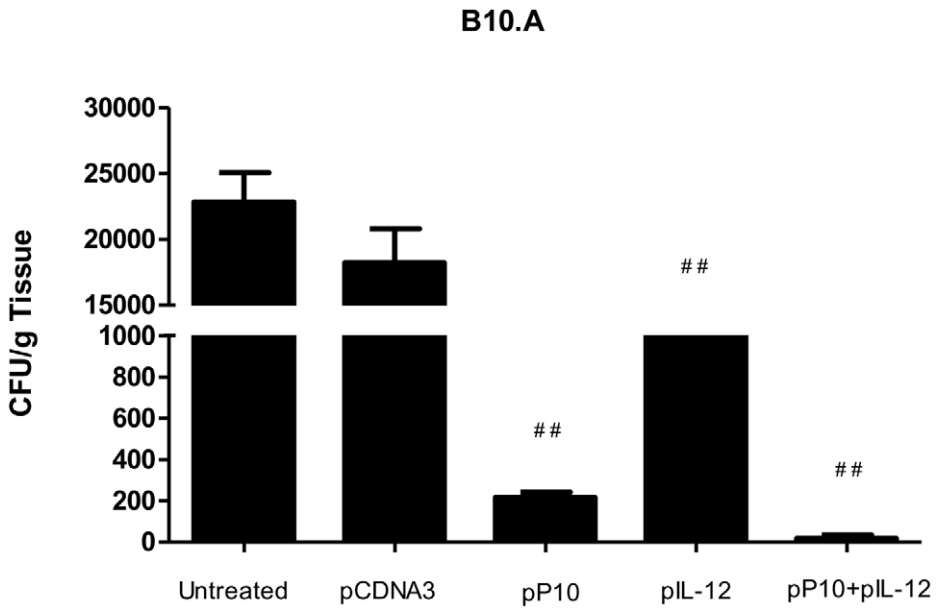

Figure 4. Long term therapeutic treatment of experimental PCM. Gene immunization started 30 days after infection and mice were sacrificed 6 months after infection. CFUs were counted in lungs of B10.A mice infected intratracheally with 3×10^5 yeast cells and immunized with vectors containing the insert encoding P10 (pP10) or IL-12 (pIL-12). Control mice were inoculated with PBS or with vector without insert. Each bar represents the average counts and standard deviations of CFU in lungs from 5 to 10 animals in each group. Experiments were carried out in triplicate, with similar results. ** $p\leq0.001$, comparing vector with and without insert; $^{\#\#}p\leq0.001$, comparing untreated and other groups.
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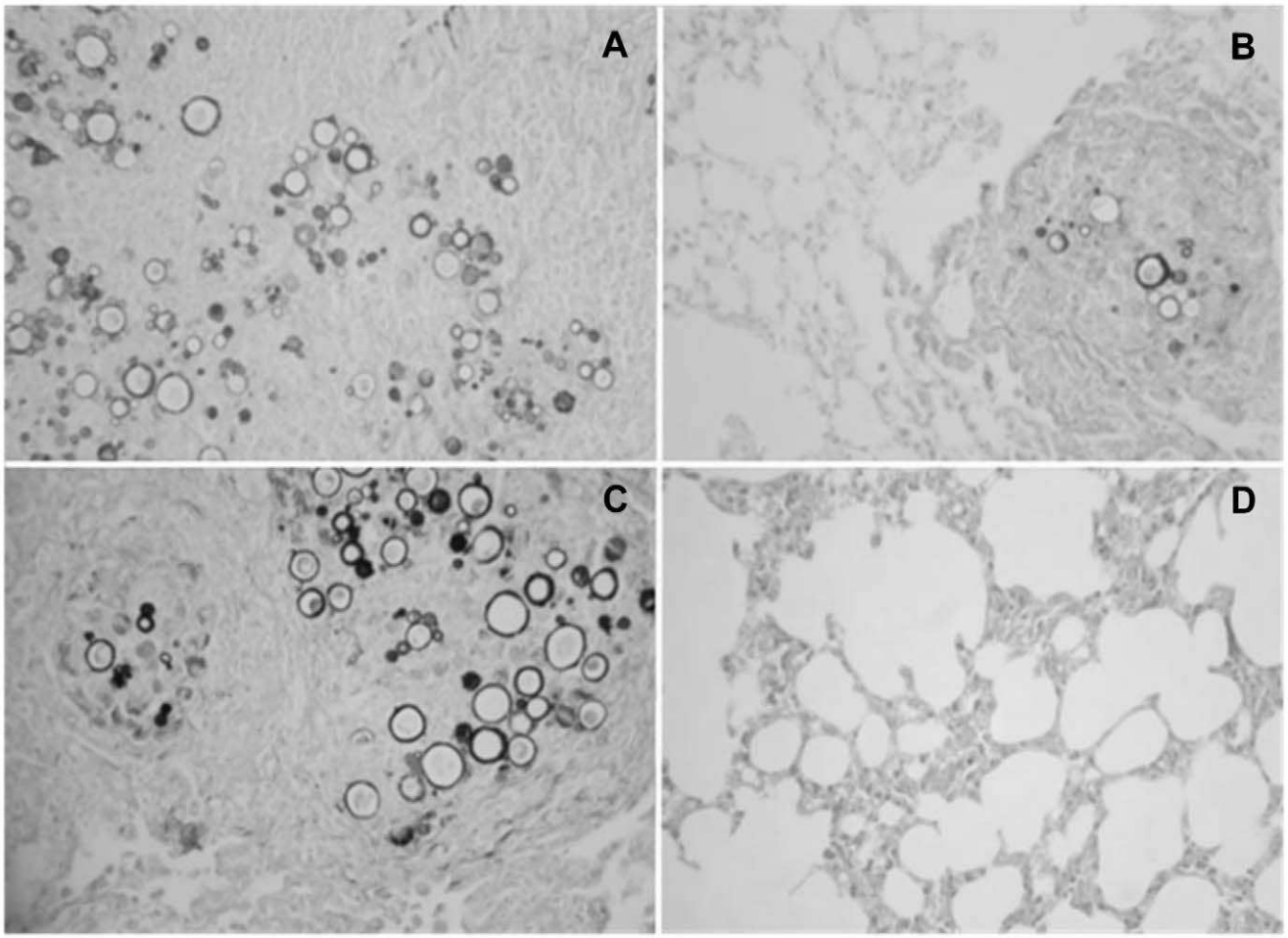

Figure 5. Histopathology of lungs from intratracheally infected B10.A mice. Animals were infected with *P. brasiliensis* for one month, treated with or without vectors carrying P10 or IL-12 DNA inserts according to protocol 3, and sacrificed 6 months after the initial infection. Infected mice treated with (**A**) control pcDNA3, (**B**) pP10, (**C**) pIL-12 DNA, and (**D**) P10 and IL-12 DNA. Gomori staining; original magnification, 40×.
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combination of pP10 and pIL-12 were mostly histologically normal and no yeast cells were identified (**Fig. 5D**).

Cytokine assays

Previous studies with BALB/c mice have established that P10 elicits a protective Th-1 immune response [11]. BALB/c and B10.A mice have different genetic backgrounds that strongly influence their response to infection by *P. brasiliensis*. Their different susceptibility to fungal infection depends in part on their capacity to produce pro-inflammatory cytokines, which are often reduced in B10.A relative to BALB/c. IL-4, IL-10, IL-12 and IFN-γ were measured in the lungs of infected B10.A mice and BALB/c. In mice subjected to the second protocol, BALB/c mice responded to pcDNA3 and pP10 gene immunization with significant increase in IFN-γ in the lung homogenate compared to untreated or pcDNA3 treated animals (data not shown). In contrast, B10.A mice produced significantly less IFN-γ after immunization with pP10 and pcDNA3 in comparison to treatment with pcDNA3 gene alone, which suggests that the increase in IFN-γ in both of these groups relative to untreated mice could be due to dendritic cell activation by plasmid CpGs. The cytokine production in the group of animals submitted to the third protocol, in which B10.A mice treated with pP10 with or without pIL-12 had undetectable yeasts in the lung tissue, is shown in **Table 1**. After 6 months post-infection, cytokine analyses in these mice showed a persistent IFN-γ production regulated by an IL-10-rich immune response, which is compatible with a protective therapeutic effect in B10.A mice.

Discussion

A vaccine against *P. brasiliensis* using plasmid DNA was first tested in 2000 [24,25]. BALB/c mice were immunized with a

Table 1. Cytokines in lung homogenates of B10.A mice infected i.t. with *P. brasiliensis* Pb 18 yeasts and submitted to gene immunization for 5 months.

Cytokines pg/ml	IL-10	IFN-γ
Untreated	12.18±4.61	4.71±1.75
pcDNA3	10.38±6.53	4.73±2.45
pP10+pcDNA3	18.71±6.10	7.64±2.46
pP10+pIL-12	12.00±2.06	7.31±1.37

Mice were sacrificed 1 month after the last dose.
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mammalian expression vector carrying the full gene of the gp43 under the control of CMV promoter with Freund's adjuvant resulting in the induction of both B and T cell-mediated immune responses characterized by a mixed Th-1/Th-2 long-lasting cellular immune response, chiefly modulated by IFN-γ. This immunization method was protective when performed in mice prior to challenge with virulent *P. brasiliensis*. When tested for immunoprotection, P10 in Freund's adjuvant was also active in the murine model of PCM, eliciting an IFN-γ-dependent Th-1 immune response [11,20]. The combined treatment of P10-vaccine in Freund's adjuvant and chemotherapy, either an azole, amphotericin B, or sulfamethoxazole, stimulated a protective Th-1 response, rich in IL-12 and IFN-γ, that was therapeutically beneficial if initiated 2 or 30 days after intratracheal infection [19]. The combined treatment was also effective in anergic animals challenged with the fungus [20].

Presently we used a DNA vaccine encoding P10 with or without a plasmid encoding IL-12 that is administered without adjuvant. We found that the pP10 vaccine, either given prior to or 1 month after intratracheal infection, induced a significant reduction in the fungal burden in the lungs of mice. Co-vaccination with murine pIL-12 significantly enhanced vaccine effectiveness, particularly in a long- term infection model in B10.A mice. The combined DNA vaccine (Protocol 3) achieved virtual sterilization after 6 months with histologically normal lungs and undetectable fungal burden. Full protection was mediated by IFN-γ production and the pro-inflammatory effect of pP10 and pIL-12 was regulated by IL-10 in these susceptible mice.

The mechanism of fungal killing by gene immunization is not solely mediated by cytokines since the empty plasmid pcDNA3 is a strong stimulator of the immune system. However, a significant protection is only achieved with pP10 or pP10+pIL-12 administration. P10 is not protective in IFN-γ-KO mice [12], indicating that this cytokine is essential for fungal killing through macrophage activation. T-CD4+ lymphocytes recognizing P10 and other cells induced by fungal infection are the main producers of IFN-γ. A role for a simultaneous induction of protective antibodies against fungal antigens [26] is also recognized.

IL-12 administration has been previously studied in experimental PCM [27]. Our current results show that IL-12 protected mice against disseminated infection. In the long term infection protocol, pIL-12 alone was only partially effective in the protection of infected mice, but the cytokine facilitated the elimination of *P. brasiliensis* when combined with pP10. This is a very encouraging result and strongly suggests that a pP10-based vaccine associated with pIL-12 could be used as a powerful adjuvant to chemotherapy.

Despite the effectiveness of chemotherapy, fatalities from invasive or systemic fungal diseases are not uncommon. Vaccines against fungal diseases are gaining increasing attention, owing to their capacity to effectively modulate the immune response (reviewed in [28]. The frequent occurrence of clinical relapses and sequellae, such as pulmonary fibrosis, following antifungal chemotherapy suggest that immunoprotective vaccines could also reduce the incidence of these complications [29].

In addition to our work with P10, there have been other notable attempts to develop vaccine strategies for the treatment of PCM. They included a cDNA encoding the antigenic protein rPb27 [30], the recombinant heat shock protein 60 emulsified in adjuvant [31], radioattenuated *P. brasiliensis* yeast cells [32] and *Mycobacterium leprae* DNAhsp65 plasmid in infected BALB/c mice [33]. Braga *et al.* [34] immunized BALB/c mice either with recombinant purified flagellins (FliC) genetically fused with P10 or with the synthetic P10 peptide mixed with purified FliC. A prevailing Th1-type immune response was obtained that reduced *P. brasiliensis* growth and lung damage in infected mice.

From a practical standpoint, the broad use of antifungal vaccines is not realistic when considering the perspective of a large number of infected people relative to the number of individuals who develop the disease. Mycoses caused by dimorphic fungi, such as PCM, coccidioidomycosis, histoplasmosis and blastomycosis, have low incidence as a deep-seated disease. Certain fungal diseases such as cryptococcosis, aspergillosis and candidiasis typically occur in immunocompromised hosts. Hence, targeted prophylactic vaccination may be a more practical approach to control disease. In the case of PCM, immunization of those at highest risk, such as farmers in highly endemic regions would be reasonable. However, we have also demonstrated that the pP10/pIL-12 combination is highly efficacious after PCM has developed. Therefore, immunization could be most useful in combination with standard therapy in PCM patients in order to enhance treatment efficacy, reduce treatment duration and, perhaps, prevent relapses.

Author Contributions

Conceived and designed the experiments: CPT LRT. Performed the experiments: GMGR JEM AFM. Analyzed the data: JDN CPT LRT. Contributed reagents/materials/analysis tools: CPT. Wrote the paper: CPT LRT JDN.

References

1. Wanke B, Londero AT (1994) Epidemiology and paracoccidioidomycosis infection. In: Franco M, Lacaz CS, Restrepo A, Del Negro G, eds. Paracoccidioidomycosis. Florida: CRC Press Boca Raton. pp 109–117.
2. Prado M, da Silva MB, Laurenti R, Travassos LR, Taborda CP (2009) Mortality in Brazil of systemic mycosis as primary cause of death or in association with AIDS: a review from 1996 to 2006. Mem Inst Oswaldo Cruz 104: 513–521.
3. Benard G (2008) An overview of the immunopathology of human paracoccidioidomycosis. Mycopathologia 165: 209–221.
4. Shikanai-Yasuda MA, Telles Filho Fde Q, Mendes RP, Colombo AL, Moretti ML (2006) Guidelines in paracoccidioidomycosis. Rev Soc Bras Med Trop 39: 297–310.
5. Puccia R, Schenkman S, Gorin PA, Travassos LR (1986) Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect Immun 53: 199–206.
6. Cisalpino PS, Puccia R, Yamauchi LM, Cano MI, da Silveira JF, et al. (1996) Cloning, characterization, and epitope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis*. J Biol Chem 271: 4553–4560.
7. Camargo ZP, Unterkircher C, Travassos LR (1989) Identification of antigenic polypeptides of *Paracoccidioides brasiliensis* by immunoblotting. J Med Vet Mycol 27: 407–12.
8. Rodrigues EG, Travassos LR (1994) Nature of the reactive epitopes in *Paracoccidioides brasiliensis* polysaccharide antigen. J Med Vet Mycol 32: 77–81.
9. Saraiva EC, Altemani A, Franco MF, Unterkircher CS, Camargo ZP (1996) *Paracoccidioides brasiliensis*-gp43 used as paracoccidioidin. J Med Vet Mycol 34: 155–161.
10. Almeida IC, Neville DC, Mehlert A, Treumann A, Ferguson MA, et al. (1996) Structure of the N-linked oligosaccharide of the main diagnostic antigen of the pathogenic fungus *Paracoccidioides brasiliensis*. Glycobiology 6: 507–515.
11. Taborda CP, Juliano MA, Puccia R, Franco M, Travassos LR (1998) Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. Infect Immun 66: 786–793.
12. Travassos LR, Taborda CP, Iwai LK, Cunha Neto E, Puccia R (2004) The gp43 from *Paracoccidioides brasiliensis*: A major diagnostic antigen and vaccine candidate. In: Domer JE, Kobayashi GS, eds. The Mycota XII, Human Fungal Pathogens. Berlin: Springer-Verlag Berlin-Heidelberg. pp 279–296.
13. Souto JT, Figueiredo F, Furlanetto A, Pfeffer K, Rossi MA, Silva JS (2000) Interferon-gamma and tumor necrosis factor-alpha determine resistance to *Paracoccidioides brasiliensis* infection in mice. Am J Pathol 156: 1811–1820.
14. Cano LE, Kashino SS, Arruda C, Andre D, Xidieh CF, et al. (1998) Protective role of gamma interferon in experimental pulmonary paracoccidioidomycosis. Infect Immun 66: 800–806.
15. Kurita N, Oarada M, Miyaji M, Ito E (2000) Effect of cytokines on antifungal activity of human polymorphonuclear leucocytes against yeast cells of *Paracoccidioides brasiliensis*. Med Mycol 38: 177–182.

16. Morais FV, Barros TF, Fukada MK, Cisalpino PS, Puccia R (2000) Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus *Paracoccidioides brasiliensis*. J Clin Microbiol 38: 3960–3966.
17. Taborda CP, Nakaie CR, Cilli EM, Rodrigues EG, Silva LS, et al. (2004) Synthesis and immunological activity of a branched peptide carrying the T-cell epitope of gp43, the major exocellular antigen of *Paracoccidioides brasiliensis*. Scand J Immunol 59: 58–65.
18. Iwai LK, Yoshida M, Sidney J, Shikanai-Yasuda MA, Goldberg AC, et al. (2003) In silico prediction of peptides binding to multiple HLA-DR molecules accurately identifies immunodominant epitopes from gp43 of *Paracoccidioides brasiliensis* frequently recognized in primary peripheral blood mononuclear cell responses from sensitized individuals. Mol Med 9: 209–219.
19. Marques AF, da Silva MB, Juliano MA, Travassos LR, Taborda CP (2006) Peptide immunization as an adjuvant to chemotherapy in mice challenged intratracheally with virulent yeast cells of *Paracoccidioides brasiliensis*. Antimicrob Agents Chemother 50: 2814–2819.
20. Marques AF, da Silva MB, Juliano MA, Munõz JE, Travassos LR, et al. (2008) Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*. Microb Infec 10: 1251–1258.
21. Souza MC, Corrêa M, Almeida SR, Lopes JD, Camargo ZP (2001) Immunostimulatory DNA from *Paracoccidioides brasiliensis* acts as T-helper 1 promoter in susceptible mice. Scand J Immunol. 54: 348–356.
22. Almeida SR, Camargo ZP, Souza MC (2005) Adjuvant effect of synthetic oligodeoxyribonucleotides (CpG-ODN) from the *Paracoccidioides brasiliensis* gp43 gene on the Th2-Th1 immunomodulation of experimental paracoccidioidomycosis. Scand J Immunol 62(4): 325–333.
23. Calich VL, Singer-Vermes LM, Russo M, Vaz CA, Burger E (1994) Immunogenetics in Paracoccidioidomycosis. In: Franco M, Lacaz CS, Restrepo A, Del Negro G, eds. Paracoccidioidomycosis. Florida: CRC Press Boca Raton. pp 151–173.
24. Travassos LR, Casadevall A, Taborda CP (2005) Immunomodulation and immunoprotection in Fungal infections: humoral and cellular immune responses. In: San-Blas G, Calderone R, eds. Pathogenic Fungi: Cellular and Molecular Biology. Norfolk: Caister Academic Press. pp 241–284.
25. Pinto AR, Puccia R, Diniz SN, Franco MF, Travassos LR (2000) DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *Paracoccidioides brasiliensis*. Vaccine 18: 3050–3058.
26. Nosanchuk JD (2005) Protective antibodies and endemic dimorphic fungi. Curr Mol Med 5: 435–442.
27. Arruda C, Franco MF, Kashino SS, Nascimento FR, Fazioli Rdos A, et al. (2002) Interleukin-12 protects mice against disseminated infection caused by *Paracoccidioides brasiliensis* but enhances pulmonary inflammation. Clin Immunol 103: 185–195.
28. Cassone A (2008) Fungal vaccines: real progress from real challenges. Lancet Infect Dis 8: 114–124.
29. Travassos LR, Taborda CP, Colombo AL (2008) Treatment options for paracoccidioidomycosis and new strategies investigated. Expert Rev Anti Infect Ther 6: 251–262.
30. Reis BS, Fernandes VC, Martins EM, Serakides R, Goes AM (2008) Protective immunity induced by rPb27 of *Paracoccidioides brasiliensis*. Vaccine 26: 5461–5469.
31. de Bastos ASR, Gomez FJ, de Almeida Soares CM, Deepe GSJ (2008) Vaccination with heat shock protein 60 induces a protective immune response against experimental *Paracoccidioides brasiliensis* pulmonary infection. Infect Immun 76: 4214–4221.
32. Martins EMN, Reis BS, de Resende MA, de Andrede AS, Goes AM (2009) Mice immunization with radioattenuated cells of *Paracoccidioides brasiliensis*: influence of the number of immunizations. Mycopathologia 168: 51–58.
33. Ribeiro AM, Bocca AL, Amaral AC, Faccioli LH, Galetti FC, et al. (2009) DNAhsp65 vaccination induces protection in mice against *Paracoccidioides brasiliensis* infection. Vaccine 27: 606–613.
34. Braga CJ, Rittner GM, Muñoz Henao JE, Teixeira AF, Massis LM, et al. (2009) *Paracoccidioides brasiliensis* vaccine formulations based on the gp 43-derived P10 sequence and the *Salmonella enterica* FliC flagellin. Infect Immun 77: 1700–1707.

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Original article

DNA vaccine encoding peptide P10 against experimental paracoccidioidomycosis induces long-term protection in presence of regulatory T cells

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Abstract

Paracoccidioidomycosis is a granulomatous systemic mycosis endemic in Brazil and other Latin America countries. A DNA vaccine encoding the immunoprotective peptide 10 (P10) significantly reduced the fungal burden in mice when given prior to or after intratracheal challenge with *Paracoccidioides brasiliensis*. Presently, the generation/expansion of $CD4^+$ $CD44^{hi}$ memory T cells as well as $Foxp3^+$ Treg cells in mice immunized with the DNA vaccine (pcDNA3-P10) before and after infection with *P. brasiliensis* was investigated. Memory $CD4^+$ $CD44^{hi}$ T cells simultaneously with $Foxp3^+$ Treg cells increased in the spleens and lungs of pcDNA3-P10 immunized mice on day 0, 30, 60 and 120 postinfection. Histopathology of the lung tissue showed minimal inflammation in immunized mice compared with the unimmunized group, suggesting a role for regulatory T cells in controlling the immunopathology. The DNA vaccine shows that the repeated immunization generates memory cells and regulatory T cells that replace the initially protective pro-inflammatory T cells conferring a long term protection while preserving the integrity of the infected tissue.

Keywords: P10 peptide; *Paracoccidioides brasiliensis*; Immunization; Regulatory T cells

1. Introduction

Paracoccidioidomycosis (PCM) is a chronic granulomatous disease caused by the thermal dimorphic fungus *Paracoccidioides brasiliensis.* It is endemic in Brazil, Colombia, Venezuela and Argentina [1,2]. PCM can inflict a high burden fungal infection with significant morbidity and mortality associated with it. The treatment for PCM is rather long (ranging from several months to more than two years) resulting in a significant number of patients self-discontinuing treatment and a high loss in patients' follow up, both associated with a high rate of disease relapse [3]. Since PCM primarily affects rural workers, the disease is associated with significant social and economic factors that mostly affect less affluent populations [2].

In order to improve the treatment effectiveness, both in terms of time shortening and protection against the disease, we have investigated disease modifying peptides. Initially, a 15 amino acid peptide (QTLIAIHTLAIRYAN, named P10 peptide) derived from the immunodominant antigen, gp43, of

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P. brasiliensis was identified [4]. This peptide is capable of protecting mice in a *P. brasiliensis* intratracheal infection model by eliciting a protective Th-1 response [4,5]. The P10 peptide, when used in combination with the standard chemotherapy regimens for experimental PCM, improved treatment efficacy with a potential to reduce the time of treatment and avoid relapses [3]. Similarly, administration of the P10 peptide entrapped within PLGA in combination with standard chemotherapy significantly reduced the fungal burden in a mouse PCM model [6]. This peptide was also administered admixed with *Salmonella enterica* FliC flagellin to mice resulting in significantly enhanced protection against intranasal challenge with *P. brasiliensis* [7].

The vaccine potential of P10 has also been explored by inserting the sequence of the P10 peptide into a plasmid vector (pcDNA3), which proved to be protective in both prophylactic and therapeutic schemes, as determined by significant reduction in fungal burden [8]. This result confirmed a previous one using as insert the whole sequence of the gp43, which showed a long-term protection of the DNA vaccine [9]. DNA vaccines present some advantages over the use of other vaccines. For instance, DNA vaccines can be rapidly produced, show thermal stability and are easily produced in large-scale [10].

An effective vaccine must be capable of generating immunological memory [11]. Memory T cells can be subdivided into two subpopulations, T effector-memory (T_{EM}) and T central-memory (T_{CM}) cells. The first migrate to inflamed peripheral tissue and are rapidly activated upon subsequent interaction with the same antigen. T_{CM} cells reside in the lymphoid tissue, where they replicate and expand, comprising effector T cells that can subsequently be released to combat specific pathogens [12,13]. In mice, both subsets express high amounts of CD44 [14]. Patients with chronic infectious diseases, such as PCM and leishmaniasis, have increased numbers of memory T cells even after treatment [15,16]. Additionally, natural $Foxp3^+$ regulatory T cells are crucial in maintaining immune homeostasis [17]. For example, they are necessary to minimize immunopathology during the host response to pathogens [18] by controlling effector responses [19]. Depending on the appropriate balance of the immune response these cells can eventually inhibit the complete eradication of pathogens from tissues, as demonstrated in an experimental model of leishmaniasis [20].

In this study, we evaluated the generation/expansion and protective efficacy of phenotypic $CD4^{\pm}$ $CD44^{hi}$ memory T cells as well as of $Foxp3^{\pm}$ Treg cells in mice immunized with the plasmid DNA encoding the P10 peptide and challenged with *P. brasiliensis* yeasts. Our results indicate that the DNA vaccine elicits a protective immune response while increasing the percentage of $CD4^+$ $CD44^{hi}$ memory T cells and $Foxp3^+$ Treg cells in the spleens and lungs of immunized mice before and after 30, 60 and 120 days of challenge. Our results reinforce the concept that the presence of T regulatory cells upon secondary antigen exposure may prevent immunopathology in the context of vaccination and favor long-term memory as discussed by Romani and Puccetti [21].

2. Materials and methods

2.1. Purification of plasmid DNA

The pcDNA3 vector (Invitrogen) used for the expression of P10 peptide sequence (pcDNA3-P10) has been previously described [8]. *Escherichia coli* DH5-α transformed with pcDNA3 (control, empty vector) or pcDNA3-P10 was grown in Luria Broth (LB, GIBCO-BRL) containing ampicillin. The purification of the plasmid DNA was accomplished using the Endofree Plasmid Purification Kit (QIAGEN AG, Basel, Switzerland) according to the manufacturer's instructions. Plasmid concentration was measured by optical density at 260 and 280 nm and by agarose gel electrophoresis relative to a standard. Purified preparations were stored in PBS at −20 °C until use.

2.2. Fungal strain

P. brasiliensis Pb18 yeast cells were used for infections. Briefly, the yeast cells were maintained on plates containing solid Sabouraud medium at 37 °C. After 7–10 days of growth, the fungus was transferred to modified Mc Veigh–Morton liquid medium and was cultivated at 37 °C for 7 days [22,23]. The yeast cells were then washed 3 times in PBS and their viability was determined using Trypan Blue. Cultures with viabilities higher than 90% were utilized.

2.3. Animal use and ethics statement

BALB/c, 6- to 8-week-old male mice, were bred at the University of São Paulo animal facility under specific pathogen-free conditions. All animals were handled in accordance with good animal practice as defined by the relevant national animal welfare authorities and all in vivo testing was approved by the Institutional Animal Care and Use Committee of the University of São Paulo.

2.4. Experimental protocols

Two different protocols were followed. In the first protocol, mice were immunized once/week for 4 consecutive weeks with 50 μg pcDNA3-P10 or control pcDNA3 (empty vector) or unimmunized. The immunization was intramuscular (i.m.) in the quadriceps. Seven days after the last immunization, the mice were euthanized and their spleens were removed for the analysis of $CD4^+$ $CD44^{hi}$ memory T cells and $Foxp3^+$ Treg cells. Splenocytes isolated from unimmunized mice were compared with those from pcDNA3-P10 and pcDNA3-immunized mice In the second protocol, mice were submitted to the previously described immunization protocol and were intratracheally infected with 3×10^5 *P. brasiliensis* yeast cells 7 days after the last immunization. For infection, mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and their tracheas were exposed and injected with yeast cells of the virulent strain Pb 18 in PBS in a total volume of 50 μl/mice. At day 0, 30, 60 or 120 postinfection,

the mice were euthanized and their spleens and lungs were collected in order to analyze the populations of $CD4^+$ $CD44^{hi}$ memory T cells and $Foxp3^+$ Treg cells.

2.5. Fungal burden assessment

The fungal burden was measured in infected mice by CFU (colony-forming units). At 30, 60 or 120 days after infection, mice were sacrificed and the lungs were removed. Lung sections were weighed and homogenized in 1 ml of PBS. 100 μl volumes of the homogenate were inoculated on brain heart infusion agar plates supplemented with 4% of fetal calf serum, 5% of *P. brasiliensis* (strain 192) culture filtrate and 19 UI/ml streptomycin/penicillin. The plates were incubated at 37 °C and colonies were counted after 20 days.

2.6. Histopathology

Lungs from immunized and infected mice were collected at 30, 60 or 120 days after infection, fixed in 10% formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin–eosin (HE).

2.7. Isolation of lung and spleen cells

Lungs of mice immunized and challenged with yeast cells were collected at 30, 60 or 120 days after infection, cut into small pieces and mechanically homogenized in PBS. The homogenate was filtered in gauze to remove major tissue fragments and then centrifuged at 1400 × *g* at 4 °C for 10 min [24,25]. Spleens were also excised and submitted to mechanical disruption in sterile RPMI 1640 followed by red blood cells lysis using RBC Lysis Buffer (e-Bioscience, EUA).

2.8. Splenocyte proliferation assays

Isolated splenocytes were counted in 0.1% Trypan blue and the cells were plated in 96-well flat-bottom plates at a concentration of 4×10^5 cells/well in RPMI 1640 supplemented with 20 mM $NaHCO_3$, 10 mM HEPES, 100 U of penicillin/ml, 100 mg of streptomycin/ml, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 5 mM sodium pyruvate, 100 mM nonessential aminoacids and 10% fetal calf serum. The spleen cells were cultured for 144 h at 37 °C under 5% CO^2 with the P10 peptide (1.02×10^{-2} mM) as a recall antigen [4]. Controls included cells stimulated with Concanavalin A (1 μg/well) or medium alone. Experiments were performed in triplicates. At the end of the incubation period, 50 μl of MTT (1 mg/ml) was added to each well, and the plates were incubated for another 4 h followed by the addition of 100 μl/well of 0.04 N isopropanol-HCl to dissolve the formazan crystals. The absorbance was measured at 596 nm in a microplate reader, according to a protocol adapted from Mosmann [26]. Culture supernatants were collected for cytokine detection.

2.9. Flow cytometry

Spleen and lung cells were prepared as follows. Fc receptors were blocked using anti-CD16/CD32 (clone 24G2) for 30 min at 4 °C in PBS containing 3% fetal calf serum. The cells were stained for surface molecules using the following mAbs: FITC-conjugated anti-CD4 (clone RM4-5) and PE-conjugated anti-CD44 (clone IM7) (0.5 μg/10^6 cells; Bd Pharmingen, San Diego, CA) and incubated for 30 min at 4 °C. Cells were washed with PBS containing 3% fetal calf serum and suspended in 300 μl of this buffer. For staining of Foxp3, cells were fixed and permeabilized using anti-mouse/rat Foxp3 Staining Set (e-Bioscience, San Diego, CA) and labeled with PE-conjugated anti-Foxp3 (clone FJK-16s, 0.75 μg/10^6 cell). Cell acquisition was performed using a FACScalibur flow cytometer (Pharmingen, BD) and the data collected was analyzed using FlowJo 7.2.4 software (TreeStar). FMO tubes were used as additional controls.

2.10. Cytokine detection in culture supernatants

Culture supernatants were analyzed for the presence of IFN-γ, IL-12, TNF-α, IL-10, IL-4 and TGF-β using ELISA kits (Pharmingen, San Diego, CA) according to the manufacturer's protocol. Standard curves were made using recombinant cytokines provided in the kit.

2.11. Statistical analysis

Data are presented as mean values ± SEM and were compared using one-way ANOVA test with Tukey multiple comparisons post-test. The unpaired Student's *t* test with Welch's correction (two-tailed) was used for comparison of two groups when the data met the assumption of *t* tests. The Graphpad Prism 5.0 software was used for the analysis. *P* values were considered significant when $p < 0.05$.

3. Results

3.1. Immunization with pcDNA3-P10 significantly decreased pulmonary fungal burden in immunized mice and reduced pulmonary tissue damage

We have previously shown that the DNA vaccine encoding P10 in experimental paracoccidioidomycosis [8] rendered a significant protective effect. Here, we show the effect of this vaccine on pulmonary fungal burden after 30, 60 and 120 days of infection (Fig. 1A). Although the empty vector pcDNA3 was partially protective, the pcDNA3-P10 was significantly more potent on every time interval examined. Histological analyses revealed that immunization with pcDNA3-P10 reduced the fungal burden and concomitantly promoted resolution of the pathological alterations induced by the infection (Fig. 1). On day 30 postinfection, the tissue samples harvested from both pcDNA3-P10 and pcDNA3-immunized mice displayed dense inflammatory infiltrates.

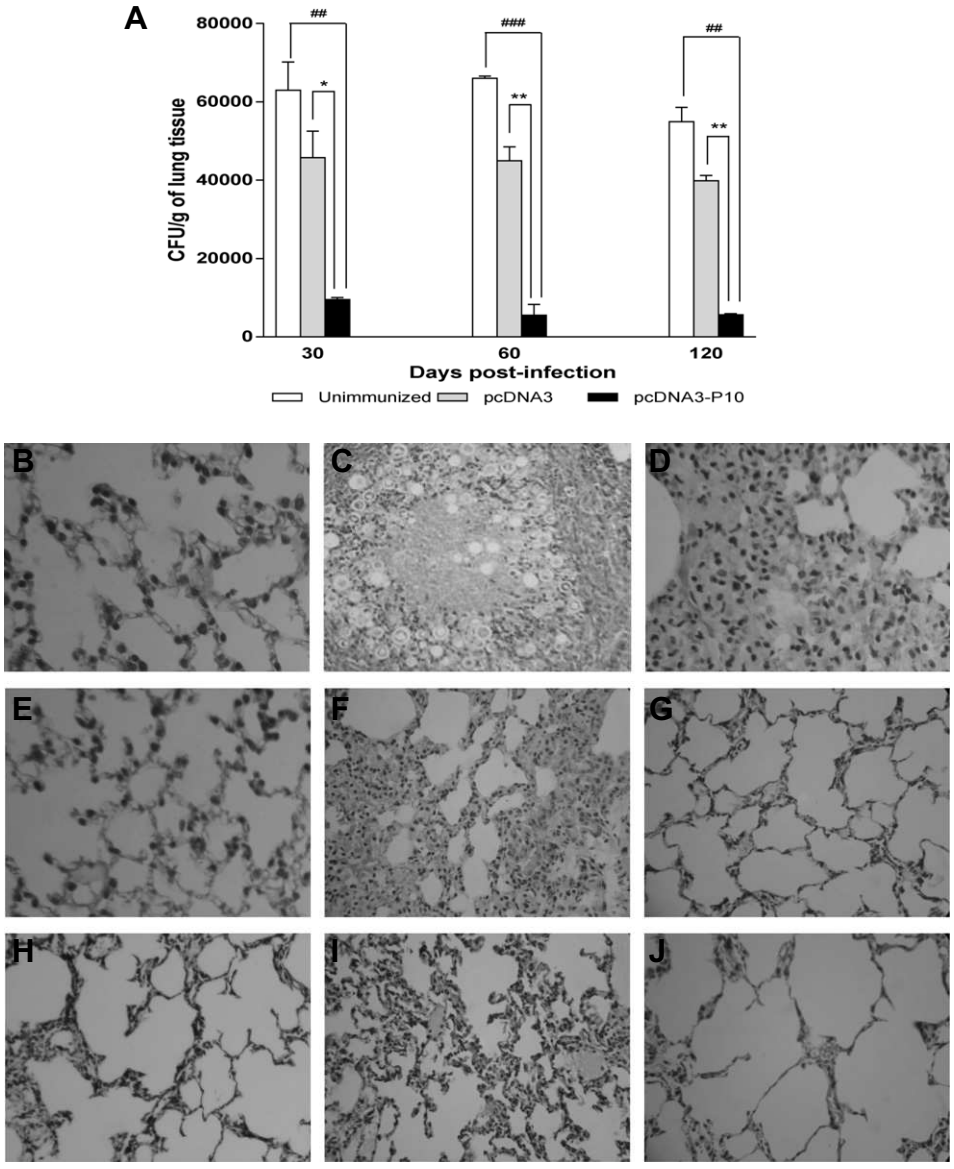

Fig. 1. Reduction of pulmonary fungal burden in pcDNA3-P10-immunized mice. A) Mice experimental groups (unimmunized, pcDNA3-P10 and pcDNA3) were challenged intratracheally with 3×10^5 *P. brasiliensis* yeast cells and sacrificed after 30, 60 and 120 days of infection. Data represent two independent experiments (total of 10 animals) with similar results and are shown as means ± SEM. * ($p < 0.05$) and ** ($p < 0.01$) comparing pcDNA3-P10 to pcDNA3 group; ## ($p < 0.01$) and ### ($p < 0.001$) comparing pcDNA3-P10 to the unimmunized group. Histology of lung sections of BALB/c mice immunized with either pcDNA3 or pcDNA3-P10 and intratracheally challenged with 3×10^5 yeast cells of *P. brasiliensis* and sacrificed after 30, 60 and 120 days of infection. Unimmunized and uninfected mice (B, E and H). pcDNA3-immunized mice at (C) 30, (F) 60 or (I) 120 days after challenge. pcDNA3-P10-immunized mice at (D) 30, (G) 60 and (J) 120 days after challenge. Slides were stained with hematoxylin–eosin; magnification 400×.

The pcDNA3-immunized mice, however, had numerous yeast cells visualized in the tissues (Fig. 1C), whereas rare yeast cells were present in tissues from pcDNA3-P10-immunized mice (Fig. 1D). Whereas tissue sections from pcDNA3-immunized mice displayed persistent dense and focal inflammation at 60 and 120 days after infection, respectively (Fig. 1F and I), lung sections from pcDNA3-P10-immunized mice showed progressively clear alveolar airspaces and minimal inflammation (Fig. 1G and J). Thus, the immunization with pcDNA3-P10 conferred protection against *P. brasiliensis* infection with a reduction in the fungal burden and a rapid resolution of pulmonary inflammation.

3.2. Immunization with pcDNA3-P10 enhances splenocyte proliferation and induces a T-cell immune response

We cultivated splenocytes from pcDNA3-P10 and pcDNA3-immunized mice and stimulated them with the P10 peptide in vitro to assess the effect of immunization on cellular proliferation. Prior to infection (day 0), the pcDNA3-P10-immunized animals showed increased splenocyte proliferation when compared to splenocytes from unimmunized group or from pcDNA3-immunized mice (Fig. 2). Similar results were obtained when the same experiment was performed with

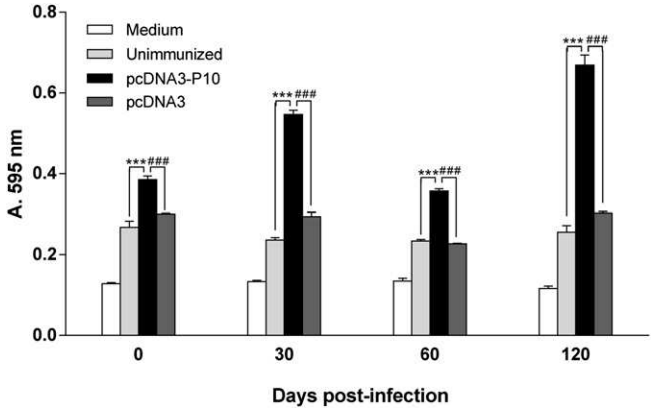

Fig. 2. Immunization with pcDNA3-P10 and P10 induced proliferation of splenocytes. Splenocytes from unimmunized, pcDNA3-P10 or pcDNA3-immunized mice were isolated on day 0, 30, 60 and 120 postinfection and incubated with the P10 peptide or medium alone for 144 h. Splenocytes cultured with Concanavalin A for 48 h gave mean absorbance values of 0.508 (data not shown). The data represent two independent experiments (total of 5 animals per group) with similar results and are shown as triplicate means ± SEM. **($p < 0.01$) and *** ($p < 0.001$) comparing pcDNA3-10 to the unimmunized group. ### ($p < 0.001$) comparing pcDNA3-10 to pcDNA3.

splenocytes obtained from unimmunized, pcDNA3 and pcDNA3-P10-immunized mice after 30, 60 or 120 days of infection. Interestingly, at 60 days after infection the splenocyte proliferation decreased to the level obtained at day 0 and then the proliferation increased to the highest level on day 120.

We also analyzed the splenocyte culture supernatants in order to evaluate the pattern of cytokines released after in vitro stimulation with P10 peptide as a recall antigen (Table 1). The immunization with pcDNA3-P10 resulted in an increase especially for IFN-γ and IL-12 but IL-10 and TNF-α also showed a small increase. When compared to unimmunized control groups the IFN-γ levels remained high in splenocytes isolated from both pcDNA3 and pCDNA3-P10-immunized mice after 30, 60 and 120 days of infection. In contrast, IFN-γ levels in unimmunized mice decreased after infection. In pcDNA3-immunized mice, IFN-γ levels increased after immunization and on days 30 and 60 after infection to a lesser degree than in the pcDNA3-P10-immunized mice. Both pcDNA3-P10 and pcDNA3 immunizations similarly induced the release of IL-12 but, although pcDNA3 treatment maintained the same response after 30 and 60 days of infection, pcDNA3-P10 immunization led to further increase in IL-12 at all time intervals after infection. Although TNF-α levels decreased in unimmunized or pcDNA3-immunized mice after infection, the pcDNA3-P10-immunized animals maintained the production of this cytokine even 60 days after infection. IL-4 levels were reduced in both pcDNA3-P10 and pcDNA3-immunized mice at 30 and 60 days postinfection, more significantly in the mice that received pcDNA3-P10. Interestingly, IL-4 levels significantly increased compared to controls in both treatment groups on day 120. The levels of IL-10 were higher in the pcDNA3-P10-immunized group at all time points analyzed compared to the unimmunized and pcDNA3 groups. The IL-10 levels increased relative to controls on day 120 after infection. TGF-β levels were similar in all groups. Taken together, these results indicate that immunization with pcDNA3-P10 can augment the proliferative capacity of splenocytes before and after infection, and demonstrate induction of a T-cell immune response characterized by the secretion of high levels of IFN-γ and IL-12.

3.3. Phenotypic $CD4^+$ $CD44^{hi}$ memory T cells are generated during immunization with pcDNA3-P10

pcDNA3-P10 immunization was able to generate memory T cells as analyzed by determining the presence of $CD4^+$

Table 1
Cytokine levels in splenocyte culture supernatants from unimmunized, pcDNA3-P10 or pcDNA3-immunized mice after stimulation with P10 peptide.

Cytokine	Experimental groups (five mice per group)	Cytokine level (pg/ml)			
		0 day inf.	30 days inf.[b]	60 days inf.	120 days inf.
IFN-γ (pg/ml[a])	Unimmunized	4070 ± 100	1102.5 ± 12.5	50 ± 40	2805 ± 15
	pcDNA3-P10	29410 ± 20 (***)	30110 ± 346 (***)	32220 ± 130 (***)	21540 ± 210 (***)
	pcDNA3	10110 ± 44 (**)	11102 ± 663 (**)	1660 ± 90 (**)	2560 ± 170
IL-12 (pg/ml)	Unimmunized	4355 ± 10	1602.5 ± 362.5	155 ± 50	3530 ± 175
	pcDNA3-P10	5680 ± 1250 (**)	21606.5 ± 733.5 (***)	16830 ± 225 (***)	14680 ± 425 (***)
	pcDNA3	5248.5 ± 125.5 (*)	5732 ± 300 (*)	6580 ± 125 (**)	3530 ± 75
TNF-α (pg/ml)	Unimmunized	488.57 ± 14.28	87. 49 ± 11.15	7.62 ± 4.76	160 ± 4.76
	pcDNA3-P10	679.04 ± 14.29	679.49 ± 86.49 (**)	688.57 ± 9.52 (***)	214.76 ± 7.14
	pcDNA3	605.08 ± 7.32	275.81 ± 24.94	281.42 ± 11.90 (**)	190.95 ± 11.9
IL-4 (pg/ml)	Unimmunized	67.84 ± 5.39	99.2 ± 1.56	119.38 ± 1.53	84.76 ± 5.38
	pcDNA3-P10	72.46 ± 2.31	2.99 ± 0.21 (***)	3.23 ± 0.77 (***)	154 ± 5.38 (**)
	pcDNA3	70.59 ± 2.06	23.72 ± 1.74 (***)	31.69 ± 4.61 (***)	255.53 ± 5.38 (***)
IL-10 (pg/ml)	Unimmunized	1775 ± 15.62	830.63 ± 30.18	787.50 ± 15.62	881.25 ± 15.62
	pcDNA3-P10	2281.25 ± 9.37 (**)	2048. 47 ± 204.52 (*)	1118.75 ± 9.37 (***)	3621.88 ± 37.5 (***)
	pcDNA3	1907.36 ± 53.04	1371.28 ± 172.64	837.50 ± 28.12	2053.13 ± 37.50 (**)
TGF-β (pg/ml)	Unimmunized	2790.5 ± 185.5	2928 ± 159	2965.5 ± 135.5	2887 ± 314
	pcDNA3-P10	3165 ± 89	3874.5 ± 112.5	3408.5 ± 121.5	2408.5 ± 78.5
	pcDNA3	3092 ± 84	3209.5 ± 222.5	2651.5 ± 292.5	3344.5 ± 314.5

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ comparing pcDNA3-P10 and pcDNA3-immunized mice to unimmunized animals.
[a] pg/ml indicates picograms per milliliter.
[b] inf. Infection.

$CD44^{hi}$ T cells in spleens by flow cytometry. The percentage of $CD4^+$ $CD44^{hi}$ T cells in the spleens from pcDNA3-P10-immunized mice progressively increased over the course of infection and the percentages were significantly greater compared to unimmunized and pcDNA3-immunized mice (Fig. 3A and C). On days 60 and 120 after infection, pcDNA3-immunized mice also had higher percentages of these cells compared to controls. Similarly, $CD4^+$ $CD44^{hi}$ T cells in the lungs of pcDNA3-P10-immunized animals increased over the course of the infection compared to unimmunized and pcDNA3-immunized groups (Fig. 3B and D) however, only at 120 days after infection the difference was statistically different. Memory T cells also significantly increased during infection in mice treated with pcDNA3 compared to unimmunized group. Thus, immunization with pcDNA3-P10 is capable of inducing phenotypic $CD4^+$ $CD44^{hi}$ memory T not only in the spleen but also in the lungs. The ability of empty vector to stimulate the immune response is probably induced by CpG sequences. In the beginning (30 and 60 days) a mixture of protective pro-inflammatory cells and Treg cells are present at higher concentration than memory cells. These experiments showed the importance of the inclusion of a group with long-term infection (120 days).

3.4. $Foxp3^+$ Treg cells are generated/expanded during immunization with pcDNA3-P10 and are recruited to the lungs during infection with P. brasiliensis

We evaluated the expansion of $Foxp3^+$ Treg cells before and after infection with *P. brasiliensis* with and without immunization using pcDNA3 or pcDNA3-P10. In unimmunized mice, the percentage of Treg cells in the spleen progressively expands. Immunization with plasmid DNA encoding P10 peptide sequence increased the percentage of $CD4^+$ $Foxp3^+$ T cells in the spleen compared to unimmunized (immunized only and at 30 and 120 days after infection) and pcDNA3-immunized (immunized only and at 30 days after infection) groups (Fig. 4A and D). The percentage of $CD4^+$ $Foxp3^+$ T cells in pcDNA3-P10-immunized mice was higher at all time points examined, although the difference was greatest on day 0 and day 30 after infection and the percentage of these cells diminished after this time point. The pcDNA3-immunized group also had an increase in the percentage of $CD4^+$ $Foxp3^+$ T cells compared to the unimmunized group, but was less than the percentage in the pcDNA3-P10-immunized mice until day 120. In the short time protocols we observed again a mixed response, the presence of Treg at the beginning of immunization followed by reduction appears to be important for reduction of tissue injury.

In contrast to the results in the spleen, pcDNA3-immunized mice had a high percentage of $CD4^+$ $Foxp3^+$ T cells in their lungs when compared to unimmunized and pcDNA3-P10-immunized mice after 30 and 60 days of infection (Fig. 5A and B). The mice immunized with pcDNA3-P10 also had increased percentages of the $CD4^+$ $Foxp3^+$ T cell compared to unimmunized mice on days 30 and 60 post-infection (Fig. 5A and B). The lower levels of Treg cell in the lung of pcDNA3-P10-immunized mice in relation to pcDNA3-immunized mice is may be mediated by cytokines produced by pro-inflammatory cells induced by the presence of P10.

4. Discussion

The need for fungal vaccines has been well defined [27]. DNA vaccines are attractive for combating mycoses since they are capable of eliciting humoral and cellular immune responses, with the latter being particularly important in fungal infections [27] including PCM [28]. Prior experimental vaccines with efficacy against *P. brasiliensis* have used plasmid DNA encoding either the gp43 [9] or the hsp65 gene from *M. leprae* [29]. We hypothesized and indeed demonstrated that a plasmid DNA containing the minigene encoding the P10 peptide, which includes the T-cell epitope of gp43 [4], could be highly effective against this deep mycosis [8]. Previously, we have shown that administration of synthetic P10 reduced the time of treatment of experimental PCM, acting as an adjuvant to standard chemotherapy [3].

Animals immunized with the pcDNA3-P10 showed a significant reduction in the pulmonary fungal burden when compared to unimmunized and pcDNA3-immunized mice after 30, 60 and 120 days of intratracheal infection. Further, immunization with pcDNA3-P10 significantly enhanced the histological resolution of the pulmonary infection.

Splenocytes from mice immunized with pcDNA3-P10 rather than pcDNA3 and unimmunized controls, isolated before or after infection and stimulated in vitro with P10, showed increased cellular proliferation at all time points. Noteworthy is that such proliferative response remained significantly high through 120 days of infection demonstrating the durability of the vaccine's effect. The high levels of IFN-γ in the culture supernatants of pcDNA3-P10-treated splenocytes compared to the other experimental groups at all time points also supports the protective capacity of our vaccine, as this cytokine activates alveolar macrophages, which are critical in the resistance to PCM [30,31]. IL-12 and TNF-α have also been associated with resistance to PCM [32,33], and the levels of these cytokines were also high in the mice immunized with pcDNA3-P10 compared to the other groups. In contrast, the pcDNA3-P10-immunized mice displayed extremely low levels of IL-4, which is associated with susceptibility to PCM [34]. The pattern of cytokines released by the splenocytes from mice immunized with pcDNA3-P10 is consistent with a Th1-biased T-cell immune response, which is predictive of a good clinical response [3,9,29].

Culture supernatants of splenocytes isolated from mice immunized with pcDNA3-P10 contained IL-10 at higher levels than the control group. In an experimental model of leishmaniasis, IL-10 is associated with persistence of the parasite [35] and anti-inflammatory responses. Notably, IL-10 modulates the differentiation process of regulatory T cells [36], and the increased secretion of IL-10 in the pcDNA3-P10-immunized group could be related to the high frequency of regulatory T cells. Mice with chronic leishmaniasis or schistosomiasis generate *n*Treg cells that are capable of secreting

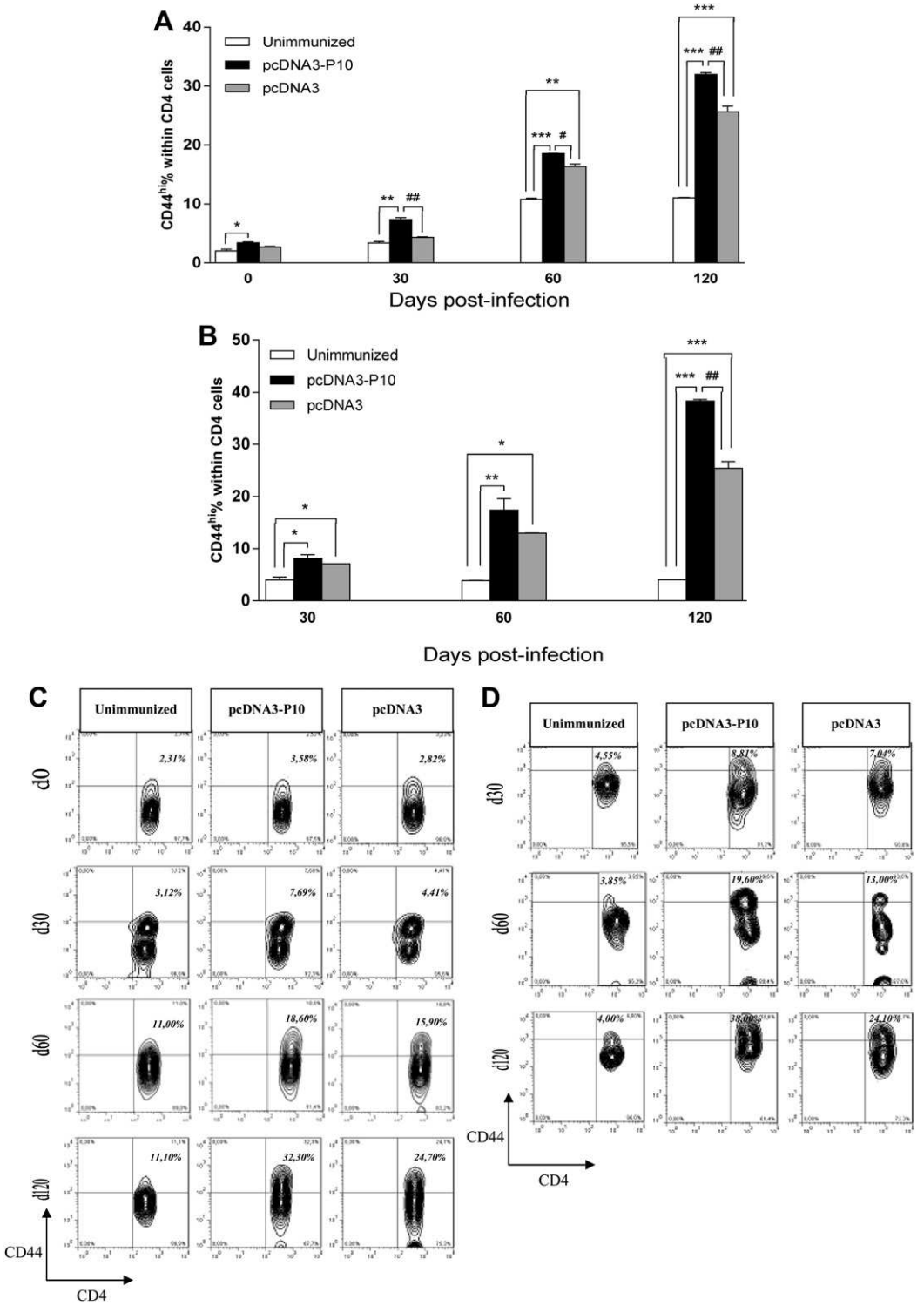

Fig. 3. Immunization with pcDNA3-P10 generates phenotypic $CD4^+$ $CD44^{hi}$ memory T cells in the spleen and lung. (A) Percentage of $CD4^+$ $CD44^{hi}$ cells in the spleens and (B) lungs of unimmunized, pcDNA3-P10 or pcDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* yeast cells. Numbers represent the means of five mice per group. The data represent two independent experiments with similar results and are shown as means ± SEM. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) comparing pcDNA3 or pcDNA3-P10 to the immunized group. # ($p < 0.05$) and ## ($p < 0.01$) comparing pcDNA3 to pcDNA3-P10. Representative counter plots demonstrate one of the experiments performed for spleen (C) and lungs (D).

IL-10 when stimulated with recall antigens [37,38]. In chronic infections, $Foxp3^+$ *n*Treg cells suppress effector immune responses against diverse pathogens, including *Candida albicans*, while contributing to their persistence in the host [39]. In the present study, we show that intratracheal infection by *P. brasiliensis* resulted in the generation/expansion of $Foxp3^+$ Tregs in the spleen and lungs of infected mice when compared to controls after 30 days of infection. Generation/expansion of

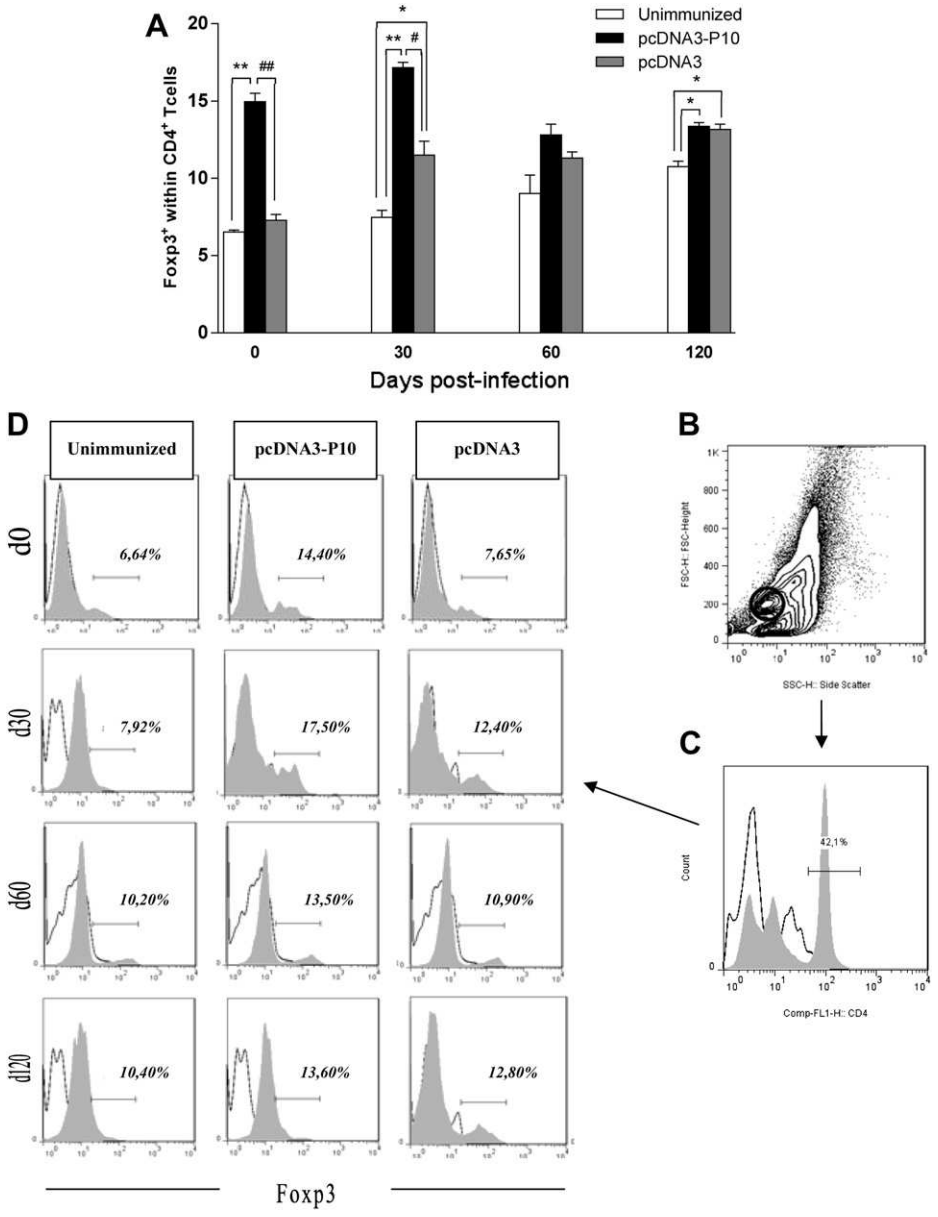

Fig. 4. Immunization with pcDNA3-P10 generates/expands $CD4^+$ $Foxp3^+$ T cells. (A) Percentage of $CD4^+$ $Foxp3^+$ cells in the spleens of unimmunized, pcDNA3-P10 or pcDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* yeast cells. Numbers represent the means of five mice per group. The data represent two independent experiments with similar results and are shown as means ± SEM. * ($p < 0{,}05$) and ** ($p < 0.01$) for pcDNA3-P10 in relation to the unimmunized group. # ($p < 0.05$) and ## ($p < 0.01$) for pcDNA3-P10 compared to pcDNA3 group. (B) Splenocytes from mice belonging to the experimental groups were gated on lymphocytes via their forward (FSC) and side scatter (SSC) properties (C) and gated on $CD4^+$ T cells. (D). Representative histograms demonstrate one of the experiments performed. The filled histograms correspond to the experimental groups and the empty histograms correspond to control FMO.

Treg cells has been shown in a broad variety of infectious diseases, as by viruses, bacteria, fungi or parasites [40–42]. In PCM, the generation of cells with the ability to suppress an effector immune response was first reported in 1988 and the effect was thought to correlate with the severity of the disease [43]. A study performed with patients suffering from the chronic form of PCM revealed that these patients had a high frequency of *n*Tregs cells in PBMC and lesions when compared to healthy individuals [44].

The impact of DNA vaccination on the long-term memory and the production of $Foxp3^+$ Treg cells, was examined in immunized and control animals before and after infection with *P. brasiliensis*. Generally, prevention of T-regulatory cells induction seems to be associated with protective immunity. Their presence, however, concomitant with repeated antigen exposure may prevent immunopathology and favor long-term memory [21].

In the present work we observed a high frequency of $Foxp3^+$ Treg cells in the spleens of mice immunized with pcDNA3-P10 compared to unimmunized and pcDNA3-immunized animals at all time points examined. In the lungs of these mice, we found that treatment with pcDNA3 induced a greater increase in $Foxp3^+$ Tregs than upon pcDNA3-P10

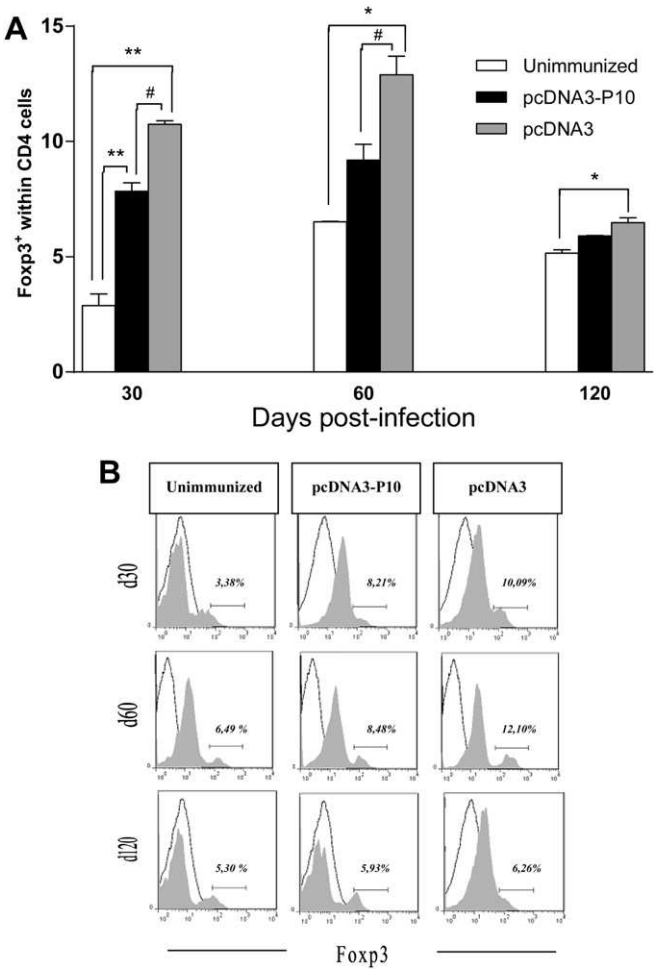

Fig. 5. $CD4^+$ $Foxp3^+$ T cells are recruited to the lungs during the course of chronic infection with PCM. Percentage of $CD4^+$ $Foxp3^+$ cells in the lungs of unimmunized, pcDNA3-P10 or pDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* yeast cells. Numbers represent the means of five mice per group. The data represent two independent experiments with similar results and are shown as means ± SEM. * ($p < 0.05$) and ** ($p < 0.01$) comparing pcDNA3 or pcDNA3-P10 to the unimmunized group. # ($p < 0.05$) comparing pcDNA3 to pcDNA3-P10. (B) Representative histograms demonstrate one of the experiments performed. The filled histograms correspond to the experimental groups and the empty histograms correspond to control FMO.

immunization, although the vaccine with P10 generated more of these cells relative to non-immunized animals. Other studies have shown that $Foxp3^+$ Treg cells are recruited to the lungs of mice with pneumocystosis and aspergillosis [45,46]. The $Foxp3^+$ Tregs cells were most likely generated in the secondary lymphoid tissues at the immunization stage with pcDNA3-P10 and recruited to the lungs after the challenge with *P. brasiliensis* where they suppress effector immune responses, thus reducing tissue damage. This hypothesis is reinforced by the histopathology which showed that pcDNA3-P10-immunized mice largely resolved their pneumonias within 60 days of infection. Treg cells are typically recruited to the lungs and other organs in response to CCL4, especially on antigen-presenting cells [47], and an efficient Treg recruitment to the lung in response to *P. brasiliensis* infection depends on CCR5 [25].

The intimate relation between Treg cells and immunological memory was first characterized in an experimental model of *Leishmania major* infection, where the presence of *n*Treg cells at the sites of infection prevented the clearance of the parasite while maintaining the immunological memory and, therefore, preventing re-infection [48]. As described, Treg cells can interfere with the host effector response and prevent the sterile cure of diverse pathogens, as demonstrated with viruses and parasites [37,49]. Nevertheless, Tregs are involved in the maintenance of immunological memory, once the pathogen is controlled, such as in a granuloma, but not eliminated. Presently, we found a high frequency of phenotypic $CD4^+$ $CD44^{hi}$ memory T cells in the spleens and lungs of mice immunized with pcDNA3-P10 compared to control and pcDNA3 groups before and after the infection at all time points. The lower numbers of $Foxp3^+$ Treg cells in the lungs of pcDNA3-P10-immunized animals could be associated with a more effective response of the $CD4^+$ $CD44^{hi}$ memory T cells in these mice. Interestingly, memory T cell expansion was also noted in spleens during infection of unimmunized animals indicating that *P. brasiliensis* infection does not suppress the generation of memory T cells. The maintenance of immunological memory seems to be closely related to the presence of Treg cells. The pcDNA3-immunized group had lower percentages of phenotypic $CD4^+$ $CD44^{hi}$ memory T cells in the lungs, but higher percentages of $Foxp3^+$ Treg cells. In fact, Treg cells can suppress other cells like $CD4^+$ $CD44^{hi}$ T cells, by inducing apoptosis mediated by cytokine deprivation [50].

In summary, our results demonstrate that vaccination with the plasmid DNA encoding the P10 peptide successfully protects mice against intratracheal infection with *P. brasiliensis* while promoting the generation of phenotypic $CD4^+$ $CD44^{hi}$ memory T cells simultaneously with the generation/expansion of $Foxp3^+$ Treg cells. Although the generation of $Foxp3^+$ Treg presumably negatively impacts on pathogen clearance, these cells contribute to reduce the immunopathology and favor long-term memory. The data strongly supports that the pcDNA3-P10 vaccine is an excellent candidate for combating *P. brasiliensis* when used in a therapeutic protocol.

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References

[1] A. Restrepo, D.L. Greer, M. Vasconcellos, Paracoccidioidomycosis: a review, Rev. Med. Vet. Mycol. 8 (1973) 97–123.
[2] M.A. Shikanai-Yasuda, F.Q. Telles Filho, R.P. Mendes, A.L. Colombo, M.L. Moretti, et al., Consenso em paracoccidioidomicose, Rev. Soc. Bras. Med. Trop. 39 (2006) 297–310.
[3] A.F. Marques, M.B. Silva, M.A.P. Juliano, L.R. Travassos, C.P. Taborda, Peptide immunization as an adjuvant to chemotherapy in mice challenged intratracheally with virulent yeast cells of *Paracoccidioides brasiliensis*, Antimicrobial Agents Chemother. 50 (2006) 2814–2819.
[4] C.P. Taborda, M.A. Juliano, R. Puccia, M. Franco, L.R. Travassos, Mapping of the T-cell epitope in th major 43-kilodalton glycoprotein of

Paracoccidioides brasiliensis which induces a Th-1 response protective against fungal infection in BALB/c mice, Infect. Immun. 66 (1998) 786–793.

[5] L.R. Travassos, G. Goldman, C.P. Taborda, R. Puccia, Insights in Paracoccidioides brasiliensis pathogenicity, in: K. Kavanagh (Ed.), New Insights in Medical Mycology, Springer, Dordrecht, The Netherlands, 2007, pp. 241–265.

[6] A.C. Amaral, A.F. Marques, J.E. Muñoz-Henao, A.L. Bocca, A.R. Simioni, A.C. Tedesco, P.C. Morais, L.R. Travassos, C.P. Taborda, M.S.S. Felipe, Poly(lactic acid-glycolic acid) nanoparticles markedly improve immunological protection by peptide P10 against murine paracoccidioidomycosis, Br. J. Pharmacol 159 (2010) 1126–1132.

[7] C.J.M. Braga, G.M.G. Rittner, J.E. Muñoz-Henao, A.F. Teixeira, L.M. Massis, M.E. Sbrogio-Almeida, C.P. Taborda, L.R. Travassos, L.C.S. Ferreira, *Paracoccidioides brasiliensis* vaccine formulations based on the gp43-derived P10 sequence and the *Salmonella enteric* FliC flagellin, Infect. Immun. 77 (2009) 1700–1707.

[8] G.M.G. Rittner, J.E. Muñoz, A.F. Marques, J.D. Nosanchuk, C.P. Taborda, L.R. Travassos, Therapeutic DNA vaccine encoding peptide P10 against experimental paracoccidioidomycosis, Plos. Negl. Trop. Dis. 6 (2012) e1519.

[9] A.R. Pinto, R. Puccia, S.N. Diniz, M.F. Franco, L.R. Travassos, DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *Paracoccidioides brasiliensis*, Vaccine 18 (2000) 3050–3058.

[10] M.A. Kutzler, D.B. Weine, DNA vaccines: ready for prime time? Natl. Rev. 9 (2008) 776–788.

[11] K.H. Ely, T. Cookenham, A.D. Roberts, D.L. Woodland, Memory T cell populations in the lung airways are maintained by continual recruitment, J. Immunol. 176 (2006) 537–543.

[12] F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cells subsets: function, generation, and maintenance, Annu. Rev. Immunol. 22 (2004) 745–763.

[13] M.K. Macleod, E.T. Clambey, J.W. Kappler, P. Marrack, CD4 memory T cells: what are they and what can they do? Semin. Immunol. 21 (2009) 53–61.

[14] R.W. Dutton, L.M. Bradley, S.L. Swain, T cell memory, Annu. Rev. Immunol. 16 (1998) 201–223.

[15] P. Scott, Immunologic memory in cutaneous leishmaniasis, Cell. Microbiol. 7 (2005) 1707–1713.

[16] A. Bozzi, B.S. Reis, M.I. Goulart, M.C.N. Pereira, E.P. Pedroso, A.M. Goes, Analysis of memory T cells in the human paracoccidioidomycosis before and during chemotherapy treatment, Immunol. Lett. 114 (2007) 23–30.

[17] S. Sakaguchi, K. Wing, M. Miyara, Regulatory T cells – a brief history and perspective, Eur. J. Immunol. 37 (2007) 116–123.

[18] Y. Belkaid, Role of Foxp3-positive regulatory T cells during infection, Eur. J. Immunol. 38 (2008) 901–937.

[19] Y. Belkaid, K. Tarbell, Regulatory T cells in the control of host-microorganism interactions, Annu. Rev. Immunol. 27 (2009) 551–589.

[20] I. Okwor, J. Uzonna, Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies, Immunol. Res. 41 (2008) 123–136.

[21] L. Romani, P. Puccetti, Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism, Trends Microbiol. 14 (2006) 183–189.

[22] A. Restrepo, M.D. Arango, In vitro susceptibility testing of *Paracoccidioides brasiliensis* to sulfonamides, Antimicrobial Agents Chemother. 18 (1980) 190–194.

[23] J.S. Handan, M.A. Resende, Lipid composition and effect of amphotericin B on yeast cells of *Paracoccidioides brasiliensis*, Mycopathologia 102 (1998) 97–105.

[24] J. Zhang, Z. Dong, R. Zhou, D. Luo, H. Wei, Z. Tian, Isolation of lymphocytes and their innate immune characterizations from liver, intestine, lung and uterus, Cell. Mol. Immunol. 2 (2005) 271–280.

[25] A.P. Moreira, K.A. Cavassani, F.S.M. Tristão, A.P. Campanelli, R. Martinez, M.A. Rossi, J.S. Silva, CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression, J. Immunol. 180 (2008) 3049–3056.

[26] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[27] A. Cassone, Fungal vaccines: real progress from real challenges, Lancet Infect. Dis. 8 (2008) 114–124.

[28] V.L.G. Calich, M. Singer-Vermes, A.M. Siqueira, E. Burger, Susceptibility and resistance of inbred mice to *Paracoccidioides brasiliensis*, Br. J. Exp. Pathol. 66 (1985) 585–594.

[29] A.M. Ribeiro, A.L. Bocca, A.C. Amaral, L.H. Faccioli, F.C.S. Galetti, C.R. Zarate-Bladés, F. Figueiredo, C.L. Silva, M.S.S. Felipe, DNAhsp65 vaccination induces protection in mice against *Paracoccidioides brasiliensis* infection, Vaccine 27 (2009) 606–613.

[30] S.S. Kashino, R.A. Fazioli, C. Cafalli-Favati, L.H. Meloni-Bruneri, C.A.C. Vaz, E. Burger, L.M. Singer, V.L.G. Calich, Resistance to *Paracoccidioides brasiliensis* infection is linked to a preferential Th1 immune response, whereas susceptibility is associated with absence of IFN-γ production, J. Interferon. Cytokine Res. 20 (2000) 89–97.

[31] G. Benard, C.C. Romano, C.R. Cacere, M. Juvenale, M.J.S. Mendes-Giannini, A.J.S. Duarte, Imbalance of IL-2, IFN-γ and IL-10 secretion in the immunosuppression associated with human paracoccidioidomycosis, Cytokines 13 (2001) 48–252.

[32] J.T. Souto, F. Figueiredo, A. Furlanetto, K. Pfeffer, M.A. Rossi, J.S. Silva, Interferon-γ and tumour necrosis factor-α determine resistance to *Paracoccidioides brasiliensis* infection in mice, Am. J. Pathol. 156 (2000) 1811–1820.

[33] C. Arruda, M.F. Franco, S.S. Kashino, F.R.F. Nascimento, R.A. Fazioli, C.A.C. Vaz, M. Russo, V.L.G. Calich, Interleukin-12 protects mice against disseminated infection caused by *Paracoccidioides brasiliensis* but enhances pulmonary inflammation, Clin. Immunol. 103 (2002) 185–195.

[34] V.L.G. Calich, S.S. Kashino, Cytokines produced by susceptible and resistant mice in the course of *Paracoccidioides brasiliensis* infection, Braz. J. Med. Biol. Res. 31 (1998) 615–623.

[35] Y. Belkaid, K.F. Hoffmann, S. Mendez, S. Kamhawi, M.C. Udey, T.A. Wynn, D.L. Sacks, The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure, J. Exp. Med. 194 (2001) 1497–1506.

[36] K. Asadullah, R. Sabat, M. Friedrich, H.D. Volk, W. Sterry, Interleukin-10: an important immunoregulatory cytokine with major impact on psoriasis, Curr. Drug Targets 3 (2004) 185–192.

[37] M. Hesse, C.A. Piccirillo, Y. Belkaid, J. Prufer, M. Mentink-Kane, M. Leusink, A.W. Cheever, E.M. Shevach, T.A. Wynn, The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells, J. Immunol. 172 (2004) 3157–3166.

[38] E. Yurchenko, M. Tritt, V. Hay, E.M. Shevach, Y. Belkaid, C.A. Piccirillo, CCR5-dependent homing of naturally occurring $CD4^+$ regulatory T cells to sites of *Leishmania major* infection favors pathogen persistence, J. Exp. Med. 203 (2006) 2451–2460.

[39] A. De Luca, C. Montagnoli, T. Zelante, P. Bonifazi, S. Bozza, S. Moretti, C. D'Angelo, C. Vacca, L. Boon, F. Bistoni, P. Puccetti, F. Fallarino, L. Romani, Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of *Rorc*, J. Immunol. 179 (2007) 5999–6008.

[40] B. Kim, N. Feng, C.F. Narvaez, X.S. He, S.K. Eo, C.W. Lim, H.B. Greenberg, The influence of $CD4^+CD25^+Foxp3^+$ regulatory T cells on the immune response to rotavirus infection, Vaccine 26 (2008) 5601–5611.

[41] M. Guilliams, G. Oldenhove, W. Noel, M. Herin, L. Brys, P. Loi, V. Flamand, M. Moser, P. Baetselier, A. Beschin, African trypanosomiasis: naturally occurring regulatory T cells favor trypanotolerance by limiting pathology associated with sustained type 1 inflammation, J. Immunol. 179 (2007) 2748–2757.

[42] A. Scholzen, D. Mittag, S.J. Rogerson, B.M. Cooke, M. Plebanski, *Plasmodium falciparum*-mediates induction of human $CD25^{hi}Foxp3^{hi}CD4$ T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGF-β, Plos Pathog. 5 (2009) e1000543.

[43] N.G.S. Mota, M.T.S. Peraçoli, R.P. Mendes, C.R. Gattass, S.A. Marques, A.M.V.C. Soares, I.C. Izatto, M.T. Rezkallah-Iwasso, Mononuclear cell subsets in patients with different clinical forms of paracoccidioidomycosis, Med. Mycol. 26 (1988) 05−111.

[44] K.A. Cavassani, A.P. Campanelli, A.P. Moreira, J.O. Vancim, L.H. Vitali, R.C. Mamede, R. Martinez, J.S. Silva, Systemic and local characterization of regulatory T cells in a chronic fungal infection in humans, J. Immunol. 177 (2006) 5811−5818.

[45] L. McKinley, A.J. Logar, F. Mcallister, M. Zheng, C. Steele, J.K. Kolls, Regulatory T cells dampen pulmonary inflammation and lung injury in an animal model of *Pneumocystis* pneumonia, J. Immunol. 177 (2006) 6215−6226.

[46] C. Montagnoli, F. Fallarino, R. Gaziano, S. Bozza, S. Bellocchio, T. Zelante, W.P. Kurup, L. Pitzurra, P. Puccetti, L. Romani, Immunity and tolerance to *Aspergillus* involve functionally distinct regulatory T cells and tryptophan catabolism, J. Immunol. 176 (2006) 1712−1723.

[47] R.S. Bystry, V. Aluvihare, K.A. Welch, M. Kallikourdis, A.G. Betz, B cells and professional APCs recruit regulatory T cells via CCL4, Nat. Immunol. 2 (2001) 1126−1132.

[48] Y. Belkaid, C.A. Piccirillo, S. Mendez, E.M. Shevach, D.L. Sacks, $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity, Nature 420 (2002) 502−507.

[49] S. Suvas, A.K. Azkur, B.S. Kim, U. Kumaraguru, B.T. Rouse, $CD4^+CD25^+$ regulatory T cells control the severity of viral immunoinflammatory lesions, J. Immunol. 172 (2004) 4123−4132.

[50] P. Pandiyan, L. Zheng, S. Ishihara, J. Reed, M.J. Lenardo, $CD4^+CD25^+Foxp3^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^+$ T cells, Nat. Immunol. 8 (2007) 1353−1362.

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DESENVOLVIMENTO DE UMA FORMULAÇÃO VACINAL EXPERIMENTAL CONTENDO PEPTÍDEOS MULTI-SOROTIPO DO VÍRUS DA DENGUE INCLUÍDOS EM PARTÍCULAS PSEUDOVIRAIS QUIMÉRICAS (VLPs) DO VÍRUS DA HEPATITE B, E TESTE EM MURINOS

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Mestre em Microbiologia.

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RESUMO

A Dengue é a doença viral infecciosa humana transmitida por artrópode de maior relevância mundial. O vírus da Dengue (DENV) é responsável por causar cerca de 50 a 100 milhões de casos anualmente e cerca de 500 mil casos das formas mais graves da doença. Apesar de alto impacto na saúde pública, as medidas profiláticas se baseiam no controle do vetor, e não existe tratamento específico da doença, fazendo com que o desenvolvimento de uma vacina, seja uma prioridade. Um dos maiores obstáculos para o desenvolvimento de uma vacina contra a dengue é que o DENV pode pertencer a quatro sorotipos (DENV-1, 2, 3 e 4) e a resposta imune induzida após infecção com um sorotipo pode não proteger, e ainda ser prejudicial, caso aconteça uma infecção por outro sorotipo. Este trabalho propôs a construção de candidatos vacinais tetravalentes baseados em dois peptídeos, conservados nos quatro sorotipos de DENV, e que representam regiões da proteína E (alvo dos anticorpos neutralizantes da infecção). Estes peptídeos foram fusionados no *loop* da proteína do core do vírus da Hepatite B (HBcAg), com a intenção de gerar partículas pseudovirais (VLPs) quiméricas que intensificassem a resposta imune. As proteínas recombinantes (HBcAg-Pep1 e HBcAg-Pep2) foram construídas em sistemas procariotos, purificadas e foram utilizadas como imunógenos vacinais separadamente ou em conjunto em camundongos C57BL/6, em protocolos do tipo dose-reforço associadas ao adjuvante Montanide ISA720. Após a primeira dose, ambas proteínas se apresentaram imunogênicas, com destaque para a HBcAg-Pep2. A segunda dose, no entanto, intensificou a resposta imune humoral de todos os protocolos vacinais. No grupo que recebeu a HBcAg-Pep2, os anticorpos específicos contra essa proteína alcançaram títulos de 1/102400, sendo que os anticorpos tipo-específicos induzidos pela HBcAg-Pep1 tiveram o título de 1/12800. Em breve serão realizados os testes funcionais das respostas imunes induzidas, incluindo ensaios de soroneutralização e de proteção contra um desafio com vírus vivo.

Palavras-chave: Dengue, formulação vacinal multi-sorotipo, proteína E, HBcAg protocolo dose-reforço

ABSTRACT

Dengue is the most relevant arthropod-borne human viral infectious disease worldwide. Dengue virus (DENV) is responsible for 50 to 100 million cases per year of the disease and 500.000 cases of the most severe forms (sometimes life-threatening) of the disease. Despite the high impact in public health, prophylactic measures are still based in controlling the mosquito vector, without any specific treatment developed to date. A vaccine is thus a priority. One of the main obstacles for developing a vaccine against dengue is the co-existence of four viral serotypes (DENV-1, 2, 3 e 4), which do not cross-protect against each other and can even act as disease-enhancers in case of an infection by a different viral serotype. In this study we have tried to develop a multivalent vaccine candidate based in two highly-conserved peptides from the sequence of the E protein (target of the virus-neutralizing antibodies). These peptides were fused to the external *loop* of the hepatitis B virus core protein (HBcAg) in an attempt to generate hepatitits B viral-like particles (VLPs) that could intensify the anti-DENV immune responses. Bacteria-produced recombinant proteins (HBcAg-Pep1 e HBcAg-Pep2) were purified and administered in Montanide ISA720 adjuvant individually or together as immunogens for C57BL/6. After the first dose, both proteins were immunogenic, in particular HBcAg-Pep2. The second dose *Boost*ed all immune responses, and groups that received HBcAg-Pep2 reached antibody titers of 1/102400, while HBcAg-Pep1 innoculated animals displayed titers of 1/12800. Regarding those animals that received HBcAg-Pep1+2 immunogens together, antibody titers reached 1/25600 and 1/1600 respectively for each immunogen. Those are promising results when considering the need of inducing high levels of antibodies to neutralize DENV. The key point now is to determine the levels of neutralizing antibodies that are present in those animals as well as their capacity to protect the animals against a challenge with live DENVs.

Key words: Dengue, multivalente vaccine formulation, E-protein, HBcAg, prime-*boost* protocol.