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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 $CD4^+$ $CD44^{hi}$ memory T cells as well as of $Foxp3^+$ 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.

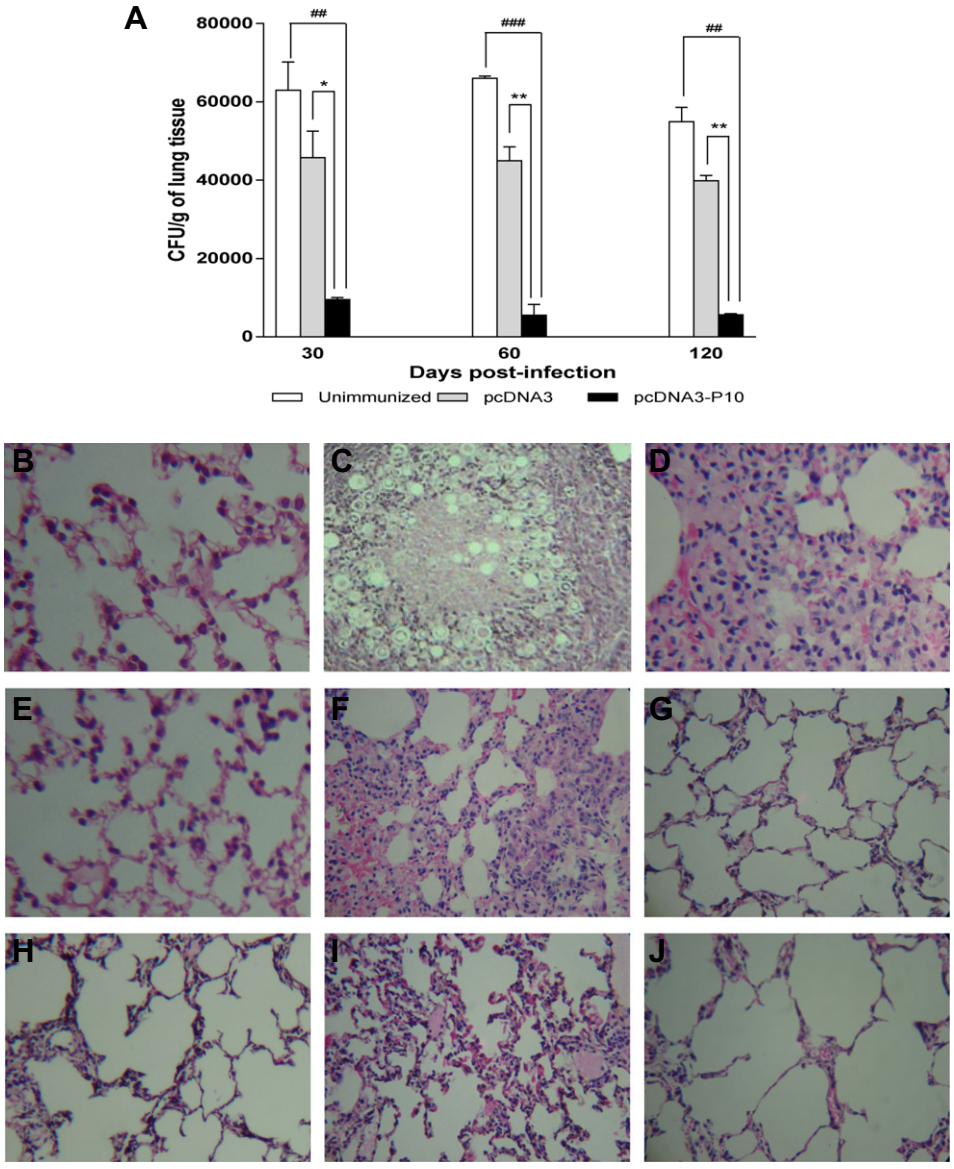

Fig. 1. Reduction of pulmonary fungal burden in pcDNA3-P10-immunized mice. A) Mice experimental groups (unimmunized, pcDNA3-P10 and pcDNA3) were challenged intratracheally with 3×10^5 *P. brasiliensis* yeast cells and sacrificed after 30, 60 and 120 days of infection. Data represent two independent experiments (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

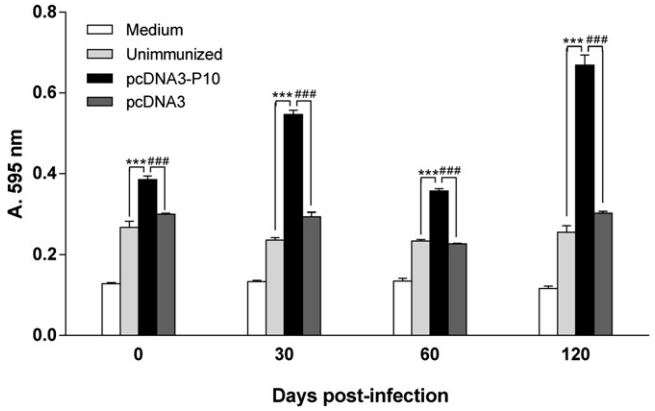

Fig. 2. Immunization with pcDNA3-P10 and P10 induced proliferation of splenocytes. Splenocytes from unimmunized, pcDNA3-P10 or pcDNA3-immunized mice were isolated on day 0, 30, 60 and 120 postinfection and incubated with the P10 peptide or medium alone for 144 h. Splenocytes cultured with Concanavalin 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

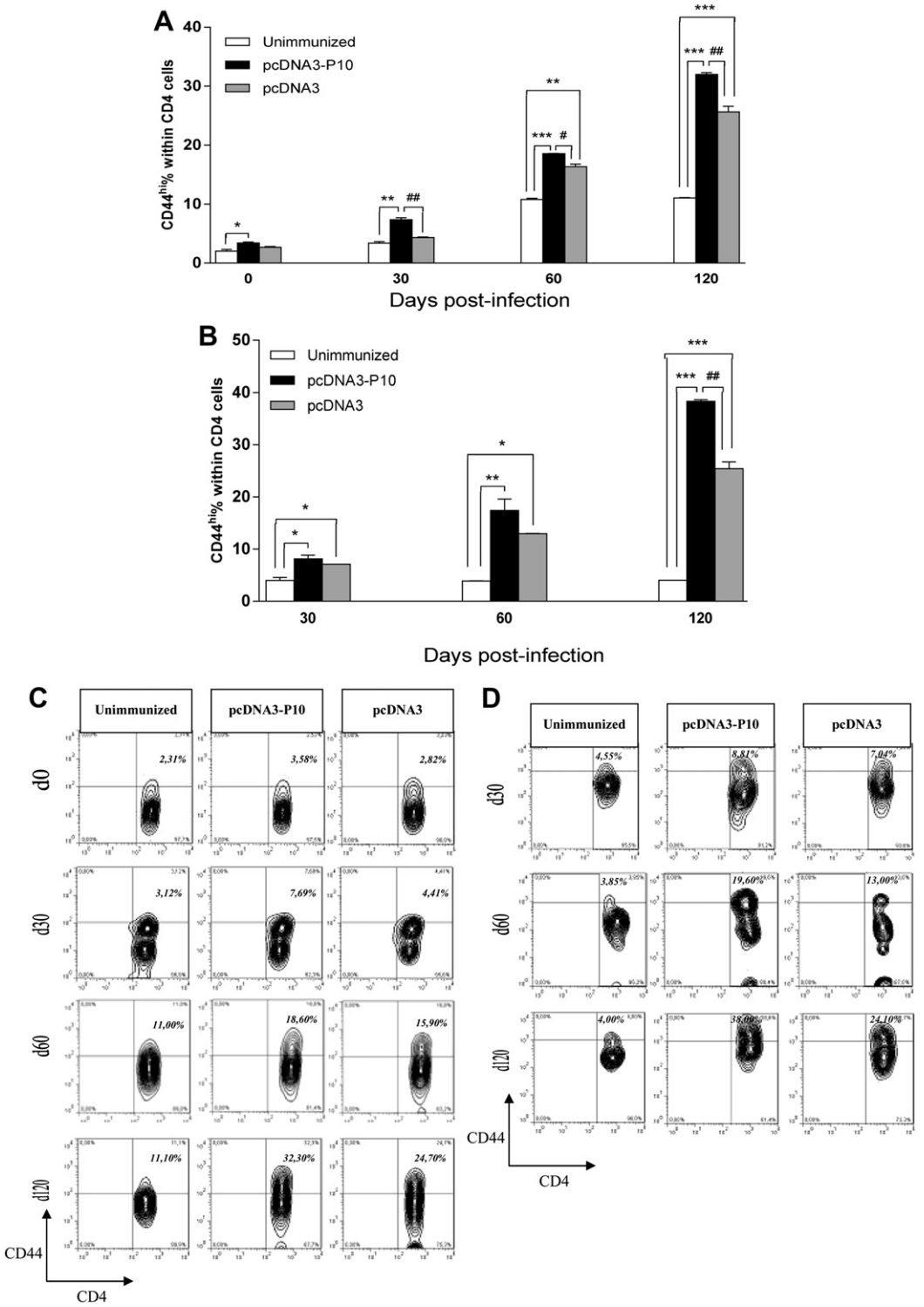

Fig. 3. Immunization with pcDNA3-P10 generates phenotypic $CD4^+$ $CD44^{hi}$ memory T cells in the spleen and lung. (A) Percentage of $CD4^+$ $CD44^{hi}$ cells in the spleens and (B) lungs of unimmunized, pcDNA3-P10 or pcDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* 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

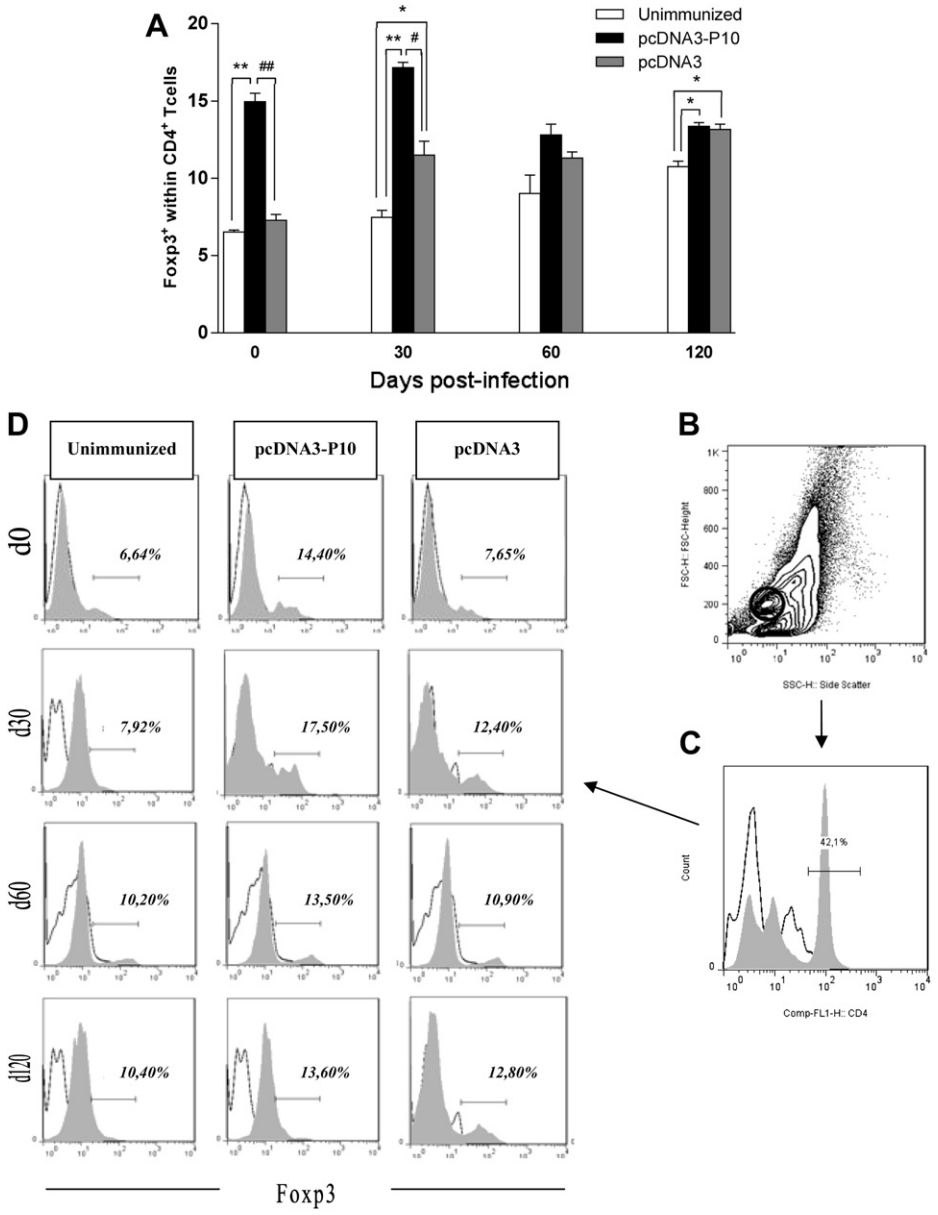

Fig. 4. Immunization with pcDNA3-P10 generates/expands $CD4^+$ $Foxp3^+$ T cells. (A) Percentage of $CD4^+$ $Foxp3^+$ cells in the spleens of unimmunized, pcDNA3-P10 or pcDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* yeast cells. Numbers represent the means 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

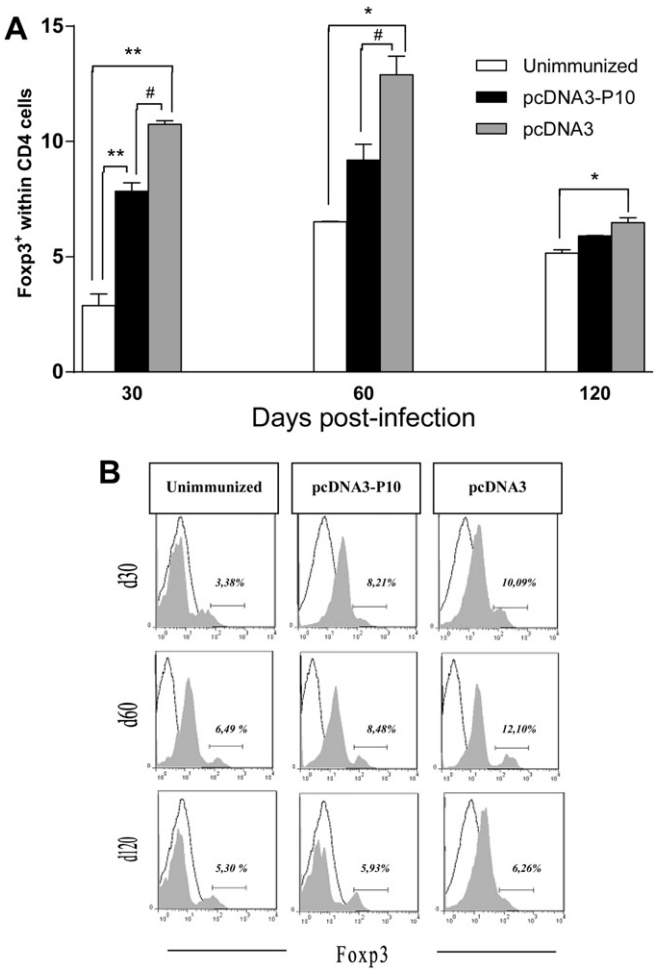

Fig. 5. $CD4^+$ $Foxp3^+$ T cells are recruited to the lungs during the course of chronic infection with PCM. Percentage of $CD4^+$ $Foxp3^+$ cells in the lungs of unimmunized, pcDNA3-P10 or pDNA3-immunized mice on day 0, 30, 60 and 120 postinfection with 3×10^5 *P. brasiliensis* yeast cells. Numbers 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.