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Therapeutic effect of DNA vaccine encoding the 60-kDa-heat shock protein from *Paracoccidioides brasiliensis* on experimental paracoccidioidomycosis in mice

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis autochthonous to Latin America and endemic to Brazil, which has the majority of the PCM cases. PCM is acquired through the inhalation of propagules of fungi from genus *Paracoccidioides* spp. and mainly affects the lungs. We have previously shown that *P. brasiliensis*-infected mice treated with single-dose of recombinant 60-kDa-heat shock protein from *P. brasiliensis* (rPbHsp60) had a worsening infection in comparison to animals only infected. In this study, we investigate whether the treatment of infected mice with *PB_HSP60* gene cloned into a plasmid (pVAX1-*PB_HSP60*) would result in efficient immune response and better control of the disease. The harmful impact of single-dose therapy with protein was not seen with plasmid preparations. Most importantly, three doses of pVAX1-*PB_HSP60* and protein induced a beneficial effect in experimental PCM with a reduction in fungal load and lung injury when compared with infected mice treated with pVAX1 or PBS. The increase of the cytokines IFN-γ, TNF, and IL-17 and the decrease of IL-10 observed after treatment with three doses of pVAX1-*PB_HSP60* appears to be responsible for the control of infection. These results open perspectives of the therapeutic use of Hsp60 in PCM.

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic infection caused by thermodymorphic fungi of the genus *Paracoccidioides.* The infection begins when the host inhales airborne fungal propagules, such as conidia or mycelial fragments, from the environment. In the lung, the fungus modifies its hyphae form to yeast one in response to the body temperature of the host [1,2], and triggers a chronic granulomatous inflammatory reaction in the host [3].

Although PCM is endemic in Latin America, from southern Mexico to northern Argentina, its distribution in these areas is not uniform [4]. In Brazil, which has the most of the cases, PCM is the eighth most common cause of death among chronic and recurrent infectious diseases, has a mortality rate higher than leishmaniasis, besides to be considered the systemic mycosis with the highest mortality rate [5].

Besides to require a long-lasting therapy, the treatment for PCM has the potential to trigger undesirable side effects. Therefore, some patients abandon the treatment, makes decreases the chances for the successful outcome [6]. Hence, several groups have been trying to find immunotherapy strategies for experimental PCM [7–9], such as the studies with a peptide of 15 amino acids derived from gp43, the most studied antigen of *P. brasiliensis*, called peptide P10, which presented as a good candidate in the therapy [10] even when was used the therapy strategy with dendritic cells primed with p10 [11]. Promising results also were obtained when recombinant Pb27 and Pb40 from *P. brasiliensis* were used for the treatment of experimental PCM in mice [12].

Our group has also been seeking in latest years to research the use of antigen- and adjuvant-based therapies in PCM [7]. Among the evaluated molecules, 60-kDa-heat shock protein from P. *brasiliensis* (PbHsp60) in either the native or recombinant form (rPbHsp60) induced a detrimental effect on PCM when a single-dose of them was administered therapeutically in infected mice.

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Antigen-treated mice had a significant increase in the fungal load, greater lung injury, and dysregulation of the immune system with production of large amounts of cytokines when compared to infected mice treated with vehicle only [8].

Although our previous results have shown that a single dose treatment with rPbHsp60 had a negative effect on PCM [8], we have not ruled out the possibility that other strategies could be useful. This idea is based on the positive impact on experimental PCM when Soares et al. [13] used rPbHsp60 prophylactically at three doses, and Ribeiro et al. [14] treated animals infected with a DNA vaccine encoding the 65-kDa heat-shock protein of *Mycobacterium leprae* (pVAX1-*ML_HSP65*). These studies motivated us to evaluate whether the use of pVAX1 subcloned with the gene of PbHsp60 (pVAX1-*PB_HSP60*) as therapeutic vaccine and the three-dose therapy of pVAX1-HSP60 or recombinant protein would result in a positive effect on the control of experimental PCM.

2. Materials and methods

2.1. Animals

Six-to-eight-week-old male BALB/c mice were obtained from the Central Animal House of the University of São Paulo (USP), Campus Ribeirão Preto and maintained under standard laboratory care in the Animal Facilities of the Department of Biology, Faculty of Philosophy, Sciences, and Letters of Ribeirão Preto, USP. All procedures were performed in accordance by the Guide for the Care and Use of Laboratory Animals of the National Research Council and approved by the Ethics Committee on Animal Use of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, USP, protocol 15.1.1770.59.0.

2.2. P. brasiliensis yeast cells

Virulent *P. brasiliensis* strain (Pb18) was kindly provided by Prof. Dr. Roberto Martinez from the Department of Internal Medicine, FMRP, USP, and maintained in DMEM medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 4% (v/v) of heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) for 7 days at 37 °C. The isolate virulence was maintained by periodical infection of *P. brasiliensis* yeasts in mice with subsequent recovery in the brain heart infusion agar (BHI, Sigma-Aldrich, St. Louis, USA) supplemented with 4% (v/v) of heat-inactivated FBS and 100 μg of ampicillin per mL for 7 days at 37 °C.

2.3. Cloning of the PB_HSP60 gene from Pb18 into pVAX1

The expression vector pET28a containing the complete gene of Hsp60 from *P. brasiliensis* (*PB_HSP60*) was used as a template to amplification and further subcloning into pVAX1 vector [8]. The amplifications were performed by polymerase chain reaction (PCR) using the following primers, forward: 5′-GCATATAAGCTTAGCATGGCACAGCGAGCTTTTACTTCCT-3′, reverse 5′- CGATATGAATTCCTACTAGAACATACCCCCGCCCATAC-3′. The addition of the Kozak sequence to the forward primer resulted in an extra "G" base after start codon. We inserted two bases (CA) to restore the reading frame, making an additional codon and consequently a new amino acid alanine as the second amino acid of the PbHsp60. PCR was performed using High Fidelity DNA Polymerase (5U/μL) (Thermo Fisher Scientific) in a C1000 Thermal Cycler (Bio-Rad Laboratories, Foster City, USA). The conditions of the reactions were: 95 °C for 2 min for template DNA denaturation and DNA activation followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 40 s, and extension at 72 °C for 2 min. Afterward, the reactions were maintained for 5 min at 72 °C. Amplification was confirmed by 0.8% agarose gel. The PCR products were subcloned into pVAX1 cloning vector following a restriction enzyme digestion using *Eco*RI and *Hind*III (Promega, Madison, USA) restriction sites that were designed into the PCR primers. Empty pVAX1 (control) and pVAX1-*PB_HSP60* were electroporated into *Escherichia coli* DH10b in a 2-mm cuvette under the following conditions: 2.5 kV, 25 μF and 200 Ω (Gene Pulser Xcell, Bio-rad, Hercules, USA). The positive transformed bacteria were selected on LB agar containing 50 μg/mL kanamycin and identified by PCR amplification and restriction enzyme digestion. All positive clones were sequenced at the Center for Human Genome Studies, Institute of Biosciences, USP, to confirm the reading frame of the cloned gene. The plasmids were purified with Endofree Plasmid Giga kit (Qiagen, Valencia, USA) following the manufacturer's protocol and suspended in sterile endotoxin-free PBS. All plasmid preparations had less than 0.1 EU/mg of the DNA, as determined using the QCL 1000 Limulus amebocyte Lysate (Lonza, Walkersville, USA).

2.4. Transfection of HEK293 cells with pVAX1-PB_HSP60 and production of antibodies against PbHsp60 and Western blot

For antibody production, four BALB/c mice were injected intramuscularly with 100 μg of pVAX1-*PB_HSP60* and two booster doses, at a 15 days interval. Seven days after the last immunization, the antisera from the animals were obtained, pooled and stored at −20 °C until to be used in the Western blot experiment. As negative controls, we used sera from animals injected with pVAX1 only and the pre-immune serum from the immunized mice.

Expression of PbHsp60 in eukaryotic cells (HEK293T cells, ATCC-293 T) was evaluated by coprecipitation of the DNA with calcium phosphate [15]. Twenty four hours after transfection, the cell suspensions were lysed with sterile frozen distilled water and centrifuged at 10,000 × *g*, for 10 min. The cell pellets were washed twice with phosphate buffered saline (PBS) and mixed with cell lysis buffer and loading buffer, and then boiled. Cells lysate proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using the Mini-Protean Tetra System (Bio-Rad Laboratories, Richmond, USA), following the manufacturer's instructions.

After blocking with 5% nonfat dry milk in Tris buffer [20 mM Tris, 150 mM NaCl, pH 7.6, and 0.05% Tween20), at 4 °C, for 16 h, the membranes were washed and incubated at room temperature with a pool of serum samples collected from naïve mice (pre-immune), pVAX1-injected mice, or pVAX1-*PB_HSP60*-injected mice. All serum pools were diluted 1:1000 in 5% skimmed milk in Tris buffer. After 4 h, the membranes were washed and incubated with peroxidase-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) at room temperature for 2 h. Antigen-antibody reactions were visualized with the 3,3′-diaminobenzidine (DAB)-hydrogen peroxide (Sigma-Aldrich).

2.5. Paracoccidioidomycosis mouse model

Mice were infected intratracheally with 100 μL of a cell suspension of 3×10^6 yeasts per mL. On day 21 postinfection, animals were divided into five groups of eight mice each and treated with one of the following preparations: pVAX1-*PB_HSP60*, pVAX1-*ML_HSP65*, pVAX-1 (plasmid control), rPbHsp60, or PBS. One hundred μg of plasmid DNA or 50 μg of rPbHsp60 both in 100 μL preparation was injected intramuscularly or subcutaneously, respectively. The protocols of treatment were composed of the administration of single-dose or three-doses at a 15 days interval. Thirty days after of the single- or last dose, the mice were euthanized for determination of fungal load and concentrations of cytokines and histological analysis. In the survival experiments,

three groups of 30 mice each were treated with three-dose of plasmids or PBS, as described above, on day 21 after intranasal infection with 10 μL of a cell suspension of 3×10^9 yeasts per mL. The animals were monitored daily for survival until the experiment was stopped on day 100 postinfection.

For determination of fungal load, the left lungs were disrupted in 1 mL sterile PBS using a tissue homogenizer (Ultra-Turrax T25 Basic IKA Works, Inc., Wilmington, USA). Lung homogenates were plated on BHI agar supplemented with 4% (v/v) of heat-inactivated FBS and 100 μg of ampicillin per mL plates in a series of diluted solutions and incubated for 7 days at 36 °C. The colony-forming units (CFU) were counted and determined as CFU/g tissue. Remaining lung homogenates were centrifuged at 5000 × *g* for 10 min at 4 °C and the supernatants stored at −20 °C until determination of cytokine concentrations. Concentrations of IL-4, IL-6, IL-10, IL-17, TNF, and IFN-γ in the lung homogenates were measured by performing capture BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD PharMingen, San Diego, USA), according to the manufacturer's protocol.

2.6. Histology

The right lungs were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. The lung sections (thickness, 5 μm) were stained with hematoxylin and eosin (H&E) by using standard protocols and analyzed by light microscopy. For morphometric measurements, images were made in the whole stained lung in a magnification of 100× using a Zeiss Axiophot photomicroscope using (Carl Zeiss, Jena, Germany) coupled with JVC TK-1270 camera (Victor Company of Japan Ltd, Tokyo, Japan). The total area of the tissue sections and inflammatory infiltrates per lung section was estimated using image analysis software (ImageJ 1.37v; National Institutes of Health, Bethesda, USA).

2.7. Statistical analysis

The difference between means was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Therapy delivery system

The success in the therapeutical use of a plasmid preparation pVAX1 subcloned with *HSP65* gene from *M. leprae* in *P. brasiliensis*-infected mice [14] allowed us to suggest that a cross-reactive immune response might have been triggered against an Hsp65 ortholog from *P. brasiliensis.* To point out most similar proteins from *P. brasiliensis* to Hsp65 from *M. leprae*, we blasted the primary sequence of Hsp65 (Genbank accession number AAA25354) against non-redundant protein sequences at NCBI using the standard protein-protein BLAST program (blastp) on *Paracoccidioides* (taxid:38946) database. Hsp65 from *M. leprae* showed higher scores and 49% identity with Hsp60s from *P. lutzii* (former strain Pb01 from *P. brasiliensis*) and *P. brasiliensis* Pb18 and Pb03 strains (Table 1). Furthermore, the multiple alignment analysis revealed that Hsp65 was highly related to Hsp60 from the *Paracoccidioides* species with many fully-conserved amino acid sequences (Fig. S1). On the basis of these data, we subcloned *PB_HSP60* gene from Pb18 strain into pVAX1 and evaluated this DNA vaccine as a treatment against experimental PCM.

Before starting the therapy experiments, we first determined the expression *in vitro* of pVAX1-*PB_HSP60* in mammalian cell line HEK293. The Western blot analysis of lysates of pVAX1-*PB_HSP60*-transfected HEK293 cells revealed the expression of PbHsp60 with the expected molecular mass, 60-kDa. When compared to bacterial recombinant protein, PbHsp60 expressed in HEK239 cells had a lower molecular mass, probably due to the presence of many additional amino acids in heterologous protein expressed in bacteria. These extra amino acids translated from the codons expressed from the polylinker region of the vector pET28a as well as of C-terminal six histidine residues were not present in the pVAX1-

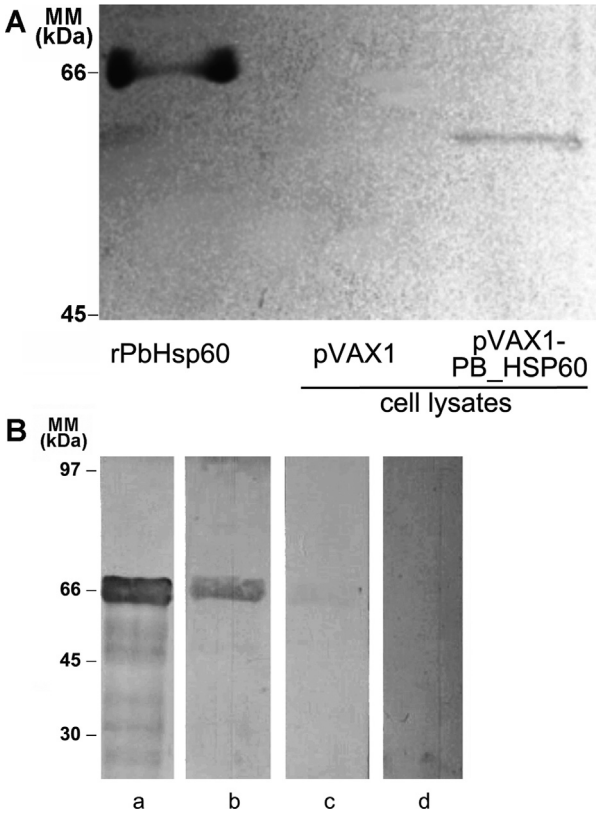

Fig. 1. pVAX1-*PB_HSP60* induces the expression of PbHsp60 in plasmid-transformed HEK293 cells and generates a specific immune response in plasmid-immunized mice. (A) HEK293 cells were transfected with pVAX1-*PB_HSP60* or empty vector (pVAX1) for 24 h. Whole cell lysate proteins or rPbHsp60 (positive control) were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with mouse polyclonal anti-rPbHsp60 antiserum. (B) Samples of rPbHsp60 separated by 12% SDS-PAGE and transferred to nitrocellulose membranes were probed with a pool of antisera from mice immunized with (a) rPbHsp60 or (b) pVAX1-*PB_HSP60*, (c) pre-immune sera from pVAX1-*PB_HSP60*-immunized mice, or (d) empty vector (pVAX1). The antigen-antibody reactions were revealed using peroxidase-conjugated rabbit anti-mouse IgG, hydrogen peroxide and DAB.

Table 1
Hsp65 from *M. leprae* (accession ID AAA25354) with closest hits by using BLASTP in *Paracoccidioides* database.

Accession ID	Definition	Score	Query cover	E-value	Identity
XP_002789992	Heat shock protein [*P. lutzii* Pb01]	487	96%	2×10^{-167}	49%
XP_010763632	hsp60-like protein [*P. brasiliensis* Pb18]	486	96%	8×10^{-167}	49%
EEH17213	hsp60-like protein [*P. brasiliensis* Pb03]	486	96%	1×10^{-166}	49%

PB_HSP60 construct. The cells transfected with the empty vector, as expected, did not reveal bands (Fig. 1A).

Since the plasmid preparation encoded the protein in mammal cells, next, we evaluated whether pVAX1-*PB_HSP60* would be capable of generating an antigen-specific immune response in mice. For this purpose, four animals were immunized with three doses of 100 μg of pVAX1-*PB_HSP60* at a gap of 15 days between each treatment. Sera from the immunized mice were pooled and tested by Western blot for the presence of anti-PbHsp60 IgG. The pool of sera from mice immunized with pVAX1-*PB_HSP60* or recombinant protein showed strong reactivity with rPbHsp60. As expected, the pool of sera from either pre-immune or empty vector-injected mice did not generate antibodies against rPbHsp60 (Fig. 1B).

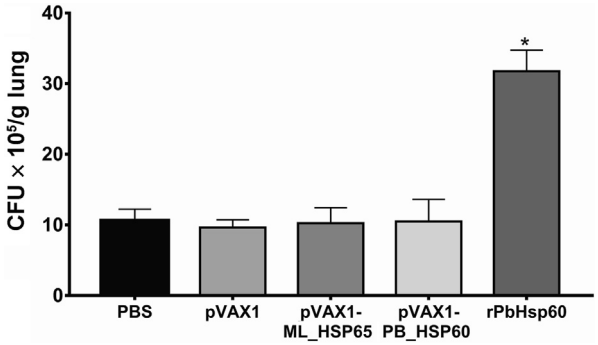

Fig. 2. Single-dose therapy with pVAX1-*PB_HSP60* in *P. brasiliensis*-infected mice did not induce a change in the fungal load. BALB / c mice were infected via i.t. with 3×10^5 P. brasiliensis yeasts and after 21 days divided into 5 groups (n = 8). The mice groups were treated with PBS or empty vector (pVAX1) as controls, pVAX1-*PB_HSP60*, pVAX1-*ML_HSP65* or rPbHsp60. On day 30 posttreatment, mice were euthanized, lungs were removed, and tissue fragments were homogenized for CFU counting. The bars represent the mean ± standard deviation of the CFU/g tissue. Asterisk indicates $p < 0.05$ relative to the control groups PBS and pVAX1.

3.2. Therapy with three doses of pVAX1-PB_HSP60 has a positive effect in P. brasiliensis-infected mice

To determine whether pVAX1-*PB_HSP60* had any effect on experimental PCM, we initially used the preparations in a single-dose therapy. Unlike the single-dose therapy with recombinant protein, which increased the fungal burden and the lung injury in *P. brasiliensis* infected mice, the pVAX1-*PB_HSP60* treatment did not induce a different outcome from that observed in infected mice treated with vehicle, pVAX1 only, or pVAX1-*ML_HSP65* (Fig. 2). The higher fungal load in infected mice treated with rPbHsp60 was followed by a significant increase in the pulmonary concentrations of IL-6, IL-17, and IL-10 when compared with the animals of the control groups (Fig. 3).

Remarkable results were obtained when we treated the infected animals with three-doses of pVAX1-*PB_HSP60*, as well as pVAX1-*ML_HSP65* or rPbHsp60. There was significantly decreased the fungal burden in the lung when compared to that obtained from control infected mice treated with pVAX1 only or PBS (Fig. 4). Histological analysis of lung sections showed that infected mice treated with three doses of pVAX1-*PB_HSP60*, pVAX1-*ML_HSP65* or rPbHsp60 (therapeutic preparations) presented compact granulomas, a reduction of inflammatory infiltrates and a

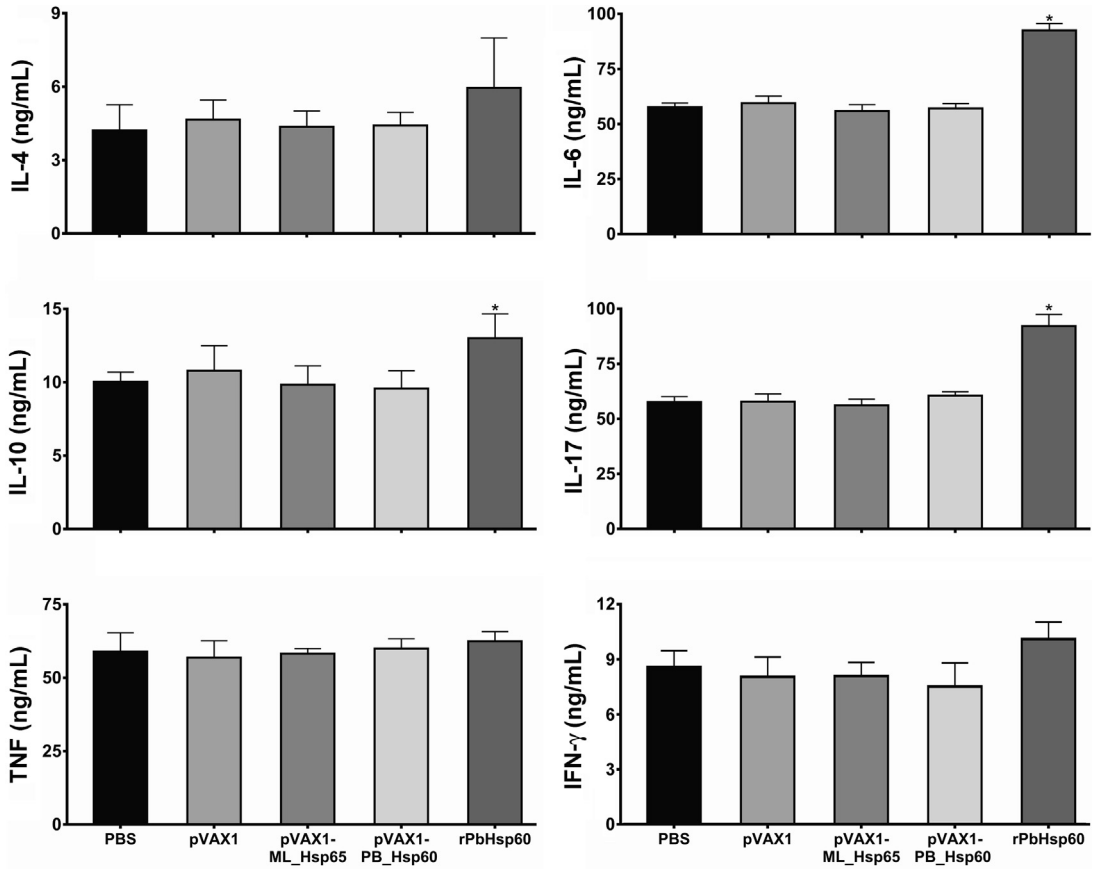

Fig. 3. Cytokine profile of infected mice treated with single-dose therapy. Concentrations of IL-4, IL-6, IL-10, IL-17, TNF, and IFN-γ were determined in the homogenates from remaining lung homogenates used to CFU analysis described in the legend of Fig. 2. Assays were performed in triplicate, and the results represent the mean ± standard deviation of at least three independent experiments. Asterisk indicates $p < 0.05$ relative to the control groups PBS and pVAX1.

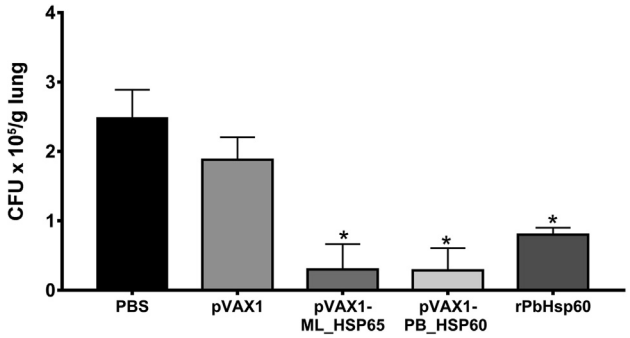

Fig. 4. Treatment of *P. brasiliensis*-infected mice with three doses of pVAX1-*PB_HSP60* induces a reduction in pulmonary fungal load. BALB/c mice were infected via i.t. with 3×10^5 *P. brasiliensis* yeasts and after 21 days divided into 5 groups (n = 8). The mice groups were treated with three doses of PBS or empty vector (pVAX1) as controls, pVAX1-*PB_HSP60*, pVAX1-*ML_HSP65* or rPbHsp60, at an interval of 15 days between each treatment. On day 30 posttreatment, mice were euthanized, lungs were removed, and tissue fragments were homogenized for CFU counting. The bars represent the mean ± standard deviation of the CFU/g tissue. Asterisk indicates $p < 0.05$ relative to the control groups PBS and pVAX1.

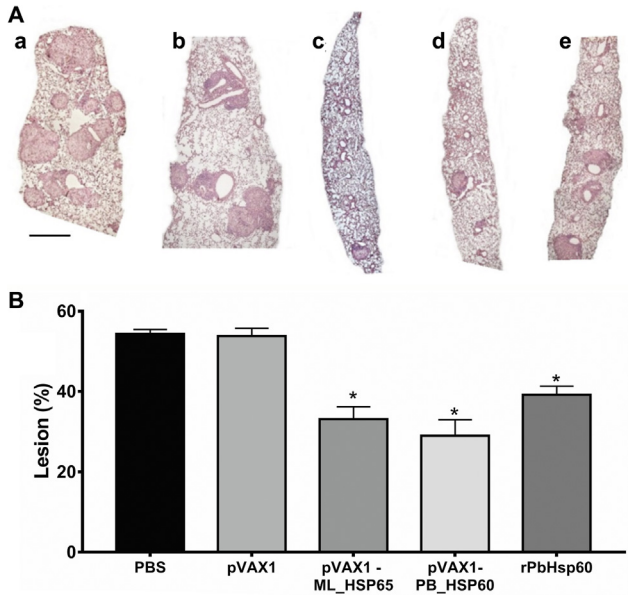

Fig. 5. Three-doses therapy of pVAX1-*PB_HSP60* in *P. brasiliensis*-infected mice decreases lung injury. (A) BALB/c mice were infected and treated as described in the legend of Fig. 5. Lung sections from *P. brasiliensis*-infected mice were obtained on day 30 after treatments and stained with hematoxylin-eosin: Treatments: (a) PBS, (b) pVAX1, (c) pVAX1-*ML_HSP65*, (d) pVAX1-*PB_HSP60*, and (e) rPbHsp60. Scale bar on panel A indicates 1 mm. **(B)** Morphometric analyses were performed using lung sections. Percentage lesion areas of the tissue sections per lung section were estimated using image analysis software. Data are expressed as mean ± standard deviation. Asterisk indicates $p < 0.05$ relative to the control groups PBS and pVAX1.

decrease in the lesion area when compared to the control infected mice treated with PBS or empty pVAX1 vector (Fig. 5A). When lung slides were microscopically scanned and the lesions quantified using image analysis software, we observed that groups treated with one of the therapeutic preparations had a significant reduction in the relative quantity of lesions when compared with the control groups (Fig. 5B).

The reduction of the fungal load and inflammatory infiltrates observed in the infected mice treated with pVAX1-*PB_HSP60* was followed by a change in the immune response, with a significant increase in the pulmonary concentrations of IFN-γ, TNF, IL-6, and IL-17 and decreased in the levels of IL-10 when compared with the animals of the control groups. The concentrations of the other evaluated cytokines had no significant changes (Fig. 6).

The beneficial effect of pVAX1-*PB_HSP60* on experimental PCM made us hypothesize that the construct could decrease the mortality rate of infected animals with a lethal dose of virulent *P. brasiliensis*. Indeed, all of the animals treated with pVAX1-*PB_HSP60* survived to the challenge up to 100 days postinfection, when the experiment was interrupted. The whole group of mice treated with PBS or pVAX1 dead up to 90 days postinfection (Fig. 7).

4. Discussion

In the current study, we reported that the therapy with three-doses of DNA vaccine pVAX1-*PB_HSP60* or pVAX1-*ML_HSP65* or rPbHsp60, but not single-dose, in *P. brasiliensis*-infected mice induced a positive effect on infection control. In the infected mice treated with these formulations, there was a significant decrease in pulmonary fungal load and tissue injury, an increase in the concentrations of Th1/Th17 cytokines and a reduction in IL-10 in the lungs when compared with controls. Moreover, pVAX1-*PB_HSP60* protected mice of succumbing to infection with a lethal dose of *P. brasiliensis* for up to 100 days. This effect was independent of possible contamination with endotoxin since pVAX1-*PB_HSP60* was prepared in the same manner as control pVAX1 and the results of both were different. Therefore, this effect is directly linked to the *PB_HSP60* gene in pVAX1.

Previously, the effect of single-dose of rPbHsp60 was evaluated as a therapy in the experimental PCM, and an unexpected negative impact was observed with increased fungal load and lung lesions [8]. Even the positive effect of treatment with complete's Freund adjuvant on experimental PCM, described by De Oliveira et al. [16] was reversed when rPbHsp60 was concomitantly administrated. Here, we showed this harmful treatment again and attempted to evaluate whether another form of PbHsp60 delivery in the immunization process could have a better effect than that observed with immunization with recombinant protein. For this purpose, therapeutic DNA vaccine was chosen because Ribeiro et al. [14] obtained good results with preparation of plasmid pVAX1 subcloned with the *M. leprae HSP65* gene, which is an ortholog of *HSP60* from species of *Paracoccidioides*. We think the success of therapy with pVAX1-*HSP65* was probably due to raised identity percentage and high homology at many sequences of amino acids between Hsp65 from *M. leprae* and Hsp60s from *Paracoccidioides* spp., when analyzed using BLASTP and ClustalW.

Unlike recombinant protein that worsened the infection, the single-dose therapy with pVAX1-*PB_HSP60* or pVAX1-*ML_HSP65* had no effect in *P. brasiliensis*-infected mice regarding the number of CFU, lung lesion and cytokine pattern in comparison to controls treated with vehicle or empty plasmid. When the infected mice were treated with three doses of pVAX1-*PB_HSP60* or pVAX1-*ML_HSP65*, we noticed a significant reduction in both fungal load and lung lesions when compared to PBS or empty vector treated controls. By these results, the protective role of the pVAX1-*PB_HSP60* preparation used in three doses is highlighted, since it protected all animals previously infected with a lethal dose of *P. brasiliensis* for up to 100 days. In contrast, about 90 days after infection, all animals in the control groups that received PBS and empty pVAX1 had already died. This new delivery protocol with three doses of DNA vaccine caused significant improvement of infection control in mice that received the treatment and allowed to question whether the same could occur with the recombinant protein. Unexpectedly, three doses treatment with rPbHsp60 decreased fungal load and lung injury when compared to the con-

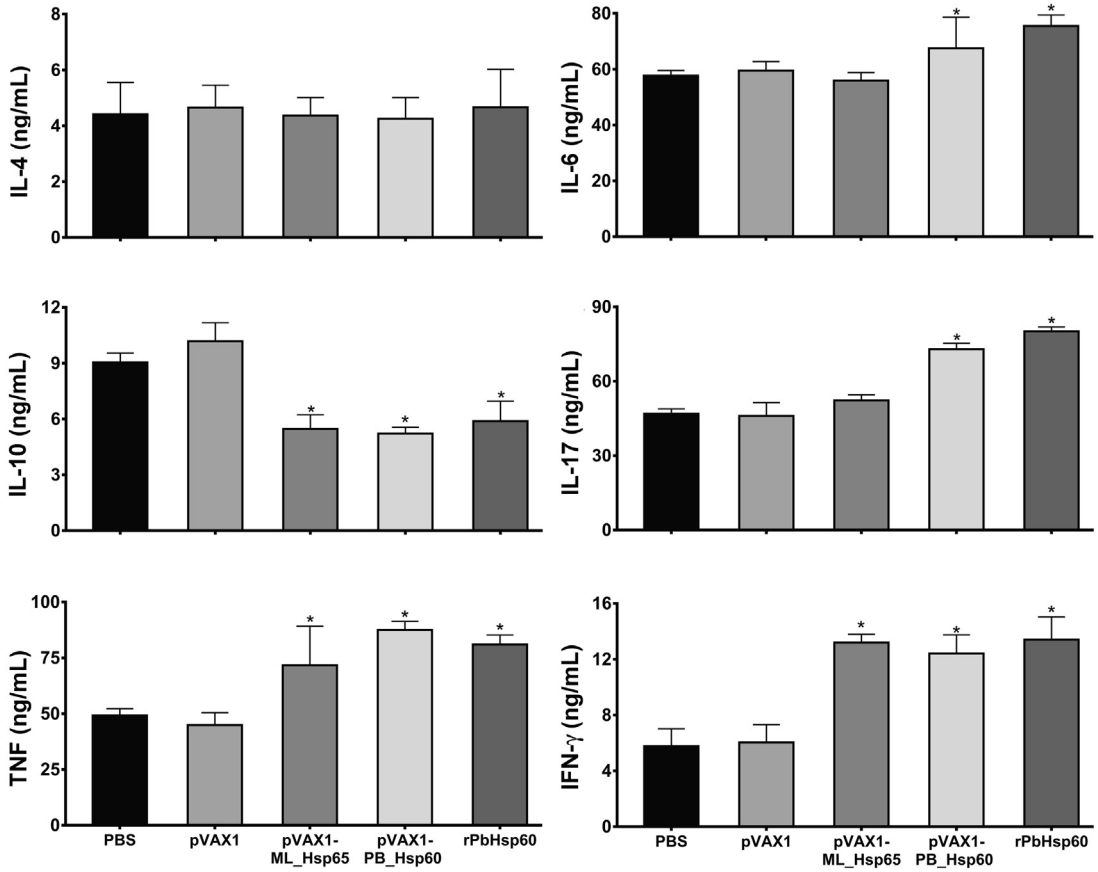

Fig. 6. Cytokine profile of infected mice treated with three-doses therapy. Concentrations of IL-4, IL-6, IL-10, IL-17, TNF, and IFN-γ were determined in the homogenates from remaining lung homogenates used to CFU analysis described in the legend of Fig. 5. Assays were performed in triplicate, and the results represent the mean ± standard deviation of at least three independent experiments. Asterisk indicates $p < 0.05$ relative to the control groups PBS and pVAX1.

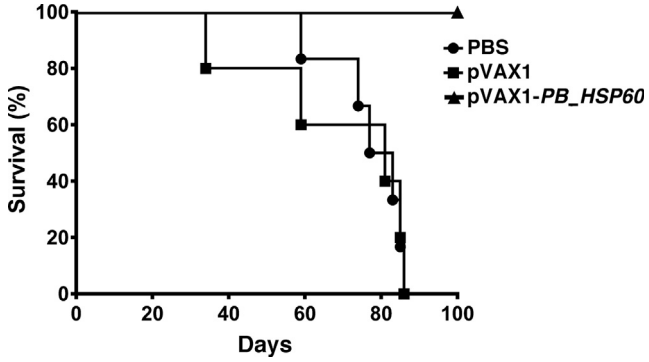

Fig. 7. Treatment with pVAX1-*PB_HSP60* prevents infected animals from succumbing to lethal infection. Animals were infected intranasally with 10 μL of a suspension of 3×10^9 *P. brasiliensis*/mL. After 21 days, the animals were divided into three groups of 10 animals. The animals were treated with three doses of pVAX1-*PB_HSP60*, pVAX1 or PBS, within 15 days between each immunization. Animals were monitored daily in all experimental groups for 100 days of infection.

trols, i.e., the number of doses of protein as therapy influenced the outcome infection.

Treatments using three injections of *pVAX1-PB_HSP60* increased the concentrations of the pro-inflammatory cytokines IL-6 and TNF, as well as IFN-γ e IL-17. These results corroborate the current knowledge about immunity in PCM where Th1 and Th17 responses to *Paracoccidioides* play a key role in the control of infection [16–21]. Interestingly, Feriotti et al. [18] have shown that the inflammatory NOD-like P3 receptor (NLRP3), which activates caspase-1 to cleave pro-IL-1β and pro-IL-18 in their mature cytokine forms, is important in protecting against pulmonary PCM by inducing responses Th1 and Th17, which reduces suppressor control mediated by regulatory T cells. In our experiments with three-doses therapy, there was a tendency to induce a Th1/Th17 protective pattern, which could be response responsible for the control of infection. The Th1 profile has been shown to induce an improvement in PCM due to an increase in NO and reactive oxygen species produced by phagocytes [22,23].

The production of IFN-γ plays a fundamental role on PCM. Cano et al. [24] demonstrated that blockade of IFN-γ with antibody leads to worsening of PCM infection in mice. Souto et al. [25], using IFN-γ-deficient animals, demonstrated that infected animals died faster than wild-type controls. It is possible that IFN-γ production stimulates the production of TNF that plays a role in the formation of compact granulomas and the attraction of cells to the site of infection [26]. Tristão et al. [20] demonstrated the importance of IL-17 and IL-6 cytokines in the formation of granulomas in *P. brasiliensis*-infected mice. They postulated that bone marrow-derived macrophages (BMDM) are essential to the differentiation of Th cells in Th17, by the production of IL-6, and Th17-associated cytokines modulate the immune response and granuloma formation on experimental PCM. In our results, although there was less lung involvement in the infected mice treated with pVAX1-*PB_HSP60*, pVAX1-*ML_HSP65*, or rPbHsp60 when compared with the controls, there was still chronic inflammation. It is worth noting that unlike of control animals, that inflammation was formed by well-defined and compact granulomas, which limit fungal replication [6]. Thus, we assume that the increase of the cytokines IFN-γ and TNF and the decrease of IL-10 in the lungs of infected

mice treated with the therapeutic preparations led to the formation of compact granulomas and restricted fungal growth.

Because the inflammatory cytokines IL-6 and IL-17 are raised in infected mice treated with both a single-dose and three doses of rPbHsp60, we suggested that anti-inflammatory effect of IL-10 could have a crucial role in the outcome of these therapies. There was a significant increase of IL-10 concentrations in mice treated with a single-dose of rPbHsp60 when compared with DNA vaccine and controls. Conversely, infected mice treated with three doses rPbHsp60 had a significant decrease of IL-10. The antagonistic effect of IL-10 to those of TNF and IFN-γ on immunological responses is well documented [27,28]. The cytokine IL-10 together with TGF-β seems to be responsible for the evasion of the fungus of the immune system and is related to the more severe forms of PCM [29–31]. Costa et al. [32] showed that the reduction of IL-10 production appears to be beneficial during PCM, because IL-10-deficient mice had an improvement in control of *P. brasiliensis* infection, provoking a reduction in fungal load in lungs without increasing the lesion area generated by the inflammation as compared to WT mice. Essentially, the elimination of the fungi associated with a reduced injury of the host tissues is dependent on a fine-tuned balance among the differentiated subsets T helper cell subsets and the balance of cytokine production that changes during different times of chronic fungal infection [33].

In conclusion, our data suggest that the increase of the cytokines IFN-γ, TNF and IL-17 and reduction of IL-10 observed after treatment with three doses of pVAX1-*PB_HSP60* led to the control of *P. brasiliensis* infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.090.

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