Saccharomyces cerevisiae Expressing Gp43 Protects Mice against Paracoccidioides brasiliensis Infection

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Abstract

The dimorphic fungus Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM). It is believed that approximately 10 million people are infected with the fungus and approximately 2% will eventually develop the disease. Unlike viral and bacterial diseases, fungal diseases are the ones against which there is no commercially available vaccine. Saccharomyces cerevisiae may be a suitable vehicle for immunization against fungal infections, as they require the stimulation of different arms of the immune response. Here we evaluated the efficacy of immunizing mice against PCM by using S. cerevisiae yeast expressing gp43. When challenged by inoculation of P. brasiliensis yeasts, immunized animals showed a protective profile in three different assays. Their lung parenchyma was significantly preserved, exhibiting fewer granulomas with fewer fungal cells than found in non-immunized mice. Fungal burden was reduced in the lung and spleen of immunized mice, and both organs contained higher levels of IL-12 and IFN-γ compared to those of non-vaccinated mice, a finding that suggests the occurrence of Th1 immunity. Taken together, our results indicate that the recombinant yeast vaccine represents a new strategy to confer protection against PCM.

Introduction

The dimorphic fungus Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), which is an endemic granulomatous chronic mycosis occurring in Latin America. Epidemiological data indicate that PCM is found with high incidence in Brazil, Argentina, Colombia, and Venezuela [1–4]. Infection occurs by inhalation of fungal spores or particles, which transform into the pathogenic yeast form after reaching the pulmonary alveolar epithelium [5]. Yeast can either be eliminated by immune-competent cells or disseminate to other tissues through lymphatic and hematogenous routes, resulting in a spectrum of clinical manifestations, which vary from asymptomatic, benign and localized to severe and disseminated forms (reviewed in [6]). Clinical and experimental evidence indicate that, similar to other
systemic mycosis, Th1 immunity exerts a singular role in the asymptomatic form of PCM, while a Th2 pattern is associated with progression to the severe disease form [7–11].

Current treatment for PCM relies on antifungal chemotherapy to control the disease. Clinically, the antifungal drugs most commonly used for PCM treatment include amphotericin B, sulfa derivatives, and azoles, but their toxicity can be a limiting factor in the treatment [12, 13]. Treatment regimens with these agents often require extended periods of maintenance therapy, which may range from months to years, and are usually associated with relapses [14].

There is a strong need for alternative clinical treatments to chemotherapy. Researchers have focused their efforts in investigating fungal components able to promote cellular immune responses and host protection. Immunization with heat-shock proteins (HSPs) from *P. brasilien sis* has been shown to provide some degree of protection against experimental disease [15–17]. The most abundant *P. brasilien sis* exocellular glycoprotein, called gp43, which is recognized by sera from virtually all *P. brasilien sis*–infected patients [18], was used to immunize mice. The whole gp43 molecule induces both CD4+ Th1 and Th2 cellular immune responses, whereas a 15-mer peptide derived from gp43, named P10, elicits IFN-γ-mediated Th1 immunity that protects mice from experimental PCM [19]. The therapeutic immunization of mice with a DNA vaccine encoding P10 and IL-12 inserts confers protection against experimental PCM, verified by reduced pulmonary fungal load [20].

It has been shown that *Saccharomyces cerevisiae* cell wall beta-glucan acts as an inherent adjuvant that activates dendritic cells required to elicit robust immune response [21]. In addition, previous studies showed that *S. cerevisiae* heat-killed yeast is able to protect mice against systemic coccidioidomycosis [22], by inducing both CD8+ and CD4+ Th1 responses in the infected mice [23]. Both responses were also activated when ovalbumin was carried as an antigen by *S. cerevisiae* [21].

On the basis of these observations, we hypothesized that *S. cerevisiae* could be a suitable vehicle for immunization against fungal infections. Here we evaluated the efficacy of immunizing mice against PCM by using *S. cerevisiae* yeast expressing gp43 as an immunogen.

### Material and Methods

#### Animal Use and Ethics statement

The Committee for Ethics in Animal Research (CETEA) of the Ribeirão Preto Medical School, University of São Paulo (USP), approved all the procedures involving the use of mice, under the protocol 137/2008. BALB/c male mice, six- to eight-week-old, were housed under approved conditions in the institutional Animal Research Facilities. All animals were provided unlimited access to food and water. The animals were monitored daily for inspection of clinical signs. Euthanasia at the completion of experiments was carried out by carbon dioxide asphyxiation or cervical dislocation while under pentobarbital anesthesia.

#### RNA extraction and production of the gp43 cDNA

Total RNA isolated from yeast cells of *P. brasilien sis* 18 was obtained by treatment with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The coding sequence of the gp43 antigen was obtained by RT–PCR. cDNAs were synthesized from total RNA from *P. brasilien sis* by using an oligo(dT) standard primer (0.5 μg) and a random primer reaction. The reaction was made using *Improm-II reverse transcriptase* (Promega Corporation, Madison, WI, USA), 5 μg of total RNA, buffer *Improm-II-1x*, 3 mM MgCl2, a mixture of dATP, dTTP, dCTP and dGTP (dNTPs) 0.5 mM to a final volume of 20 μL reaction.
Construction of pBG1805_Gp43

The cDNA synthesized from the complete coding sequence of gp43 was amplified using specific primers for cloning into the plasmid pCR 2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA, USA). The 5’ primers (5’ AGTACTATGAATTATTTAGTCTCTTAAACCTGG 3’) had a direct initiator present in the sequence whereas the 3’ primers (5’ TCACCTGCATCCACCATCTT 3’) contained a stop codon. The PCR reaction was made to a final volume of 50 μL: Buffer Platinum Taq DNA polymerase High Fidelity (Invitrogen) (600 mM Tris-SO4 pH 8.9, 180 mM ammonium sulfate); 2 mM MgSO4; dNTPs 0.2 mM; 5 pmol of each primer and 1.25 units Platinum Taq DNA polymerase High Fidelity. The reaction included one cycle of 94°C (5 min), 29 cycles of 94°C (1 min), 50°C (1min), 68° C (1min) and one cycle of 68°C (10 min).

The PCR fragment generated a product with 1251 base pairs and was purified from the agarose gels by using the Kit GFX PCR DNA and Gel Band Purification (GE Healthcare). PCR products were cloned into plasmid pCR 2.1-TOPO (Invitrogen) and were sequenced in both directions.

The gp43 ORF was amplified with other specific primers to recombine in the Gateway System (Invitrogen). The forward oligonucleotides contained 14 nucleotides derived from the attB1 site and 25 nucleotides contained in the gp43 ORF near to the ATG translation initiator (5’ CAAAAAGCAGGCTTCATGAATTTAGTTCTCTTAAACCTGG-3’). The reverse oligonucleotides contained 17 nucleotides from the attB2 site and 18 nucleotides in the gp43 ORF near the terminator site but did not carry the stop codon (5’ GTACAAAGACAGTGGCTTCCATCGACCTACCATCTT 3’). Oligonucleotides had additional nucleotides to maintain the open reading frame. The reaction included one cycle of 94°C (5 min), 24 cycles of 94°C (1 min), 51°C (1min), 68° C (1min) and one cycle of 68°C (10 min). PCR products were recombined into the Gateway vector pDONR 201 using BP Clonase (Invitrogen). The recombinant products were transformed in Escherichia coli. After a plasmid extraction, ORF containing plasmid DNAs were recombined into a destination vector pBG1805 (described in [24]), using LR Clonase (Invitrogen).

E. coli was transformed with the recombined plasmid pBG1805_Gp43. This plasmid was used to transform Saccharomyces cerevisiae (strain Y258). The resulting strain (yMAgp43) was used for protein overexpression.

Expression and detection of the gp43 protein in S. cerevisiae

For overexpression in S. cerevisiae, the gp43 ORF cloned into the vector pBG1805 was expressed as previously described [24]. Yeast cells were induced with YP +2% galactose medium (yeast extract 10 g/L, peptone 20 g/L, galactose 20 g/L). The ORF was expressed under control of the GAL1 promoter with their C-terminus fused to a complex tag containing 6xHIS, HA epitope followed by a 3C site and the ZZ domain of protein A. After 4 and 16 hours of induction, the cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C, washed and the pelleted cells suspended in PBS.

The induction of expression was monitored in 10% polyacrylamide gel by SDS-PAGE, and the preparation from total protein extract was done as previously described [24]. To confirm the expression, protein was electrotransferred from polyacrylamide gels to nitrocellulose membranes (Hybond-P, GE Healthcare Biosciences, Pittsburgh, PA, USA), at 75 V for two hours using transfer buffer (Tris-Base 1.895%, 9.09% Glycine). The membrane was rinsed with TBS-T 1X (Tris-HCl 60.5 g/L, NaCl 87.6 g/L, 0.05% Tween 20) with 5% skim milk, for 14–16 hours at 4°C, to block nonspecific interactions. Membranes were then incubated for 1 hour at room temperature, with anti-gp43 monoclonal antibodies (1:50), kindly provided by Ebert Hanna, or with monoclonal primary anti-HA (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) that reacts with the HA C-terminal tag. Three subsequent washes of 10 minutes were done.
with TBS-T plus 5% skimmed milk, at room temperature. The membranes were incubated with secondary antibody anti-mouse IgG conjugated with peroxidase (ECL western blotting reagent detection and Analysis System) diluted 1:4000 in TBS-T. After additional series of washes, development was done following manufacturer instructions (GE Healthcare).

Aliquots of 2x10^7 recombinant yeasts expressing the gp43 protein (yMAgp43) were killed by heating at 56°C for 1 h and stored at -80°C until used for mouse immunization. The same procedure was done with yeasts carrying the empty plasmid (yMA).

**Vaccine administration**

To evaluate the prophylactic effect of recombinant *S. cerevisiae* upon *P. brasiliensis* infection, groups of BALB/c mice were immunized intraperitoneally (i.p.) with 2x10^7 yeast cells given weekly for three times (day 0, 7, and 14). These cells were previously harvested by centrifugation, suspended in PBS and killed by heating (56°C by 1h). One group was immunized with recombinant yeast expressing the gp43 protein (yMAgp43; Vaccine group), and the other two groups of mice were used as controls: one group was immunized with yeast cells carrying an empty plasmid (yMA; Vector Group) and the other with vehicle only (PBS Group).

**Cultivation of *P. brasiliensis***

Yeast cells from *P. brasiliensis* 18 strain were collected after growth in YPD liquid medium (Difco) at 37°C for 10 days. The viability of the yeasts were determined as previously described [25]. Pb18 strain was used based on its high virulence and ability to induce a strong granulomatous reaction [26].

**Experimental Infection**

One week after the last immunization with yMAgp43, yMA or PBS, BALB/c mice were infected intravenously (i.v.) with 1x10^6 *P. brasiliensis* yeast cells in 100 μL of PBS, through the ophthalmic plexus. The course of infection was evaluated 30 and/or 60 days post-infection.

**Histopathological examination of infected mice**

Fragments of the right lobe of the lung from all mice, obtained on day 30 or 60 post infection, were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours and processed for paraffin embedding. They were serially cut into 5-μm-thick-sections and sequentially stained with hematoxylin and eosin (H&E), for analysis of granulomatous lesions and inflammatory infiltrates, or with Grocott’s methenamine silver impregnation, to detect polysaccharides in the fungal cell wall based in an oxidation reaction. The granuloma count (number of granuloma/mm²) in lung sections was determined by using an optical microscope with an integrator lens (Carl Zeiss, Germany). The granuloma area (mm²) was measured by with a KS-100 program (Carl Zeiss, Germany).

**Assay for Organ Colony-Forming Units**

Mice from all experimental groups were euthanized 30 or 60 days post-infection and fungal burden was measured by colony-forming units (CFU). Lung and splenic fragments were aseptically collected, weighed, homogenized in 1 mL of sterile PBS, and serially diluted. Aliquots of 100 μL were dispensed, in duplicates, into Petri dishes containing brain heart infusion agar (BHI, Difco Laboratories, Detroit, MI, USA), supplemented with 4% (v/v) heat-inactivated fetal bovine serum. After 14 days incubation at 37°C, the colonies were counted and the numbers of CFU per gram of tissue were calculated.
Quantification of cytokines

To determine the organ contents of IL-12 and IFN-γ cytokines, lung and spleen homogenates were centrifuged at 2000 x g for 15 minutes, and the supernatants were analyzed by ELISA (OptEIA set; Pharmingen, San Diego, CA, USA), according to the manufacturer’s recommendations.

Statistical analysis

Statistical differences between means of experimental groups were performed with analysis of variance (ANOVA) with GraphPad Prism5 version 3.01 using a post hoc Tukey test for multiple comparisons. Values were considered significant when p < 0.05. All experiments were performed at least three times.

Results

Construction and Expression of recombinant gp43 in S. cerevisiae

To obtain the recombinant gp43 protein, the gp43 ORF was amplified from TOPO_Gp43. The amplified fragment with specific primers (GP43_F, GP43_R) was recombined into Gateway vector pDONR 201 using BP Clonase (Invitrogen). A homologous recombination reaction was taken with fragment encoding gp43 (pDONR 201_Gp43) and then cloned into the expression plasmid BG1805 using LR Clonase (Invitrogen). In the C-terminal portion of the recombinant plasmid pBG1805_Gp43, the protein gp43 was fused in tandem with 6X His, HA, a cleavage site of protease 3C and ZZ domain of protein A (Fig. 1A).

The recombinant plasmid pBG1805_Gp43 was transformed into strain Y258 of S. cerevisiae Y258. Western blot analysis of the protein extracts of S. cerevisiae yeast cells expressing tagged gp43 (yMAgp43) at 4 and 16 hours after induction with galactose showed a band of apparent molecular mass of 66kDa, which corresponds to the predicted size of gp43 in fusion with a HA-His tag plus the protein A ZZ domain (Fig. 1B). This recombinant protein is detected either by anti-HA (Fig. 1B, left) or anti-gp43 polyclonal antibodies (Fig. 1B, right).

Lung Histopathology

To evaluate whether prophylactic administration of S. cerevisiae expressing gp43 affects the course of experimental PCM, groups of BALB/c mice were inoculated i.p. with recombinant yeast expressing the gp43 protein; with yeast cells carrying an empty plasmid; or with vehicle only, on days 0, 7, and 14. Challenge was performed one week after the third immunization, by i.v. inoculation with P. brasiliensis yeast. Thirty days after infection, the lungs were examined for inflammation and fungal presence, by staining the tissue sections with hematoxylin and eosin (HE), or with silver methenamine. The lung parenchyma of PBS group mice was largely replaced by large and loose granulomas (Fig. 2A). In the rare preserved areas, thickening of alveolar septa were observed. The granuloma, in its central area, presented phagocytic cells ordered around numerous yeasts (Fig. 2B and 2C). The presence of yeast cells were extended to the periphery of some granulomas, an area that is characterized by intense basophilia due to the presence of plasmocyte nuclei. Some budding yeasts were seen inside the granulomas (Fig. 2B and 2C). The lung of mice of the empty vector group exhibited less inflammatory infiltrates and fewer granulomas (Fig. 2D), when compared to the PBS group (Fig. 2A), differences that were not confirmed by the morphometric analysis (Fig. 2J and 2K). Notably, in the Vaccine group, lung architecture was more extensively preserved, showing thin alveolar septa. In the fewer and more compact granulomas, yeast cells were less frequent and restricted to the central area of the granuloma (Fig. 2G-I).
Fig 1. Gp43 gene cloning and expression of the gp43 protein by the transformed S. cerevisiae. (A) Representative scheme of recombinant gp43 cloned into the vector pBG1805 by Gateway system. The recombinant gp43 is fused to 6X His, HA, a cleavage site of protease 3C and ZZ domain of protein A in C-terminal site, besides being on promoter induction of GAL1. B1 and B2 are recombinant sites of the pBG1805. (B) Detection of recombinant gp43 protein by Western blotting. Overexpression of the gp43 fusion protein in S. cerevisiae yeast cells was induced by adding galactose to the YPD media. The cells grew for 4 hours (lanes 2 and 5) or overnight (3 and 6). Non-induced samples were used as controls (lanes 1 and 4). Total protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and reacted with anti-HA or anti-gp43 antibodies. A polypeptide of approximately 66 kDa, which is the predicted molecular weight of the gp43-tagged fusion protein, was detected either by an anti-HA (Fig. 1B, left) or anti-gp43 polyclonal antibodies (Fig. 1B, right). The bands of molecular weight lower than 66 kDa may correspond to degradation products.

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The pulmonary morphometric analysis showed that vaccinated mice, compared to the controls, had a lower number of granulomas (Fig. 2J), which occupied a smaller parenchymal area (Fig. 2K). On the other hand, there was no significant difference between empty vector group and PBS group regarding the density of granulomas and the area they occupied. Notably, the PBS group presented 2.64 granulomas/mm², whereas the vaccine group presented 1.21 granulomas/mm² (Fig. 2J), corresponding to a 54% reduction in the granuloma number. Besides the lower number of granulomas found in mice of the vaccine group, the total area occupied by the granulomas was significantly lower than that verified in the PBS group (Fig. 2K).

Fig 2. Pulmonary histopathology and morphometric analysis of the granulomas. The pulmonary histopathology of immunized mice infected with P. brasiliensis were analyzed thirty day post infection. Lungs of mice immunized with yMAg43 (Vaccine Group) presented discrete granulomatous infiltrates (HE, 10X, G) if compared to controls (Vector and PBS Groups, D and A). Silver methenamine staining revealed that the lungs in the Vaccine Group (H and I) presented less yeast cells and granulomas when compared to controls (B, C, E and F). Panel J: The number of granulomas/mm² in the tissue was obtained using an optical microscopy with the aid of an integrator lens (Carl Zeiss Jena, Germany). Each bar represents the average ± SD of lung sections from 4 animals. Panel K: the granuloma area (mm²) was determined by means of KS-100 program (Carl Zeiss Jenamed 2, USA). The data represent the average ± SD of lung sections from 4 animals, where: *P < 0.05 in relation to the PBS group.

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The granulomas of the vaccinated mice occupied the total area of 0.58mm², which is 3-fold lower than the total area occupied by granulomas in control animals. These results show that the vaccination with *S. cerevisiae* expressing the gp43 recombinant protein promoted a significant reduction in the pulmonary damage caused by *P. brasiliensis* infection.

Fungal burden in immunized mice

In order to determine whether vaccination confers protection against *P. brasiliensis* infection, lung and spleen fragments of immunized and control (Vector and PBS groups) mice, challenged with 1X10⁶ *P. brasiliensis* yeasts, were obtained 30 or 60 days post-infection and analyzed for fungal CFU. On day 30 post-infection, the number of CFU recovered from the organs of mice from the vaccine group was significantly lower than that of the PBS group. The reductions attained 50% and 84% in the lung and in the spleen, respectively (Fig. 3A and 3B). These differences were more pronounced on day 60 post-infection, and extended to the empty vector group, whose fungal burden was 4- and 8-fold lower than the provided by the lung and the spleen of mice from the PBS group. As on day 30 post infection (Fig. 3A and 3B), the protection conferred by the yeast expressing gp43 was higher than that achieved by yeast with the empty vector group on day 60 post-infection (Fig. 3C and 3D). The observed reductions in fungal burden were statistically significant, with *P* < 0.05, **P** < 0.01, and ***P*** < 0.001 in relation to the PBS Group.

**Fig 3.** Quantification of fungal burden in the lung and spleen of recombinant yeast-immunized mice, after infection with 1x10⁶ yeasts of *P. brasiliensis*. Bars represent CFU levels in lungs (A and C) and spleens (B and D). BALB/c mice were intraperitoneally inoculated with PBS (PBS group), or yMA (Vector Group), or yMAgp43 (Vaccine group) and intravenously infected with *P. brasiliensis*. One and two months after infection, all mice were sacrificed. The Vaccine group mice presented reduced levels of colony forming units (CFU) when compared with the PBS groups in both organs and both time (A-D). In addition, sixty days post infection, the CFU number in the Vector Group mice was significantly lower than PBS group (C and D). Data are representative of a typical experiment independently repeated three times, where: * P<0.05, **P<0.01, and ***P<0.001 in relation to the PBS Group.

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load indicate that vaccine promoted efficient *P. brasiliensis* yeasts clearance, which is importantly favored by the adjuvant effect provided by *S. cerevisiae*.

**Cytokines content in organs of immunized mice**

To investigate the mechanism by which immunization with yMAgp43 has conferred protection against *P. brasiliensis* infection, the levels of cytokines contained in the organs of vaccinated (yMAgp43) and control mice (Vector and PBS) were quantified 30 days after fungal challenge. Significantly higher concentrations of IL-12 were detected in the pulmonary tissue, and of both IL-12 and IFN-γ, in the pulmonary and splenic tissues of mice vaccinated with yMAgp43 in comparison to those found in mice of either control group (PBS or Vector) (Fig. 4). This finding suggests that the vaccination of mice with yMAgp43 elicited a Th1-immune response, which is known to account for protection against the fungus. The slightly higher cytokine content observed in the vector group compared to PBS may be due to the adjuvant effect conferred by yeast. This is consistent with protection presented by the vector group as evaluated by histopathology and CFU measurements (Figs. 2 and 3). The significantly higher protection conferred by the vaccine group (yMAgp43) may be a result of a specific response against gp43 in combination with the yeast adjuvant effect.
Discussion

In this study we show that immunization with *S. cerevisiae* expressing the gp43 recombinant protein (yMAgp43) prior to challenge with *P. brasiliensis* protects mice against systemic PCM. This protection was revealed by more effective fungal clearance from the lungs of immunized mice, as well as by reduction in density of pulmonary granulomatous lesions, associated with the presence of few viable yeast. These beneficial effects are likely due to the development of Th1 immunity, featured by augmented pulmonary levels of IL-12 and IFN-γ.

The analysis of the pulmonary tissue of *P. brasiliensis* infected mice showed that those mice vaccinated with yMAgp43 had less frequent and more compact granulomas, which are the prominent inflammatory lesions in human and experimental PCM, and known to restrict fungal growth and avoid dissemination throughout the host organism [27, 28]. These roles are not played by loose granulomas, which were extensively found in non-vaccinated mice. In addition, vaccinated mice showed fewer silver stained fungi in the center of pulmonary granulomas, a finding that is consistent with the significantly lower fungal burden presented by these mice in comparison those of the control groups, and reflects the protection level provided by the vaccination procedure. Moreover, the protection conferred by the immunization with yMAgp43 was equivalent to the previously reported for immunization with gp43 or its derived 15 amino-acid peptide, named P10, when administered in association with adjuvants [19]. Administration of the non-expressing gp43 *S. cerevisiae* yeast (Vector group) did not reduce significantly the granulomas density nor the fungal burden, examined at the 30th day post-infection. However, at the 60th day the empty vector group showed fungal burden as low as the vaccinated group, a result that is consistent with the reported use of non-recombinant *S. cerevisiae* yeasts as a vaccine against coccidioidomycosis and other fungal infections [22]. As a result of its adjuvant properties, *S. cerevisiae* yeast may induce a certain grade of protection, as provided by therapy with complete Freund’s adjuvant against murine PCM, and the protection may be induced by cross-protective antigens [29].

Extensive studies have shown that *S. cerevisiae* successfully delivers proteins into dendritic cells [21, 30–32], which are uniquely able to shape antifungal immunity by initiating naïve T cell responses [33, 34]. The stimulus of IL-12 produced by activated dendritic cells accounts for the development of Th1 effector cells [35], which release high concentration of IFN-γ. This cytokine, in turn, induces the antifungal effector function of phagocytes. As a result, this sequence of events explains why a dominant Th1 response correlates with the occurrence of protective immunity to fungal infection [34]. Because we found significant increase of the IL-12 and IFN-γ contents in organs of the vaccinated mice when challenged with *P. brasiliensis*, we postulate that mice immunized with *S. cerevisiae* yeasts expressing the gp43 antigen have used efficiently the mechanisms mentioned before, involved in resistance against PCM.

Our postulation is supported by studies showing that severe PCM in humans and experimental hosts is associated with depression of cell-mediated immune responses [2, 36], while resistance to *P. brasiliensis* is often related with the production of IFN-γ [9, 37–39]. Indeed, IFN-γ production is required for resistance against several infections by pathogenic fungi, such as *Cryptococcus neoformans* [40, 41], *Histoplasma capsulatum* [42], *Blastomyces dermatitidis* [43]. Furthermore, the protective effect against *P. brasiliensis* infection conferred by immunization with the peptide P10 was related to the *in vitro* demonstrated peptide ability of inducing an IFN-γ secreting Th1-lymphocyte population [19].

Immunotherapy with P10 has been effective using adjuvants, P10-primed dendritic cells, and especially a combination of plasmids encoding P10 and IL-12 gene [44], in a demonstration that an additional stimulus to induce T cells, beyond the peptide itself, is necessary to confer protection against the fungus. We hypothesize that in our study the protection induced by...
immunization with the whole gp43, which encompasses P10 and several Th2 epitopes as mapped by Taborda et al. (1998), was made possible by the ability of the *S. cerevisiae* as a vehicle to support Th1 immunity.

Heat-killed *S. cerevisiae* has been successfully used as a vaccine against several fungal infections, such as systemic aspergillosis, coccidioidomycosis, candidiasis and cryptococcosis [22, 45–47]. The immune response elicited by the heat-killed yeast is characterized mainly by high IFN-γ production, which is similar to that obtained by administration of live yeasts [23] and coincident with the already mentioned profile detected here in mice immunized with the *S. cerevisiae* expressing gp43. Since the *S. cerevisiae* yeast itself also conferred a significant protection against *P. brasiliensis* infection, it is reasonable to envisage further studies to better explore the eventual resistance that may be conferred by *S. cerevisiae* derived preparations.

In summary, we tested a new approach to immunoprophylaxis against PCM that uses *S. cerevisiae* as a vaccine vehicle. Further yeast vaccine refinements may be implemented for use as a therapeutic tool against PCM.

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Author Contributions

Conceived and designed the experiments: MAA-M AFO LPR MCR-B PSRC. Performed the experiments: MAA-M AFO LPR TFR. Analyzed the data: MAA-M AFO LPR MCR-B PSRC. Wrote the paper: MAA-M AFO LPR MCR-B PSRC.

References

1. Franco M. Host-parasite relationships in paracoccidioidomycosis. Medical Mycology. 1987; 25(1):5–18. PMID: 3553526
2. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev. 1993; 6(2):89–117. Epub 1993/04/01. PMID: 8472249
3. San-Blas G, Nino-Vega G, Iturriaga T. Paracoccidioides brasiliensis and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Med Mycol. 2002; 40(3):225–42. PMID: 12146752
4. Blotta MH, Mamoni RL, Oliveira SJ, Nuer SA, Papaioannou PM, Goveia A. et al. Endemic regions of paracoccidioidomycosis in Brazil: a clinical and epidemiologic study of 584 cases in the southeast region. Am J Trop Med Hyg. 1999; 61(3):390–4. Epub 1999/09/25. PMID: 10497977
5. Restrepo-Moreno A. Paracoccidioidomycosis. Fungal infections and immune responses: Springer; 1993. p. 251–76.
6. Borges-Walmsley Mi, Chen D, Shu X, Walmsley AR. The pathobiology of *Paracoccidioides brasiliensis*. Trends in microbiology. 2002; 10(2):80–7. PMID: 11827809
7. Ruaas LP, Bernardes ES, Fermino ML, de Oliveira LL, Hsu DK, Liu F-T, et al. Lack of galectin-3 drives response to *Paracoccidioides brasiliensis* toward a Th2-biased immunity. PLoS One. 2009; 4(2): e4519. doi: 10.1371/journal.pone.0004519 PMID: 19223338
8. Karhawi A, Colombo A, Salomao R. Production of IFN-γ is impaired in patients with paracoccidioidomycosis during active disease and is restored after clinical remission. Medical Mycology. 2000; 38-(3):225–9. PMID: 10892991
9. Cano LE, Kashino SS, Arruda C, Andre D, Xidieh CF, Singer-Vermes LM, et al. Protective role of gamma interferon in experimental pulmonary paracoccidioidomycosis. Infect Immun. 1998; 66(2):800–6. Epub 1998/02/07. PMID: 9453644
10. Benard G, Romano CC, Cacere CR, Juvenale M, Mendes-Giannini MJ, Duarte AJ. Imbalance of IL-2, IFN-gamma and IL-10 secretion in the immunosuppression associated with human paracoccidioidomycosis. Cytokine. 2001; 13(4):248–52. Epub 2001/03/10. PMID: 11237434
11. Oliveira SJ, Mamoni RL, Musatti CC, Papaioordanou PM, Biotta MH. Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls. Microbes Infect. 2002; 4(2):139–44. Epub 2002/03/07. PMID: 11880044

12. Mendes RP, Negroni R, Arechavala A. Treatment and control of cure. In: Franco M, Lacaz CS, Restrepo A, del Negro G, editors. Paracoccidioidomycosis: Boca Raton, CRC Press; 1994. p. 373–92.

13. Hahn RC, Morato Conceicao YT, Santos NL, Ferreira JF, Hamdan JS. Disseminated paracoccidioidomycosis: correlation between clinical and in vitro resistance to ketoconazole and trimethoprim-sulfamethoxazole. Mycoses. 2003; 46(8):342–7. Epub 2003/09/03. PMID: 12950907

14. Shikanai-Yasuda MA, Telles-Filho Q, Mendes RP, Morelli ML. Guidelines in Paracoccidioidomycosis. Revista da Sociedade Brasileira de Medicina Tropical. 2006; 39:297–310. PMID: 16906260

15. Soares CMA, Mendes-Giannini MJ, Filippe MSS, Chaturvedi V. A centennial: discovery of Paracoccidioides brasiliensis. Mycopathologia. 2008; 165:179–81. PMID: 18777627

16. Ribeiro AM, Bocca AL, Amaral AC, Faccioli LH, Galetti FC, Zaraté-Blades CR, et al. DNAhsp65 vaccination induces protection in mice against Paracoccidioides brasiliensis infection. Vaccine. 2009; 27:606–13. doi: 10.1016/j.vaccine.2008.10.022 PMID: 19028537

17. Ribeiro AM, Bocca AL, Amaral AC, Souza AC, Faccioli LH, Coelho-Castelo AA, et al. HSP65 DNA as therapeutic strategy to treat experimental paracoccidioidomycosis. Vaccine. 2010; 28:1528–34. doi: 10.1016/j.vaccine.2009.11.062 PMID: 20045500

18. Puccia R, Schenkman S, Gorin PA, Travassos LR. Exocellular components of Paracoccidioides brasiliensis: identification of a specific antigen. Infect Immun. 1986; 53(1):199–206. Epub 1986/07/01. PMID: 2424841

19. Taborda CP, Juliano MA, Puccia R, Franco M, Travassos LR. 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. 1998; 66(2):786–93. Epub 1998/02/07. PMID: 9453642

20. Rittner GM, Munoz JE, Marques AF, Nosanchuk JD, Taborda CP, Travassos LR. Therapeutic DNA vaccine encoding peptide P10 against experimental paracoccidioidomycosis. PLoS Negl Trop Dis. 2012; 6(2):e1519. Epub 2012/03/06. doi: 10.1371/journal.pntd.0001519 PMID: 22389734

21. Stubbs AC, Martin KS, Coeshott C, Skaates SV, Kuritzkes DR, Bellgrau D, et al. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. Nat Med. 2001; 7(5):625–9. Epub 2001/05/01. PMID: 11329066

22. Capilla J, Clemons KV, Liu M, Levine HB, Stevens DA. Saccharomyces cerevisiae as a vaccine against coccidioidomycosis. Vaccine. 2009; 27(27):3662–8. Epub 2009/05/26. doi: 10.1016/j.vaccine.2009.03.030 PMID: 19464548

23. Liu M, Clemons KV, Bigos M, Medovarska I, Brummer E, Stevens DA. Immune responses induced by heat killed Saccharomyces cerevisiae: a vaccine against fungal infection. Vaccine. 2011; 29(9):1745–53. Epub 2011/01/12. doi: 10.1016/j.vaccine.2010.12.119 PMID: 21219976

24. Gelperin DM, White MA, Wilkinson ML, Kon Y, Kung LA, Wise KJ, et al. Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev. 2002; 4(2):139–44. Epub 1994/05/12. PMID: 16906260

25. Kashino SS, Mendes RP, Arechavala A. Treatment and control of cure. In: Franco M, Lacaz CS, Restrepo A, del Negro G, editors. Paracoccidioidomycosis: Boca Raton, CRC Press; 1994. p. 373–92.
therapeutic antitumor responses. Clin Cancer Res. 2008; 14(13):4316–25. Epub 2008/07/03. doi: 10.1158/1078-0432.CCR-08-0393 PMID: 18594015

32. Bernstein MB, Chakraborty M, Wansley EK, Guo Z, Franzusoff A, Mostbock S, et al. Recombinant Saccharomyces cerevisiae (yeast-CEA) as a potent activator of murine dendritic cells. Vaccine. 2008; 26(4):509–21. Epub 2007/12/25. PMID: 18155327

33. Roy RM, Klein BS. Dendritic cells in antifungal immunity and vaccine design. Cell Host Microbe. 2012; 11(5):436–46. Epub 2012/05/23. doi: 10.1016/j.chom.2012.04.005 PMID:22607797

34. Borghi M, Penga G, Puccetti M, Okonomou V, Palmieri M, Galos P, et al. Antifungal Th immunity: growing up in family. Frontiers in Immunology. 2014.

35. Scott P, Kaufmann SH. The role of T-cell subsets and cytokines in the regulation of infection. Immunology today. 1991; 12(10):346–8. PMID:1683536

36. Mota FT, de Franco MF. [Study of anti-Paracoccidioides brasiliensis IgM antibodies using immunofluorescence of the serum of patients with paracoccidioidomycosis]. Rev Inst Med Trop Sao Paulo. 1979; 21(2):82–9. Epub 1979/03/01. PMID: 384494

37. Souto JT, Aliberti JC, Campanelli AP, Maffei CM, Ferreira BR, et al. Chemokine Production and Leukocyte Recruitment to the Lungs of Paracoccidioides brasiliensis-Infected Mice Is Modulated by Interferon-y. The American journal of pathology. 2003; 163(2):583–90. PMID: 12875978

38. Brummer E, Hanson LH, Stevens DA. Gamma-interferon activation of macrophages for killing of Paracoccidioides brasiliensis and evidence for nonoxidative mechanisms. International journal of immunopharmacology. 1988; 10(8):945–52. PMID: 3145925

39. Gonzalez A, de Gregori W, Velez D, Restrepo A, Cano LE. Nitric oxide participation in the fungicidal mechanism of gamma interferon-activated murine macrophages against Paracoccidioides brasiliensis conidia. Infect Immun. 2000; 68(5):2546–52. Epub 2000/04/18. PMID: 10768942

40. Mody CH, Syme RM. Effect of polysaccharide capsule and methods of preparation on human lymphocyte proliferation in response to Cryptococcus neoformans. Infect Immun. 1993; 61(2):464–9. Epub 1993/02/01. PMID: 8423074

41. Allendoerfer R, Deepe GS Jr. Intrapulmonary response to Histoplasma capsulatum in gamma interferon knockout mice. Infect Immun. 1997; 65(7):2564–9. Epub 1997/07/01. PMID: 9199420

42. Majumder T, Liu M, Chen V, Martinez M, Alvarado D, Clemons KV, et al. Killed Saccharomyces cerevisiae Protects Against Lethal Challenge of Cryptococcus grubii. Mycopathologia. 2014; 178(3-4):189–95. Epub 2014/08/15. doi: 10.1007/s11046-014-9797-6 PMID:25129421

43. Liu M, Clemons KV, Johansen ME, Martinez M, Chen V, Stevens DA. Saccharomyces as a vaccine against systemic candidiasis. Immunol Invest. 2012; 41(8):847–55. Epub 2012/06/13. doi: 10.3109/08920139.2012.692418 PMID:22686468

44. Stevens DA, Clemons KV, Liu M. Developing a vaccine against aspergillosis. Med Mycol. 2011; 49 Suppl 1:S170–6. Epub 2010/07/09. doi: 10.3109/13693786.2010.497775 PMID:20908783