Mycopathologia (2013) 175:463–475
DOI 10.1007/s11046-012-9599-7

DNA-hsp65 Vaccine as Therapeutic Strategy to Treat Experimental Chromoblastomycosis Caused by *Fonsecaea Pedrosoi*

Isaque Medeiros Siqueira · Alice Melo Ribeiro · Yanna Karla de Medeiros Nóbrega · Karina Smidt Simon · Ana Camila Oliveira Souza · Márcio Souza Jerônimo · Florêncio Figueiredo Cavalcante Neto · Célio Lopes Silva · Maria Sueli Soares Felipe · Anamélia Lorenzetti Bocca

Received: 18 September 2012 / Accepted: 4 November 2012 / Published online: 22 November 2012

Abstract Chromoblastomycosis (CBM) is a chronic subcutaneous mycosis, caused by several dimorphic, pigmented dematiaceous fungi. Patients with the disease are still considered a therapeutic challenge, mainly due to its recalcitrant nature. There is no "gold standard" treatment for this neglected mycosis, but rather there are several treatment options. Chemotherapy alternatives include 5-flucytosine, itraconazole, terbinafine, fluconazole, thiabendazole, ketoconazole and amphotericin B, although the healing of severe cases is still uncommon. However, several studies have reported the DNA vaccine to be promising in the treatment for fungal infections; this vaccine allows the host to restore depressed cellular immunity, minimizing the toxic effects from conventional antifungal therapies. This work was therefore carried out aiming to establish a suitable model for experimental CBM, suggesting also new therapies, including DNA-hsp65 vaccine. By analyzing the morphometrical and histopathological aspects and by quantifying the fungal burden, the results showed the establishment of a chronic, although transitory, experimental CBM model with lesions similar to those presented in humans. A treatment regimen using intralesional itraconazole or amphotericin B was effective in treating experimental CBM, as was a therapy using naked DNA-hsp65 vaccine. It has also been shown that chemotherapy associated with DNA-hsp65 vaccine is promising in the treatment for CBM.

Keywords Chromoblastomycosis · Itraconazole · Amphotericin B · DNA vaccine · DNA-hsp65

I. M. Siqueira · K. S. Simon · A. C. O. Souza · M. S. Jerônimo
Molecular Pathology Post-Graduate Program, Faculty of Medicine, University of Brasília, Brasília, DF, Brazil

A. M. Ribeiro · A. L. Bocca (✉)
Biology Institute, University of Brasília, Asa Norte, Brasília, DF 70910-900, Brazil
e-mail: albocca@unb.br

Y. K. de Medeiros Nóbrega
Faculty of Health Science, University of Brasília, Brasília, DF, Brazil

F. F. C. Neto · A. L. Bocca
Faculty of Medicine, University of Brasília, Brasília, DF, Brazil

C. L. Silva
Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

M. S. S. Felipe
Genomic Science and Biotechnology Post-Graduate Program, Catholic University of Brasília, Brasília, DF, Brazil

Introduction

Chromoblastomycosis (CBM) is a chronic cutaneous and subcutaneous mycosis caused by several dematiaceous fungi worldwide [1]. Commonly, CBM

develops after transcutaneous inoculation of the etiologic agent into the subcutaneous tissues, but it usually takes decades before clinical symptoms develop. The infection is characterized by erythematous papules, which develop with varying morphology, and it is also a granulomatous, suppurative and sometimes debilitating mycosis [2]. Once installed in the tissue, the fungus adheres to epithelial cells, then turning into its pathogenic form (muriform cells), which resist destruction by host effector cells, resulting in a chronic disease [2]. The most common species associated with the disease are *Fonsecaea pedrosoi*, *Phialophora compacta*, *Phialophora verrucosa*, *Rhinocladiella aquaspersa* and *Cladosporium carrionii*, which are found as saprophytes in soil and plants [1].

The granulomatous reaction in CBM is associated with macrophages, in different degrees of maturation and activation, and high levels of neutrophils [3]. The latter destroy fungal cells by the production of reactive oxygen intermediates (ROI) and by myeloperoxidase production. Activated macrophages have shown only fungicidal activity against *F. pedrosoi*, and there is evidence that some fungal structure, such as melamine, may negatively modulate certain microbicidal activities in macrophages that are important in the elimination of fungal cells [4–6].

The mechanisms of adaptive immunity in CBM include both humoral and cell-mediated immune responses. In patients with the disease, specific antibodies are produced, such as IgG1, IgM and IgA [7]. As seen with other chronic diseases caused by fungal infections, the humoral immune response (Th2) does not seem to play a protective role when compared with cell-mediated immunity (Th1) [2]. Patients with severe CBM have elevated IL-10 and low IFN-γ serum levels. In the same way, those patients demonstrate an inefficient T cell proliferation, as a typical Th2 response. On the other hand, in patients with the mild form, there is intense production of IFN-γ, low levels of IL-10 and efficient T cell proliferation, as a typical Th1 response [8]. Studies using athymic mice infected with *F. pedrosoi* showed diffuse and confluent granulomas with random distribution of the fungus, strengthening the role of cellular immune response in CBM [9].

Patients with CBM are still a real therapeutic challenge for clinicians due to the recalcitrant nature of the disease, especially in the severe clinical forms. There is still no "gold standard" treatment for this mycosis, but rather a series of therapeutic options, including physical treatment, chemotherapy and combination therapy. Amputations are rarely performed, and spontaneous resolution of the disease is uncommon [1, 2]. Chemotherapy alternatives include 5-flucytosine, itraconazole, terbinafine, fluconazole, thiabendazole, ketoconazole and amphotericin B. All these drugs are associated with severe adverse effects when used in long-term treatment, including renal and hepatic toxicity [10, 11].

Recently, much has been said about the use of DNA vaccines as a therapeutic strategy in the treatment of various diseases. In animal models, DNA vaccines have been successfully employed in treating various diseases, such as those caused by *Trypanosoma cruzi* [12]; *Leishmania major* [13, 14]; *Giardia lamblia* [15], hepatitis B virus [16] and *Mycobacterium tuberculosis* [17]. DNA vaccines have also been used successfully in immunotherapy against cancer and tuberculosis [17, 18].

DNA vaccines are capable of modulating both humoral and cell-mediated immunity by stimulating CD4 and CD8 cytotoxic cells. These vaccines are already showing promise in the treatment for fungal infections, in that they allow the host to restore depressed cellular immunity, minimizing the toxic effects from conventional antifungal therapies. Several proteins from the family of Heat Shock Proteins (HSP) were effective in the prophylactic treatment and/or therapeutic treatment of numerous diseases including tumors, autoimmune diseases and various types of infections, including mycoses [19–21]. Reports showed that recombinant HSP60 and 90 (rHSP60 and rHSP90) conferred protection on mice against infection with *Histoplasma capsulatum* and *Candida albicans*, respectively [20, 22, 23]. Among the HSPs, previous studies have showed the immunomodulatory potential of HSP65 derived from *Mycobacterium leprae* (*M. leprae*) [24, 25]. This HSP was tested as a DNA vaccine (DNA-hsp65) and was capable of conferring protection on animals infected with *Mycobacterium tuberculosis*, *Leishmania major* and others [26–29]. Ribeiro and colleagues applied the DNA-hsp65 successfully in the prevention [28] and treatment [29] of experimental paracoccidioidomycosis, showing it to be promising for use in the treatment of other chronic mycoses.

After the direct intramuscular administration of plasmid DNA vaccine containing a gene coding

HSP65, it is disseminated throughout the body and its distribution is dose-dependent. HSP65 expression signals were detected for over 15 days after immunization with the vaccine. Another important aspect is that DNA-hsp65 does not replicate in mammalian cells and does not integrate with the DNA of those being immunized/treated [30, 31].

Considering these data, the goal of the current study was to test new strategies in CBM treatment, using DNA-hsp65 vaccine, alone or in association with current drugs such as itraconazole and amphotericin B. Our results suggest that treatment regimens using itraconazole or amphotericin B, intralesionally, are effective in treating experimental CBM, as is a therapy using naked DNA-hsp65 vaccine. More importantly, the chemotherapy associated with DNA-hsp65 vaccine is more efficient in the treatment of experimental CBM, accelerating the healing process.

Materials and Methods

DNA-hsp65 Plasmid Construction and Purification

DNA plasmid HSP65 was derived from the vector pVAX1 (Invitrogen, Carlsbad, CA, USA), as previously described [29].

Animals

Male Wistar rats (*Rattus norvegicus albinus*), 7–9 weeks old, were purchased from the University of Brasilia (UnB, Brasilia, DF, Brazil) and maintained under standard laboratory conditions. All experiments involving animals were approved by the Bioethical Committee of the University of Brasilia and conducted in accordance with their guidelines.

Fonsecaea Pedrosoi Strain and Infection

Fonsecaea pedrosoi strain ATCC 46428 was cultivated in Sabouraud Dextrose Agar medium (SDA, Himedia, Mumbai, India) supplemented with 100 mg l^{-1} chloramphenicol at 37 °C. To enhance fungal virulence and to adapt the strain to an animal host, the *F. pedrosoi* strain was inoculated twice into the footpad of the experimental animals at 10^7 cells ml^{-1} and recovered 30 days later in SDA medium. Virulent *F. pedrosoi* propagules containing hyphal fragments, conidiogenous cells and conidia were grown in potato dextrose medium (10 % potato and 1 % dextrose, supplemented with 100 mg l^{-1}) over 14 days in a rotary shaker (190 rpm) at 37 °C and were obtained after mechanical breakage followed by 10 min decantation of larger fragments. Supernatant was centrifuged at 3,000*g,* and a suspension was prepared, containing 10^7 viable cells ml^{-1} in sterile Phosphate buffered saline (PBS). Animals were then inoculated in each footpad with 200 µl of this suspension. The injured tissue was measured with a caliper 15, 30, 45, 60 and 90 days after inoculation for morphometric analysis.

Treatment

After 30 days of infection, the treatment was started by intralesional administration. Rats were divided into eight groups (8 animals per group): group I—rats non-infected and non-treated (HEALTHY); group II—rats infected and treated with PBS (INFECTED); group III—rats infected and treated with 50 µl of amphotericin B at 5 g ml^{-1}, every 3 days (AMB); group IV—rats infected and treated with 50 µl of itraconazole at 12 g ml^{-1}, every 3 days (ITZ); group V—rats infected and treated with 30 µl of DNAhsp65 at 0.3 g ml^{-1}, every 15 days (DNA-hsp65); group VI—rats infected and treated with 30 µl of pVAX1 at 0.3 g ml^{-1}, every 15 days (pVAX); group VII—rats infected and treated with a combination of the treatment in group III and V (AMB + DNA-hsp65); group VIII—rats infected and treated with a combination of the treatment in group IV and V (ITZ + DNA-hsp65). The animals were euthanized in CO_2 chamber 15 and 30 days after treatment started, and the injured tissue was measured with a caliper. Lesion tissue and serum samples were collected for sequential analysis.

Histopathology, Fungal Quantification and Fungal Burden

To evaluate lesion progress, injured tissue fragments were fixed in 10 % formalin for 6 h, dehydrated in alcohol and embedded in paraffin. Serial 5-µm

sections were stained with: hematoxylin–eosin (HE) to visualize the fungus and granulomatous appearances and Masson's trichrome to detect collagen fibers. For fungal quantification, the histological slides were analyzed under optical microscope and with the aid of a reticle coupled to the ocular it was possible to determine the amount of *F. pedrosoi* per mm^2 of injured tissue. Fungal burden in the lesion was measured by quantitative counts of colony-forming units (CFU) of *F. pedrosoi*. Injured tissues were homogenized in 1.0 mL of PBS (pH 7.2) and then plated onto SDA medium, supplemented with 100 $mg.l^{-1}$ chloramphenicol and cultivated at 37 °C for 7 days. Results were expressed as number of CFU ± standard error of the mean (SEM) per lesion.

Biochemistry Assay

For evaluation of the treatment's toxicity, aspartate aminotransferase (AST/SGOT) and alanine aminotransferase (ALT/SGPT) were measured from serum samples collected from all animals by cardiac puncture in an evacuated tube collector. AST and ALT were measured by spectrophotometry in a Modular P (Roche®), using reagents from the same brand.

Measurement of Nitric Oxide (NO) Concentration

NO production was evaluated by measuring the concentrations of NO_3 in serum. The serum nitrate concentration was determined by reducing nitrate to nitrite enzymatically, using nitrate reductase as described previously [32]. The total amount of nitrite was then determined using the Griess method [33]. The results are reported as μM of NO_3.

Experimental Reproducibility and Statistical Analysis

The results were obtained by averaging data in two independent assays. Differences between experimental groups were analyzed by one-way ANOVA followed by Bonferroni post-test method. All calculations and plotting were done using the statistical program GraphPad PRISM, version 5.0, GraphPad Software, San Diego, California, USA. P values were considered significant at $P < 0.05$. All values are mean ± SEM.

Results

Adjustment of Experimental Chromoblastomycosis

One of the biggest challenges in the study of CBM, especially concerning the development of new therapies and treatment combinations, was the absence of a reproducible animal model with lesions similar to those presented in humans. To establish a suitable experimental model for CBM, Wistar rats were infected with *F. pedrosoi* in the footpad so that after 15 days they developed lesions similar to those reported in humans, and the peak of the inflammatory response occurred, on average, 30 days after infection (Fig. 1a). At that time, an ulcerative lesion was observed, featuring a black crust at the inoculation point (Fig. 1c–d), when compared with the healthy mice (Fig. 1b). The fungal burden analyses confirmed the inability of the host to reduce fungal load until 60 days of infection. Only after 60 days were it possible to detect a significant decrease in fungal load in the infected tissue (Fig. 1h, i). Histopathological analysis revealed the presence of muriform cells surrounded by multinucleated giant cells, one of the main characteristics of chromoblastomycosis (Fig. 1l), even after 90 days of infection, although very few muriform cells remained viable (Fig. 1i). As a result, the chronic nature of the disease could be observed with the persistence of the lesion (Fig. 1e, f), with muriform cells in the damaged tissue, for at least 60 days after infection (Fig. 1m, n). After 90 days, the macroscopic aspect of the footpad was similar to that of healthy animals (Fig. 1g).

In order to characterize the course of the disease, histopathological examination of the infected tissue was carried out 30, 60 and 90 days after infection, making it possible to produce a detailed description of the histological features in experimental CBM.

Histological assessment during progress of the disease revealed histopathological variations with a progressive reduction in tissue composition and organizational elements, being involutive over time. In addition to the presence of muriform cells surrounded by multinucleated giant cells, all specimens showed the presence of a predominantly histiocytic inflammatory reaction in the footpad, associated with neutrophilic exudate foci and the presence of

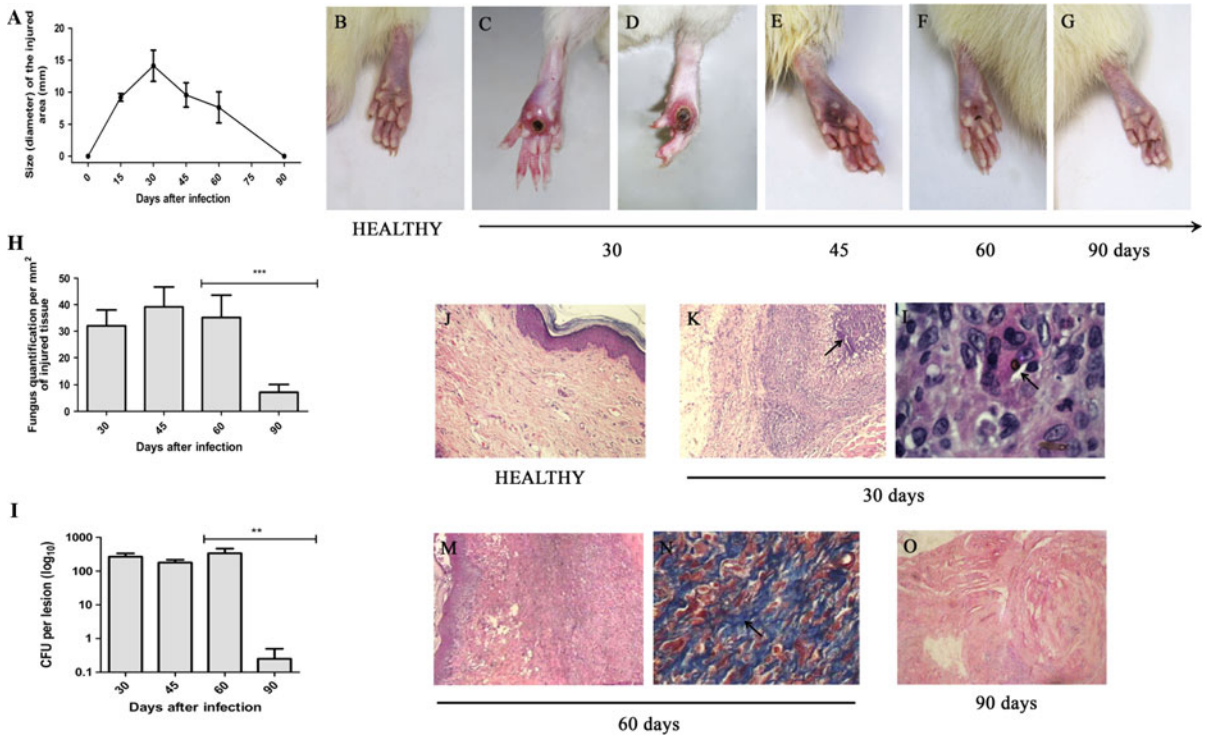

Fig. 1 Clinical evolution of CBM in rats infected with 2×10^6 propagules of the fungus *F. pedrosoi.* **a** Morphometry of the lesion; **b–g** morphological aspect of the disease over time; **b** healthy animal, **c–d** onset of ulceration 30 days after infection; **e** necrosis and fibrosis in the injured tissue after 45 days of the onset of infection; **f–g** scar healing after 60 days of infection; **h–i** quantification of fungal loads and fungal burden in damage tissue; **j–o** histopathology of the lesion 30, 60 and 90 days after infection, showing the presence of necrotic areas permeated by polymorphonuclear leukocytes and cellular debris (**k**, *arrow*) and the presence of muriform cells surrounded by multinucleated giant cells (**l**, *arrow*); **j–k**, **m**, **o** HE, ×100; (l) HE, ×400; (n) Masson's trichrome, ×400; Results were expressed as mean ± SEM (** $P < 0.01$, *** $P < 0.001$)

fibroblast proliferation areas, collagen deposition and vascular proliferation.

After 30 days of infection (Fig. 1k, l), the animals showed a histiocytic reaction, displaying cells in different stages of morphological differentiation. It became possible to observe monocytes, epithelioid histiocytes and multinucleated giant cells surrounding muriform cells (Fig. 1l). The exudative areas exhibited necrotic material (Fig. 1k) in which fungi forms such as muriform cells and hyphae were surrounded and permeated by polymorphonuclear leukocytes and cellular debris. These areas were more superficial, causing skin ulceration, particularly of the epidermis, when compared with the healthy mice.

At 60 days of infection (Fig. 1m, n), the lesions were less intense, more localized, showing fewer exudations, with hyalinization foci (Fig. 1n) and fewer newly formed vessels. Exudative foci were observed, as well as necrosis, both being limited to the dermis. The less intense histiocytic reaction was characterized by the presence of loose granulomas, although self-limited, but with epithelioid and multinucleated giant cells dissociated. There was no evidence of a lymphocytic cuff delimiting the lesions, even though diffuse neutrophilic infiltrate often appears permeating them. Giant cells of different sizes and shapes have been identified, many with the appearance of foreign body giant cells containing fungal particles in the cytoplasm.

By contrast, after 90 days of infection (Fig. 1o), there was a significant reduction in the elements described above, so that large areas of tissue repair became visible (with similar features to those found in uninfected animals). However, isolated necrosis foci were still observed, showing fungal forms such as muriform cells and fewer hyphae between leukocyte exudates.

Evaluation of Treatment Regimens

Once the experimental CBM model had been established, infected animals were then subjected to treatment with itraconazole or amphotericin B, as well as therapy using naked DNA-hsp65 vaccine, alone or in combination with the aforementioned drugs. After 15 and 30 days of treatment, the effectiveness of the tested treatment regimens was evaluated by observing the clinical aspect of the lesions and by macroscopic quantification of the lesion size with the aid of a caliper. It was possible to measure the progress of local inflammation, also a characteristic of CBM, as well as the tissue damage in the footpad of treated animals (Fig. 2).

Animals treated with PBS and pVAX, 15 and 30 days after treatment began, exhibited lesions with dimensions and clinical aspects similar to those described for only INFECTED animals, after 45 and 60 days of infection, respectively (data not shown).

After 15 days of treatment, except for the ITZ + DNA-hsp65 group (Fig. 2c), no significant variation in the diameter of the injured tissue was observed when comparing other treatment regimens to the untreated animals (Fig. 2a, c). However, after 30 days of treatment, all groups containing naked DNA vaccine showed a significant reduction in lesion size compared with the INFECTED group. On the other hand, animals treated only with amphotericin B (Fig. 2b) or itraconazole (Fig. 2c) showed no significant differences when compared with those animals treated with PBS (Fig. 2b, c). Likewise, it was observed that the footpad of animals treated with naked DNA vaccine was more similar to that found in healthy animals than to those only treated with drugs (Fig. 2a–c), indicating a possible modulation of the inflammatory response mediated by the DNA-hsp65 vaccine, which seems to have contributed to a significant reduction in tissue damage.

In order to confirm the significant reduction in tissue damage shown in animals treated with DNA vaccine, as well as to analyze the evolution of other histopathological aspects of the disease in animals of different groups, histopathological analysis of the lesion was evaluated after 15 (Fig. 3a, c, e, h, j, k) and 30 days (Fig. 3b, d, f, g, i, l) of treatment.

Animals treated with PBS or pVAX showed the same histopathological characteristics observed in animals infected and described above (data not

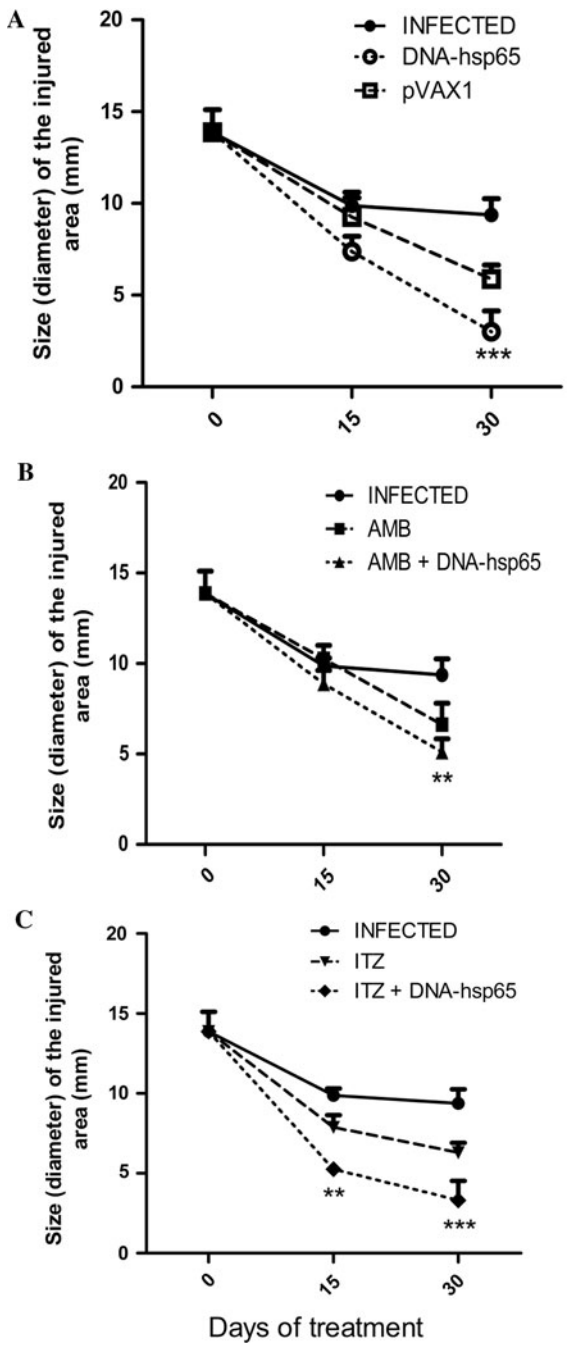

Fig. 2 Morphometric aspects of the lesion 15 and 30 days after treatment with: PBS solution (INFECTED), amphotericin B (AMB) and itraconazole (ITZ) alone or in association with DNA vaccine (DNA-hsp65); healthy animals and treatment using the vaccine vector (pVAX1) were used as control. **a** Animals treated with DNA-hsp65; **b** animals treated with AMB, alone or in association with DNA-hsp65; **c** animals treated with ITZ, alone or in association with DNA-hsp65. Results were expressed as mean ± SEM (* $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$ compared with infected animals)

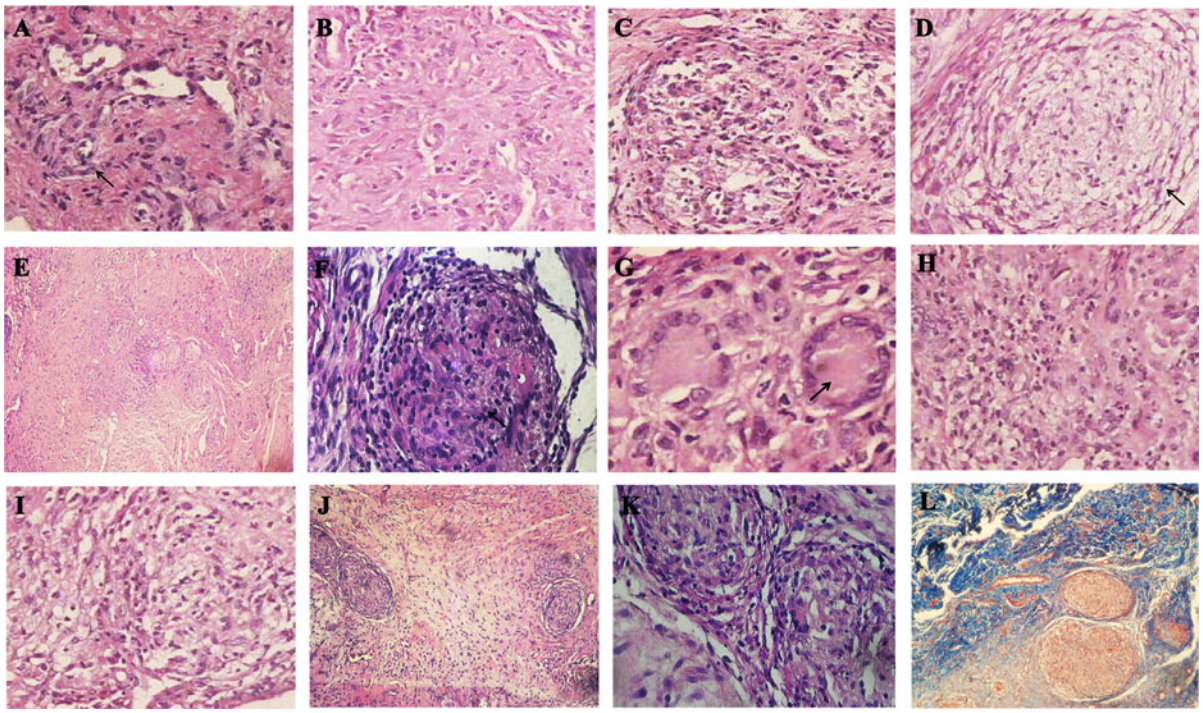

Fig. 3 Histopathological analysis 15 (**a**, **c**, **e**, **h**, **j**–**k**) and 30 days (**b**, **d**, **f**, **g**, **i**, **l**) after the treatment begins. **a**, **b** Animals treated with DNA-hsp65 showing small granulomas and inflammatory response with diffuse lymphocytic infiltrate (*arrow*); **c**, **d** animals treated with AMB showing **c** granulomas barely delimited after 15 days and **d** granulomas better delimited with no lymphocytic halo (*arrow*); **e**–**g** animals treated with AMB associated with DNA-hsp65 showing **e** intense mononuclear infiltration after 15 days and **f**, **g** a remarkable organization of the aspects that compose the lesion with well-defined granulomas after 30 days (*arrow*) and presence of Langerhans multinucleated giant cells (*arrow*); **h**, **i** animals treated with ITZ **h** after 15 days showing a diffuse inflammatory reaction with focal areas and **i** after 30 days exhibiting, eventually, giant cells alone or in small groups with no well-defined granulomas; **j**–**l** animals treated with ITZ associated with DNA-hsp65 exhibiting **j**, **k** morphological patterns with a predominance of epithelioid cells in well-defined lesions after 15 days and **l** well-defined granulomas rich in epithelioid and giant cells with dense collagen matrix separating them; **a**, **f**, **g**, **k** HE, ×400; **b**–**d**, **h**, **i** HE, ×200; **e**, **j** HE, ×100; **l** Masson's trichrome, ×200

shown). The lesions observed in the footpad of animals treated with DNA vaccine were characterized by the presence of histiocytes formation of granulomas and hyalinization areas. At 15 days of treatment, the group treated only with DNA-hsp65 (Fig. 3a) showed exudative granulomatous lesions, with extensive damage limited to the dermis, and small granulomas and inflammatory response with diffuse lymphocytic infiltrate (Fig. 3a). The exudative areas were delimited by the granulomatous process or by fibroblast reaction culminating in new collagen deposition vessels and presence of neutrophils.

In these group, at 30 days of treatment, the lesion had a clearly granulomatous aspect, with confluent lesions, widely and densely infiltrated by lymphocytes and histiocytes (Fig. 3b). There were no neutrophils in exudation; the granulomatous reaction was rich in epithelioid cells and multinucleated giant cells. These lesions were richly vascularized and circumscribed or permeated by collagen deposition that acquired a dense aspect as hyalinization took place. These granulomas extend from dermis to epidermis, with lymphocytes closely linked to the epithelial cells, giving rise to a lymphoepithelial lesion.

The animals treated with amphotericin B showed lesions similar to the INFECTED group after 15 days of infection, although reductions in those characteristics were observed after 30 days of treatment. Within 15 days of treatment with amphotericin B, the lesion showed few cells and exudative areas, dense collagenization and little neovascularization (Fig. 3c). Exudation areas presented a predominance of necrotic material, fewer polymorphonuclear cells limited to the dermis and little involvement of the epidermis. Within

30 days of treatment, granulomas were better delimited and can be described as isolated, showing no lymphocytic halo (Fig. 3d). Few multinucleated giant cells were shown, displaying a reduction in differentiated histiocytic cells and exhibiting fewer epithelioid cells compared with the more immature cells. A peculiar aspect of this group and at this time of infection is the small amount of polymorphonuclear infiltration.

After 15 days of treatment, the combination of amphotericin B and DNA vaccine induced diffuse lesions, with intense mononuclear infiltration (Fig. 3e). There were histiocytes, epithelioid cells and multinucleated giant cells. At 30 days of treatment, there was a remarkable organization of the aspects that compose the lesion, showing different features, with granulomas well defined (Fig. 3f), exudation foci with a central necrosis, usually delimited by histiocytic reaction with broad cytoplasm cells and roundish nuclei, and also surrounded by lymphocytes, fibroblasts, multinucleated giant cells and dense collagen. Granulomas appeared epithelioid, showing Langerhans multinucleated giant cells in the center surrounded by lymphocytic halo, often confluent (Fig. 3g).

The lesions observed in the footpad of animals treated with itraconazole are distinguished by a diffuse inflammatory reaction with focal areas. In its composition can be identified: histiocytes, epithelioid cells, multinucleated giant cells, also neutrophils and lymphocytes. In addition, fibroblasts, collagen deposition and newly formed vessels are recognized after 15 days of treatment (Fig. 3h). Fibroblasts are found in diffuse lesions associated with dense collagen, vascular slits, lymphocytes, histiocytes and some polymorphonuclear cells. The giant cells, taken together, were associated with areas where fungal cells are identified. In these areas, polymorphonuclear cells were also present, giving the lesion a loose but circumscribed aspect. After 30 days, collagen deposition areas were predominant with remodeling rather vascularized foci, eventually exhibiting giant cells alone or in small groups with no well-defined granulomas (Fig. 3i). At this stage, it was possible to observe exudative areas between collagen, in which histiocytes became thicker in the center and fungal forms were surrounded by giant cells. Exudation was bounded by collagen matrix and polymorphonuclear leukocytes (neutrophils) were scarce, being concentrated in exudative foci, as described.

Animals treated with the combination of itraconazole and DNA vaccine evidenced granulomatous lesions after 15 days of treatment, with the presence of usual cells. These injuries exhibited morphological patterns with a predominance of epithelioid cells in well-defined lesions (Fig. 3j). Multinucleated giant cells were observed in lesions of varying sizes with scarce exudative foci. There was still lymphocytic infiltrate, and in some areas, fibroblasts were prominent and appeared to individualize the granulomas. Within 30 days of treatment, there were well-defined granulomas, rich in epithelioid and giant cells, bordered by lymphocytes associated with a histiocytic infiltrate, and there were areas of histiocytes with a large and vacuolated cytoplasm (Fig. 3j, k) and also dense collagen matrix separating granulomas (Fig. 3l).

Fungal Quantification and Fungal Burden

To evaluate how treatment regimens are effective in eliminating fungal cells and therefore contributing to resolving the disease, quantification of histological fungal cells and fungal burden was carried out. After 15 days of treatment, there was a significant reduction in fungal cells in the histopathology of all animals treated with amphotericin B, itraconazole and a DNA vaccine, alone or in combination (Fig. 4a). Similar results occurred after 30 days, and a marked reduction in the number of fungi could be noted in all treated groups (Fig. 4a).

The fungal burden recovery confirmed histological quantification of the fungal cells in the lesion. The number of CFUs was reduced in all groups treated with amphotericin B, itraconazole and DNA vaccine, alone or in combination, both 15 and 30 days after treatment (Fig. 4b). In these same groups, there was a remarkable reduction in fungal viability after 30 days, whereas CFU count was not detectable in the group treated with the combination of itraconazole and DNA vaccine at these time.

Therefore, treatments using amphotericin B, itraconazole and a DNA vaccine were equally effective in reducing fungal load in infected tissue, although drugs associated with the DNA vaccine showed a tendency to maximize each pharmacological potential to eliminate the fungus (Fig. 4b).

NO Production and Biochemistry Assay

The NO_3 levels present in the serum from animals submitted to different treatment regimes were significantly reduced after 15 and 30 days, in animals

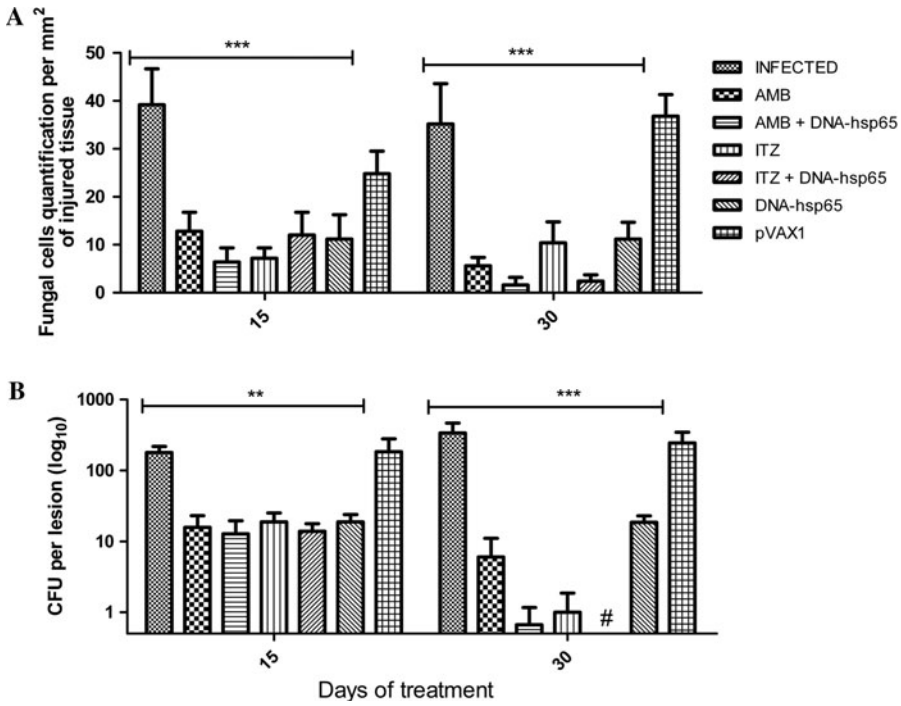

Fig. 4 Fungal cell quantification 15 and 30 days after treatment. **a** Fungal load per mm^2 of infected tissue and **b** CFU quantification per lesion in animals treated with: PBS (INFECTED), amphotericin B (AMB), amphotericin B associated with DNA vaccine (AMB + DNA-hsp65), itraconazole (ITZ), itraconazole associated with DNA vaccine (ITZ + DNA-hsp65), DNA vaccine alone (DNA-hsp65) and vaccine vector (pVAX1). # *Bar* equal to zero. Results were expressed as mean ± SEM (** $P < 0.01$, *** $P < 0.001$ compared with infected animals)

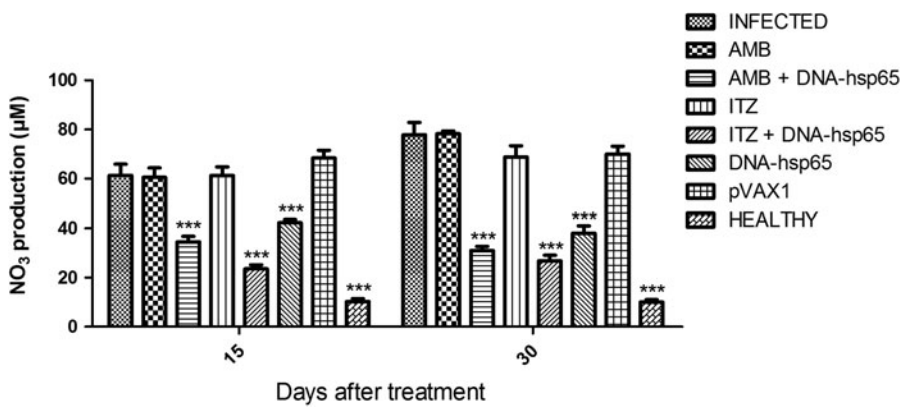

Fig. 5 NO_3 production after 15 and 30 days of the treatment begins. Animals were treated with PBS (INFECTED), amphotericin B (AMB), amphotericin B associated with DNA vaccine (AMB + DNA-hsp65), itraconazole (ITZ), itraconazole associated with DNA vaccine (ITZ + DNA-hsp65), DNA vaccine alone (DNA-hsp65) and vaccine vector (pVAX1). Results were expressed as mean ± SEM (*** $P < 0.001$ compared with infected animals)

treated with DNA vaccine alone, or in combination with itraconazole or amphotericin B, compared with animals treated with PBS, pVAX or itraconazole and amphotericin alone (Fig. 5).

In order to evaluate possible cytotoxic damage to the liver due to treatment regimens, parameters for liver toxicity were investigated, quantifying AST and ALT. As shown in Table 1, serum ALT and AST in all treated animals, at both 15 and 30 days after treatment, produced results that were very similar to those obtained in the INFECTED group, showing no significant differences when compared with other treatment groups. Although the literature shows hepatotoxicity during prolonged treatment with amphotericin B and itraconazole, there was no significant difference in treatment regimens tested in this work.

Table 1 Evaluation of liver toxicity indicators (AST and ALT), 15 and 30 days after treatment

	AST/SGOT (U/L)		*ALT*/SGPT (U/L)	
	15 days	30 days	15 days	30 days
HEALTHY	163 ± 43		39 ± 1	
INFECTED	306 ± 85	197 ± 31	53 ± 13	55 ± 10
AMB	169 ± 26	186 ± 25	68 ± 10	77 ± 2
AMB + DNA-hsp65	179 ± 20	268 ± 49	56 ± 9	71 ± 2
ITZ	173 ± 18	187 ± 12	57 ± 4	67 ± 1
ITZ + DNA-hsp65	235 ± 39	424 ± 191	64 ± 7	92 ± 18
DNA-hsp65	194 ± 24	200 ± 27	55 ± 2	55 ± 8
pVAX1	234 ± 102	182 ± 6	64 ± 8	55 ± 8

The results are expressed as U/L ± SEM

Discussion

Chromoblastomycosis is a subcutaneous chronic fungal disease that is difficult to treat, particularly in long-standing cases. Although oral itraconazole showed good results in short schedule treatments, in long-term follow-up, no single treatment is considered as the "gold standard." The long period of treatment required may cause patients to quit medication, resulting in recurrence of the disease. Immune response was not reestablished after 1 year of treatment with itraconazole, terbinafine or association of both. There was no increase in T cell proliferation and IFN-γ production. The high levels of TNF-α and IL-10 were not challenged after the treatment [34]. Therefore, the challenge in treating cutaneous and systemic mycosis is to develop better therapies and improve immune response.

In the present study, animals infected with *F. pedrosoi* developed typical CBM lesions in the footpad, very similar to those observed in humans, just after 30 days of infection. In the same way, histopathological aspects were like those observed in human CBM with the presence of muriform cells, exudative areas with necrotic material, abscesses rich in neutrophils and a characteristic granulomatous reaction, as described in literature [2, 3, 35]. Similar results were achieved by Xie and colleagues [36], a study which succeeded in the establishment of chronic injuries of CBM, for up to 4 months in Wistar rats infected with *Fonsecaea monophora*. Machado and colleagues [37] also succeeded in developing lesions very similar to those of human CBM, in the footpad of mice (BALB/c) infected with *F. pedrosoi*, but injuries did not become chronic.

Combinations of antifungal agents and immunotherapics have been successfully described for *P. brasiliensis* [38, 39]. Peptide P10 was identified in the glycoprotein Gp43 from *P. brasiliensis* and elicited the secretion of IFN-γ and other Th1 type cytokines. The combination of P10 and antifungal agents improved the therapeutic effectiveness of all drugs used, reflected in organ histological areas with very few or undetectable yeast cells [38, 40]. DNA-hsp65 vaccine also contributed to a reduction in pulmonary fungal burden, less lung tissue compromised by collagen deposition, and an increase in Th1 immune response in *P. brasiliensis* infected mice in both immunization and treatment approaches [28, 29].

The treatment with DNA-hsp65 and itraconazole reduced the lesion at all-time points analyzed. When evaluating the morphometric aspects of experimental CBM after 30 days of treatment, animals treated with DNA-hsp65 vaccine reduced lesions to levels below those shown by animals treated with itraconazole and amphotericin B alone (Fig. 2). Histopathological analyses confirmed the effectiveness of DNA-hsp65 vaccine in the treatment of experimental CBM alone, or in combination with tested drugs, contributing to a well-defined granuloma formation, with increased involvement of lymphocytes and neutrophils during infection (Fig. 3). Neutrophils have been related to innate resistance to fungi infections. Neutrophil deficient animals were more susceptible to fungal infections, proving the importance of these cells to host defenses [41]. Moreover, it has been demonstrated by ultrastructural analysis that neutrophils are able to destroy *F. pedrosoi* cells upon degranulation [42]. The lymphocytes, in their turn, play an equally important role in the control of fungal infections, so that athymic mice are highly susceptible to *F. pedrosoi* infection. In these animals, disease regressed after adoptive transfer of lymphocytes [9].

HSPs are recognized as important molecules in the modulation of the immune system, being able to activate a cellular immune response [43]. Several studies have demonstrated a potential use of HSP65 from *Mycobacterium leprae* as an immunomodulator [24, 25]. DNA-hsp65 vaccination protects mice against challenge with tuberculosis by establishing a cellular immune response that is dominated by

antigen-specific T lymphocytes that both produce IFN-γ and are cytotoxic toward infected cells (a type-1 cellular immune response). Both of these functions are probably required for maximally effective antimycobacterial immunity in mice and in humans. These responses are particularly favored by DNA vaccines [44]. In contrast, antigen-specific T cells that produce interleukin-4 (IL-4) and are not cytotoxic are abundant during infection with *M. tuberculosis* [45]. The treatment with DNA-hsp65 induced an increase in levels of Th1 cytokines, a standard protective response to PCM [28]. In the murine model of PCM, resistant mice are assumed to direct their immune response toward a preferential Th1 activation with production of IFN-γ, and efficient macrophage activation is able to contain fungal dissemination and disease progression [46].

The efficiency of treating chromoblastomycosis with DNA-hsp65 was also confirmed after fungal cell quantification and recovery, in that animals treated with DNA vaccine showed equally effective results to those obtained by groups of animals treated with amphotericin B and itraconazole. Animals treated with DNA-hsp65 associated with amphotericin B or itraconazole, particularly the association ITZ + DNA-hsp65, showed a tendency, albeit not statistically significant, to eliminate fungal cells completely from injuries (Fig. 4).

Analyzing NO_3 levels present in the serum of animals submitted to different treatment regimes, there was a significant reduction, after 15 and 30 days, in animals treated with DNA vaccine alone or in combination with itraconazole or amphotericin B, compared with animals treated with PBS, pVAX, itraconazole or amphotericin B (Fig. 5). However, as noted above, this reduction did not result in inefficiency in removing the pathogen, resulting, on the other hand, in less tissue damage. Nitric oxide (NO) is an important immune mediator in the removal of infectious agents while at higher doses it contributes to increased tissue damage during inflammatory response and immunosuppression [47]. NO decomposes spontaneously into nitrite (NO_2) and nitrate (NO_3). NO can be produced by phagocyte activation in the presence of IFN-γ, which induces nitric oxide synthase (iNOS), and it is determined by the presence of nitrate in animals' serum.

The reduction of tissue injury observed in groups treated with DNA vaccine is supported by the reduction in NO production. This fact that does not involve increased fungal load, which, instead, tends to be better controlled. It is noteworthy that NO_3 levels, detected in all groups that received the DNA vaccine, remain constant throughout the treatment, demonstrating the immunomodulatory action of the vaccine.

From a toxicological point of view, there were no hepatotoxic effects detected by biochemical analyses in drug concentrations and times tested. Possibly the dose and duration of treatment, likewise the route of administration, were essential to the absence of adverse effects in tested treatment regimens.

Given the above, it was found that itraconazole and amphotericin B, intralesionally, were effective in treating experimental CBM as well as the therapy using naked DNA-hsp65 vaccine. It was also shown that chemotherapy associated with DNA-hsp65 vaccine is even more efficient in the treatment of experimental CBM while accelerating the healing process of the disease, and in turn, becoming able to tone down the toxic effects from prolonged chemotherapy.

Acknowledgments The authors would like to thank the Fundação de Apoio à Pesquisa do Distrito Federal (FAPDF), Conselho Nacional de Pesquisa (CNPq) and Decanato de Pesquisa e Pós-Graduação da Universidade de Brasília (DPP/UnB) for financial support, CAPES for graduate students grants and Viviane Monteiro Leal for technical assistance in the immunohistochemical assay.

References

1. López Martínez R, Méndez Tovar LJ. Chromoblastomycosis. Clin Dermatol. 2007;25:188–94.
2. Queiroz-Telles F, Esterre P, Perez-Blanco M, Vitale RG, Salgado CG, Bonifaz A. Chromoblastomycosis: an overview of clinical manifestations, diagnosis and treatment. Med Mycol. 2009;47:3–15.
3. Esterre P, Peyrol S, Sainte-Marie D, Pradinaud R, Grimaud JA. Granulomatous reaction and tissue remodelling in the cutaneous lesion of chromomycosis. Virchows Archiv A Pathol Anat Histopathol. 1993;422:285–91.
4. Farbiarz SR, de Carvalho TU, Alviano C, De Souza W. Inhibitory effect of melanin on the interaction of *Fonsecaea pedrosoi* with mammalian cells in vitro. J Med Vet Mycol. 1992;30:265–73.
5. Bocca AL, Brito PPMS, Figueiredo F, Tosta CE. Inhibition of nitric oxide production by macrophages in chromoblastomycosis: a role for *Fonsecaea pedrosoi* melanin. Mycopathologia. 2006;161:195–203.
6. Cunha MML, Franzen AJ, Seabra SH, Herbst MH, Vugman, NV, Borba LP, De Souza W, Rosental S. Melanin in *Fonsecaea pedrosoi*: a trap for oxidative radicals. BMC Microbiol. 2010;10:80.

7. Esterre P, Jahevitra M, Andriantsimahavandy A. Humoral immune response in chromoblastomycosis during and after therapy. Clin Diagn Lab Immunol. 2000;7:497–500.
8. Gimenes MF, Souza MG, Ferreira KS, Marques SG, Gonçalves AG, Santos VCL. Pedroso e Silva CDM, Almeida SR. Cytokines and lymphocyte proliferation in patients with different clinical forms of chromoblastomycosis. Microbes Infect. 2005;7:708–13.
9. Ahrens J, Graybill JR, Abishawl A, Tio FO, Rinaldi MG. Experimental murine chromomycosis mimicking chronic progressive human disease. Am J Trop Med Hyg. 1989;40:651–8.
10. Lopes CF, Alvarenga RJ, Cisalpino EO, Resende MA, Oliveira LG. Six years' experience in treatment of chromomycosis with 5-fluorocytosine. Int J Dermatol. 1978;17:414–8.
11. Diaz M, Negroni R, Montero-Gei F, Castro LG, Sampaio SA, Borelli D, Restrepo A, Franco L, Bran JL, Arathoon EG. A Pan-American 5-year study of fluconazole therapy for deep mycoses in the immunocompetent host. Pan-American Study Group. Clin Infect Dis. 1992;14(Suppl 1):S68–76.
12. Arce-Fonseca M, Ramos-Ligonio A, López-Monteón A, Salgado-Jiménez B, Talamás-Rohana P, Rosales-Encina JL. A DNA vaccine encoding for TcSSP4 induces protection against acute and chronic infection in experimental chagas disease. Int J Biol Sci. 2011;7:1230–8.
13. Domínguez-Bernal G, Horcajo P, Orden JA, De La Fuente R, Herrero-Gil A, Ordóñez-Gutiérrez L, Carrion J. Mitigating an undesirable immune response of inherent susceptibility to cutaneous leishmaniosis in a mouse model: the role of the pathoantigenic HISA70 DNA vaccine. Vet Res. 2012;43:59.
14. Wu W, Huang L, Mendez S. A live Leishmania major vaccine containing CpG motifs induces the de novo generation of Th17 cells in C57BL/6 mice. Eur J Immunol. 2010;40:2517–27.
15. Abdul-Wahid A, Faubert G. Mucosal delivery of a transmission-blocking DNA vaccine encoding *Giardia lamblia* CWP2 by *Salmonella typhimurium* bactofection vehicle. Vaccine. 2007;25:8372–83.
16. Gu Q-L, Huang X, Ren W-H, Shen L, Liu B-Y, Chen S-Y. Targeting hepatitis B virus antigens to dendritic cells by heat shock protein to improve DNA vaccine potency. World J Gastroenterol. 2007;13:5911–7.
17. Okada M, Kita Y. Tuberculosis vaccine development: the development of novel (preclinical) DNA vaccine. Hum Vaccin. 2010;6:297–308.
18. Li JL, Liu HL, Zhang XR, Xu JP, Hu WK, Liang M, Chen SY, Hu F, Chu DT. A phase I trial of intratumoral administration of recombinant oncolytic adenovirus overexpressing HSP70 in advanced solid tumor patients. Gene Ther. 2009;16:376–82.
19. Oglesbee MJ, Pratt M, Carsillo T. Role for heat shock proteins in the immune response to measles virus infection. Viral Immunol. 2002;15:399–416.
20. Scheckelhoff M, Deepe GS. The protective immune response to heat shock protein 60 of *Histoplasma capsulatum* is mediated by a subset of V beta 8.1/8.2 + T cells. J Immunol. 2002;169:5818–26.
21. Ferraz JC, Stavropoulos E, Yang M, Coade S, Espitia C, Lowrie DB, Colston MJ, Tascon RE. A heterologous DNA priming-Mycobacterium bovis BCG boosting immunization strategy using mycobacterial Hsp70, Hsp65, and Apa antigens improves protection against tuberculosis in mice. Infect Immunol. 2004;72:6945–50.
22. Matthews RC, Burnie JP, Howat D, Rowland T, Walton F. Autoantibody to heat-shock protein 90 can mediate protection against systemic candidiasis. Immunology. 1991;74:20–4.
23. Matthews R, Hodgetts S, Burnie J. Preliminary assessment of a human recombinant antibody fragment to hsp90 in murine invasive candidiasis. J Infec Dis. 1995;171:1668–71.
24. Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, Colston MJ, Hewinson RG, Moelling K, Silva CL. Therapy of tuberculosis in mice by DNA vaccination. Nature. 1999;400:269–71.
25. Silva CL, Bonato VLD, Coelho-Castelo AAM, De Souza AO, Santos SA, Lima KM, Faccioli LH, Rodrigues JM. Immunotherapy with plasmid DNA encoding mycobacterial hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice. Gene Ther. 2005;12:281–7.
26. de Paula L, Silva CL, Carlos D, Matias-Peres C, Sorgi CA, Soares EG, Souza PRM, Bladés CR, Galleti FC, Bonato VL, Gonçalves ED, Silva EV, Faccioli LH. Comparison of different delivery systems of DNA vaccination for the induction of protection against tuberculosis in mice and guinea pigs. Genet Vaccines Ther. 2007;5:2.
27. Coelho EA, Tavares CA, Lima KM, Silva CL, Rodrigues JM Jr, Fernandes AP. *Mycobacterium* hsp65 DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against *Leishmania* (*Leishmania*) *major* infection. Parasitol Res. 2006;98:568–75.
28. Ribeiro AM, Bocca AL, Amaral AC, Faccioli LH, Galetti FCS, Zárate-Bladés CR, Figueiredo F, Silva CL, Felipe MS. DNAhsp65 vaccination induces protection in mice against *Paracoccidioides brasiliensis* infection. Vaccine. 2009;27:606–13.
29. Ribeiro AM, Bocca AL, Amaral AC, Souza ACO, Faccioli LH, Coelho-Castelo AAM, Figueiredo F, Silva CL, Felipe MS. HSP65 DNA as therapeutic strategy to treat experimental paracoccidioidomycosis. Vaccine. 2010;28:1528–34.
30. Coelho-Castelo AAM, Trombone AP, Rosada RS, Santos RR, Bonato VLD, Sartori A, Silva CL. Tissue distribution of a plasmid DNA encoding Hsp65 gene is dependent on the dose administered through intramuscular delivery. Genet Vaccines Ther. 2006;4:1.
31. Coelho-Castelo AAM, Santos Júnior RR, Bonato VLD, Jamur MC, Oliver C, Silva CL. B-lymphocytes in bone marrow or lymph nodes can take up plasmid DNA after intramuscular delivery. Hum Gene Ther. 2003;14:1279–85.
32. Schmidt HH, Wilke P, Evers B, Böhme E. Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol. Biochem Bioph Res Com. 1989;165:284–91.
33. Green LC, Tannenbaum SR, Goldman P. Nitrate synthesis in the germfree and conventional rat. Science. 1981;212:56–8.
34. Gimenes VMF, Criado PR, Martins JEC, Almeida SR. Cellular immune response of patients with chromoblastomycosis

undergoing antifungal therapy. Mycopathologia. 2006;162: 97–101.
35. de Melo-Júnior MR, de Lima-Neto RG, Lacerda AM, Beltrão EIC. Comparative analysis of extracellular matrix and cellular carbohydrate expression in the sporotrichosis and chromoblastomycosis. Mycopathologia. 2011;171:403–9.
36. Xie Z, Zhang J, Xi L, Li X, Wang L, Lu C, Sun J. A chronic chromoblastomycosis model by *Fonsecaea monophora* in Wistar rat. Med Mycol. 2010;48:201–6.
37. Machado AP, Silva MRR, Fischman O. Local phagocytic responses after murine infection with different forms of *Fonsecaea pedrosoi* and sclerotic bodies originating from an inoculum of conidiogenous cells. Mycoses. 2011;54: 202–11.
38. Marques AF, da Silva MB, Juliano MAP, Travassos LR, Taborda CP. Peptide immunization as an adjuvant to chemotherapy in mice challenged intratracheally with virulent yeast cells of *Paracoccidioides brasiliensis*. Antimicrob Agents Chemother. 2006;50:2814–9.
39. Amaral AC, Marques AF, Muñoz JE, Bocca AL, Simioni AR, Tedesco AC, Morais PC, Travassos LR, Taborda CP, Felipe MS. Poly(lactic acid-glycolic acid) nanoparticles markedly improve immunological protection provided by peptide P10 against murine paracoccidioidomycosis. Br J Pharmacol. 2010;159(5):1126–32.
40. Marques AF, da Silva MB, Juliano MAP, Munhõz JE, Travassos LR, Taborda CP. Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*. Microbes Infect. 2008;10(12–13):1251–8.
41. Zelante T, Montagnoli C, Bozza S, Gaziano R, Bellocchio S, Bonifazi P, Moretti S, Moretti S, Fallarino F, Puccetti P, Romani L. Receptors and pathways in innate antifungal immunity: the implication for tolerance and immunity to fungi. Adv Exp Med Biol. 2007;590:209–21.
42. Rozental S, Alviano CS, de Souza W. Fine structure and cytochemical study of the interaction between *Fonsecaea pedrosoi* and rat polymorphonuclear leukocyte. J Med Vet Mycol. 1996;34:323–30.
43. Fałkowska-Podstawka M, Wernicki A. Heat shock proteins in health and disease. Pol J Vet Sci. 2003;6(1):61–70.
44. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol. 1997;15:617–48.
45. Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J Immunol. 1993;151:518–25.
46. Cano LE, Kashino SS, Arruda C, André D, Xidieh CF, Singer-Vermes LM, Vaz CA, Burger E, Calich VL. Protective role of gamma interferon in experimental pulmonary paracoccidioidomycosis. Infect Immunol. 1998;66:800–6.
47. Bocca AL, Hayashi EE, Pinheiro AG, Furlanetto AB, Campanelli AP, Cunha FQ, Figueiredo F. Treatment of *Paracoccidioides brasiliensis*-infected mice with a nitric oxide inhibitor prevents the failure of cell-mediated immune response. J Immunol. 1998;161:3056–63.