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Vaccination with Heat Shock Protein 60 Induces a Protective Immune Response against Experimental *Paracoccidioides brasiliensis* Pulmonary Infection[▽]

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Paracoccidioides brasiliensis **causes a chronic granulomatous mycosis prevalent in Latin America. The successful resolution of infection with this fungus is dependent on the activation of cellular immunity. We previously identified heat shock protein 60 (HSP60) as a target of the humoral response in paracoccidioidomycosis. Herein we expressed the gene encoding HSP60 in *Escherichia coli* and analyzed the immunological activity of this recombinant antigen. The immunization of BALB/c mice with recombinant protein emulsified in adjuvant stimulated a cellular immune response. Splenocytes from immunized mice proliferated in response to antigen and released interleukin-12 and gamma interferon (IFN-γ). Vaccination with HSP60 reduced the fungal burden in mice given 10^6 or 10^7 yeasts and protected mice from a lethal challenge. The efficacy of the vaccination was blunted by the neutralization of IFN-γ. $CD4^+$ cells were necessary for the efficacy of the vaccination in both the afferent and efferent phases. Thus, we have demonstrated that this immunodominant antigen is a candidate for the development of a vaccine against this fungus.**

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM), a deep mycosis endemic to Latin America, including Brazil (43). An infection is caused by the inhalation of airborne conidia. PCM exhibits a wide spectrum of clinical and pathological manifestations, ranging from benign and localized forms to severe disseminated disease (10, 34). In most cases, the infection is restricted primarily to the lungs but can disseminate to other organs (43).

Experimental (14, 15, 45) and clinical (12, 36, 37, 38) studies have indicated the relevance of humoral and/or cellular immune responses in the pathogenesis and evolution of PCM. Specific T-cell-mediated immune responses play a fundamental role in the resistance to *P. brasiliensis*. Compared to individuals with localized disease, patients with systemic PCM display several defects in cellular immunity characterized by impaired in vivo delayed type hypersensitivity reactivity, alterations in the ratio of $CD4^+$/$CD8^+$ cells, an imbalance in cytokine levels, and a depressed in vitro T-cell proliferation to fungal antigens (2, 5, 6, 7, 11, 37, 38).

Heat shock protein 60 (HSP60) from fungal, protozoal, and bacterial pathogens has been identified as a prominent antigen recognized by $CD4^+$ cells. Recombinant preparations of pathogen-derived HSP60 have been successfully used as vaccines in infection models (18, 24, 31, 35, 39, 40, 42, 48, 49). Vaccination with recombinant or native *Histoplasma capsulatum* HSP60 reduces the fungal burden in mice given a sublethal inoculum of yeast cells and improves survival in animals challenged intranasally with a lethal inoculum (20, 24). A surface form of *H. capsulatum* HSP60 is involved in the adhesion of parasite yeast cells to host macrophages via interaction with CD18 integrin receptors (32).

Intrigued by the immunological properties of the highly conserved family of HSP60 proteins, our group isolated the complete coding cDNA of HSP60 from *P. brasiliensis* (PbHSP60). Initially, we studied the humoral immune responses to this antigen among human subjects. By immunoblot analysis, recombinant PbHSP60 was recognized by antibodies in 72 of 75 sera from paracoccidioidomycosis patients. No cross-reactivity was detected with individual sera from patients with aspergillosis, sporotrichosis, cryptococcosis, and tuberculosis. Reactivity to HSP60 was observed in sera from 9.52% of control healthy individuals and 11.5% of patients with histoplasmosis (17, 26).

In the present study, we developed a murine model of pulmonary PCM in order to examine the cellular immune responses against PbHSP60 antigen. We endeavored to delineate the protective immune response elicited by recombinant HSP60 in the inductive and expressive phases of vaccine-induced immunity and identified the cytokines and T-cell subpopulations responsible for HSP60 protective properties. Our results suggest that vaccination with the recombinant protein PbHSP60 conferred protection in mice against a pulmonary PCM. The depletion of $CD4^+$ cells during the inductive and expressive phases of vaccination abrogated the protective activity of PbHSP60.

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MATERIALS AND METHODS

Animals. BALB/c (*H-2^d*) mice were purchased from Jackson Laboratory, Bar Harbor, ME. The animals were housed in isolator cages and were maintained by the University of Cincinnati Department of Laboratory Animal Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Medicine. All animal experiments were done in accordance with the Animal Welfare Act guidelines of the National Institutes of Health.

Preparation of *P. brasiliensis* yeast cells and infection of mice. *P. brasiliensis* isolate 01 (ATCC MYA-826) was cultured at 37°C in liquid Ham's F-12 medium supplemented with 18 g/liter of glucose, 0.6% HEPES, 1% cystine, 0.1% L-glutamic acid at 37°C in an orbital incubator at 200 rpm. After 7 days, log-phase cultures were harvested by centrifugation and washed three times with Hanks balanced salt solution (HBSS; Mediatech, Inc., Herndon, VA), and yeasts were enumerated in a hemocytometer. Experimental infection was initiated by lightly anesthetizing the animals with 3% isoflurane, followed by intranasal inoculation with 1×10^6, 1×10^7, or 3×10^7 *P. brasiliensis* yeast cells in a volume of 50 μl.

PbHSP60 production. To generate recombinant protein, the intron-free PbHSP60 was incubated with the following set of primers (the positions of the sense bases are given in relation to the ATG starting codon): NH_2-terminal sense (bases 1 to 17; 5′-GCGGCCGCCATATGCAGCGAGCTTTTAC-3′) and carboxy-terminal antisense (bases 1764 to 1776; 5′-GCGGCCGCCAGATCTCTG AACATACCCCCG-3′). The sense primer introduces an NdeI site (underlined), and the carboxy-terminal antisense primer introduces a BglII site (underlined). The material was amplified by PCR using the following conditions: 94°C for 60 s and 72°C for 3 min for 35 cycles with High Fidelity *Taq* polymerase (Invitrogen, San Diego, CA). The gene product was gel purified and cloned into the NdeI and BamHI sites of pET19b (Novagen, Madison, WI). The plasmids were transformed into BL21(DE3) lysogen host cells. To express the recombinant protein, transformed *Escherichia coli* cells were grown in Luria-Bertani medium (1,000 ml) at 37°C in a shaking incubator with the Overnight Express autoinduction system (Novagen, Madison, WI) at 200 rpm overnight. The cells were harvested by centrifugation at 5,000 × *g*. *E. coli* pellets were resuspended in a buffer consisting of 10 mM imidazole, 500 mM NaCl, and 50 mM Tris-Cl (pH 7.9) and lysed by a freeze-thaw cycle followed by sonication. The soluble and insoluble fractions were separated by centrifugation at 20,000 × *g*. The insoluble pellet fraction was found to contain the recombinant product when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

HSP60 purification. The insoluble pellet fraction was resuspended by sonication in a denaturing solubilization buffer consisting of 4 M urea, 500 mM NaCl, 10 mM imidazole, and 50 mM Tris (pH 7.9). The denatured material was recovered in the supernatants after centrifugation at 20,000 × *g* and filtered through a 0.22-μm-pore-size cellulose acetate membrane to remove particulate material. The protein was purified by metal chelate affinity chromatography with a Ni^{2+}-Sepharose affinity column (His-Bind; Novagen) and washed with 60 mM imidazole. The recombinant product was eluted with 140 mM imidazole in the same buffer. The eluted material was dialyzed against 20 mM Tris (pH 7.4)-200 mM NaCl containing decreasing concentrations of urea. The eluate was concentrated by ultrafiltration, and the protein concentration was determined by the Bradford method (9).

Immunization of mice with PbHSP60. Groups of mice were immunized subcutaneously at the base of the tail with PbHSP60 or an equal amount of bovine serum albumin (BSA). Antigens were suspended in adjuvant containing monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and cell wall skeleton (Corixa, Hamilton, MT). The cell wall skeleton was derived from streptococcal species. The animals were injected subcutaneously with 0.1 ml of emulsion (10 μg of protein) twice. The injections were separated by 2 weeks. The BSA was purchased from Sigma-Aldrich (St. Louis, MO).

Depletion of T-cell subsets and IFN-γ neutralization. To determine the influence of $CD4^+$ or $CD8^+$ cells on the inductive phase of vaccination, the mice were injected intraperitoneally with 100 μg of monoclonal antibody (MAb) to CD4 or CD8 on days −7 and −3 and on the day of the first immunization. This amount of MAb achieved a ≥95% depletion of both cell populations as determined by flow cytometry. Subsequently, the mice received 100 μg of MAb each week until the end of the vaccination. For studies of the expressive phase, treatment with MAb was begun after vaccination and continued until the experiments were terminated. The mice were challenged with *P. brasiliensis* 2 weeks postvaccination. In some experiments, the mice were treated with MAb to gamma interferon (IFN-γ) (rat immunoglobulin G2b [IgG2b] cell line XMG-1.2) purchased from the National Cell Culture Center (Minneapolis, MN) or rat IgG. The mice received 1 mg of antibody every week.

Organ culture for *P. brasiliensis*. Groups of four to six mice of each treatment were sacrificed at 2, 4, and/or 6, 8, or 12 weeks postinfection. The lungs, livers, and spleens were removed aseptically and homogenized individually in 10 ml of sterile saline solution by a Teflon tissue grinder. Homogenates were diluted serially and 0.1 ml of each dilution was plated in duplicate onto brain heart infusion agar (Difco; 5% agar [wt/vol] supplemented with 4% [vol/vol] fetal bovine serum [Atlanta Biologicals]) and 10 μg of gentamicin per ml. The plates were incubated at 37°C, and the colonies were counted daily until no increase in counts was observed. The fungal burden was expressed as the mean number of CFU per whole organ ± the standard error of the mean (SEM). The limit of detection was 10^2 CFU.

Splenocyte preparation. The spleens from mice were teased apart between the frosted ends of two glass slides. The cells were washed three times with HBSS (BioWhittaker, Walkersville, MD) and resuspended at a concentration of 2.5×10^6 cells per ml in RPMI medium, containing 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 10 μg of gentamicin/ml.

Proliferation assay. Male BALB/c mice were subcutaneously immunized twice with 10 μg or 100 μg of PbHSP60 in an adjuvant containing monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and cell wall skeleton (Corixa, Hamilton, MT). The control mice were injected with BSA alone. The injections of antigen were separated by 2 weeks. Splenocytes were prepared as described above. To each well of a microtiter plate, 5×10^5 splenocytes in 0.1 ml of medium were added (RPMI 1640 containing 10% fetal bovine serum, 10 mg of gentamicin/ml); 50 μl of either medium or antigen (PbHSP60) was added to each well in different concentrations (0.5, 2.5, 5, 25, or 50 μg/ml). The cells were incubated for 120 h. The cells were pulsed 18 h before being harvested with the addition of 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, MA). The cells were harvested to glass fiber filter paper, and the incorporation of radioactivity was measured by a liquid scintillation counter. Activity was analyzed as the stimulation index: the counts per minute of cells stimulated with antigen divided by the counts per minute of cells in media alone. Reactivity to PbHSP60 was defined as a stimulation index, indicating a ≥2.5-fold increase in the proliferation of cells compared to that of cells that were cultured in medium alone. The experiments were performed at least three times.

Determination of cytokine production in vitro. Splenocyte suspensions were prepared from BALB/c mice immunized with PbHSP60 or BSA at serial intervals after vaccination. A total of 5×10^5 splenocytes per well of suspension was added to each well of a 24-well plate. The cells were exposed to 0, 0.5, 1.0, or 10 μg of PbHSP60 in a volume of 25 μl or were incubated with an equal volume of buffer. The cell suspensions were cultured for 24 h at 37°C in 5% CO_2, and the supernatants from the cultures were harvested, filter sterilized, and stored at −70°C until assayed. The cytokine concentrations in the splenocyte supernatants were determined by antigen capture enzyme-linked immunosorbent assay (ELISA). We measured IFN-γ, interleukin-4 (IL-4), IL-12p70, tumor necrosis factor alpha (TNF-α; Endogen, Cambridge, MA), and IL-10 (BD Biosciences, San Diego, CA.). We expressed the results as the change in the level of cytokine by subtracting the amount of cytokine detected in nonstimulated splenocytes from that found in the supernatant of antigen-stimulated cells.

Preparation of lung leukocytes. Lungs were teased apart with the frosted ends of two glass slides and digested with collagenase (1 mg/ml) and DNase (20 μg/ml; Sigma-Aldrich). The solution was filtered through 60-μm nylon mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA) and washed three times with HBSS. Leukocytes were isolated by gradient density centrifugation using Lympholyte-M (Cedarlane Laboratories, Hornby Ontario, Canada).

Reagents and flow cytometry. The following antibodies were purchased from BD Biosciences: CD62L- and CD11c-allophycocyanin; B220-, Mac3-, and Ly-6G-phycoerythrin; Ly-6C-biotin; and CD8α conjugated to streptavidin-peridin-chlorophyll protein. CD205-phycoerythrin was purchased from Miltenyi Biotec (Auburn, CA). The cells (10^6/ml) were incubated with 0.5 μg of antibody in staining buffer (1% BSA in phosphate-buffered saline) for 30 min at 4°C. Nonspecific staining was blocked by preincubation with MAb to CD16 (BD Biosciences). The cells were washed in staining buffer, and fluorescence was measured using a FACScaliber flow cytometer (BD Biosciences). Between 50,000 to 100,000 events were counted.

Histology. The lungs were inflated, excised, fixed in 10% formalin, and embedded in paraffin blocks. Sections (5 μm) were stained with hematoxylin and eosin. Analysis of the sections was performed in a blinded fashion.

Statistical analysis. The protective activity of PbHSP60 was determined by comparing the organism burden in organs from immunized and control animals using analysis of variance (ANOVA). A P value of <0.05 was considered significant. The levels of cytokine production were compared between mice immunized with PbHSP60 or with BSA and analyzed by ANOVA.

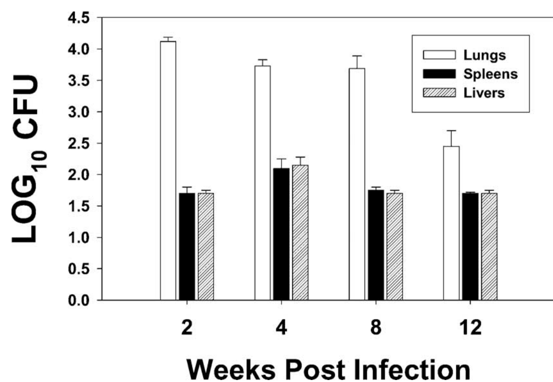

FIG. 1. Time course of *P. brasiliensis* infection. Mice were infected i.n. with 1×10^6 *P. brasiliensis* isolate 01 yeast cells, and groups of mice were euthanized at weeks 2, 4, 8, and 12 postinfection. Each bar represents the mean (± SEM) of the results from six animals. Range bars represent SEM. One of two experiments is shown.

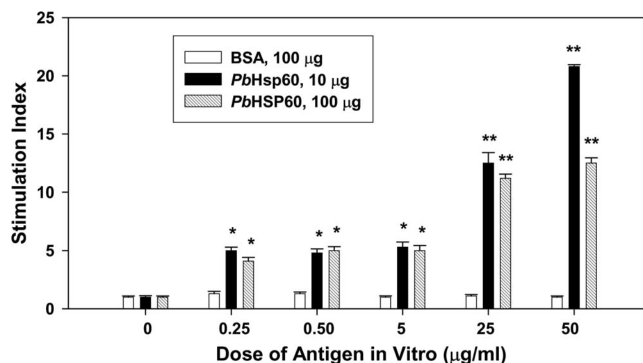

FIG. 2. PbHSP60 is a target of the cellular immune response to *P. brasiliensis*. Increasing concentrations of PbHSP60 (0.5, 2.5, 5, 25, or 50 μg/ml) were incubated with 5×10^5 splenocytes from BSA-immunized mice or mice immunized with 10 or 100 μg of *P. brasiliensis* recombinant HSP60. [^{3}H]thymidine incorporation was measured after 6 days of culture. Means (± SEM) of the results from three experiments are illustrated. *, *P* of <0.05 by ANOVA compared to controls; **, *P* of <0.01 by ANOVA compared to controls.

RESULTS

***P. brasiliensis* pulmonary infection and dissemination.** Initially, we sought to establish the course of infection in BALB/c mice infected with *P. brasilieusis* 01. Mice were infected with a putative nonlethal inoculum of 10^6 yeasts intranasally. The burden of infection was monitored by CFU counts in the lungs, livers, and spleens at 2, 4, 8, and 12 weeks postinfection. The numbers of CFU in the lungs of BALB/c mice at weeks 2, 4, and 8 postinfection were similar. Thereafter, the CFU counts in the lungs declined by week 12. Yeast cells were recovered from the livers and spleens during weeks 2 to 12, and their numbers remained constant over that interval (Fig. 1). Thus, we had established that isolate 01 could infect and disseminate in BALB/c mice and that 10^6 yeasts could disseminate to lymphoid and visceral organs from the lungs.

Splenocytes from mice injected with yeast cells or with PbHSP60 proliferate in response to PbHSP60. To determine if PbHSP60 elicits a specific cellular immune response, BALB/c mice were immunized subcutaneously twice with 10 μg or 100 μg of PbHSP60 or with BSA as a control. Figure 2 shows that splenocytes from animals immunized with 10 μg of PbHSP60 or with 100 μg of PbHSP60 proliferate when exposed to the protein in vitro, while splenocytes from control mice did not. The proliferative response by cells from animals injected with 10 μg of antigen exceeded that of mice immunized with 100 μg of PbHSP60 at the higher in vitro concentrations.

Since the response to the higher amount of PbHSP60 led to a decrease in proliferation, we analyzed whether this amount was toxic to cells. We incubated splenocytes from mice vaccinated with BSA or with 10 μg or 100 μg of PbHSP60 and examined the percentage of viable cells following incubation for 5 days in microtiter plates. In cells from BSA-immunized mice, viability was 68.3% ± 4.7% (n = 3) in cells incubated with the smaller amount and 66.8% ± 6.3% in cells incubated with 100 μg of antigen. In splenocytes from PbHSP60-immunized mice, cell viability was 88.3% ± 5.9% in 10 μg and 85.6% ± 3.9% in 100 μg of PbHSP60. Thus, toxicity did not account for the differences.

PbHSP60 vaccination specifically enhances the in vitro production of IL-12 and IFN-γ. Splenocytes from mice injected with PbHSP60 or BSA, as a control, were stimulated with 10 μg of PbHSP60 or an equal amount of BSA in vitro, respectively. The production of IFN-γ, IL-4, IL-10, IL-12, and TNF-α by splenocytes was assessed by ELISA. The production of IL-12 and IFN-γ by splenocytes from mice immunized with PbHSP60 was significantly greater ($P < 0.01$) than that of the control group (Fig. 3). IL-12 production peaked at day 7 following the second vaccination. IFN-γ production exceeded that of the controls at each time point and was maximal on day 7 after the second vaccination. On the other hand, we failed to detect elevations during this period of vaccination in the levels of IL-4, IL-10, and TNF-α (data not shown).

Protective activity of PbHSP60. We sought to determine whether the observed Th1 T-cell recognition of PbHSP60 an-

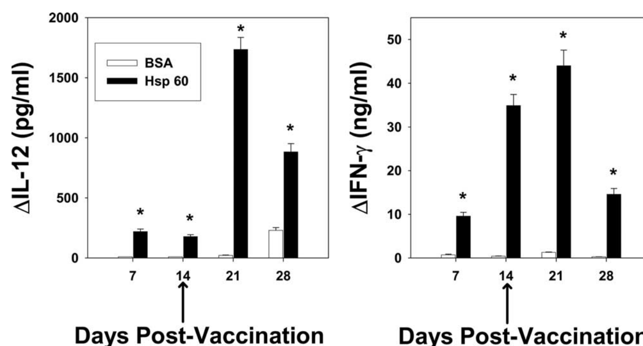

FIG. 3. Cytokine levels in supernatants from the splenocytes of mice injected with BSA or PbHSP60 and stimulated with cognate antigen. At serial intervals after immunization, spleen cells from mice were stimulated for 48 h with equal amounts of BSA, or PbHSP60, and the levels of IL-12 and IFN-γ in the supernatants were determined by ELISA. The arrows under the *x* axes indicate the time of the second injection of antigen. The results represent the means ± SEM for eight mice at each time point. *, *P* of ≤0.01 compared to BSA. The results were pooled from two independent experiments.

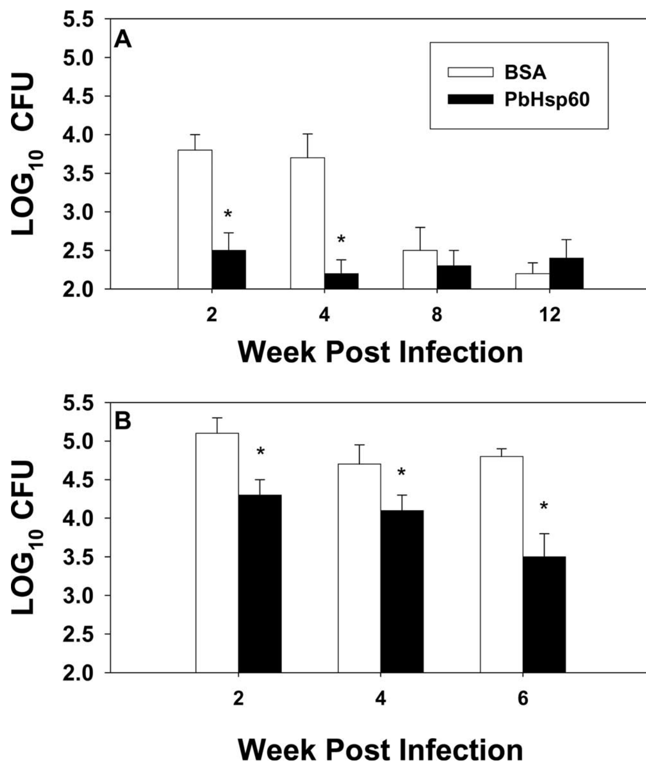

FIG. 4. Protective efficacy of PbHSP60. Groups of BALB/c mice were immunized twice with 10 μg of PbHSP60 or with an equal amount of BSA. Two weeks after the last injection of antigen, the animals were challenged with 1×10^6 or 1×10^7 yeast cells i.n. (A) Time course of animals immunized with PbHSP60 or BSA and infected with 1×10^6 yeast cells. (B) Time course of animals immunized with PbHSP60 or BSA and infected with 1×10^7 yeast cells. *, P of <0.01 compared to BSA.

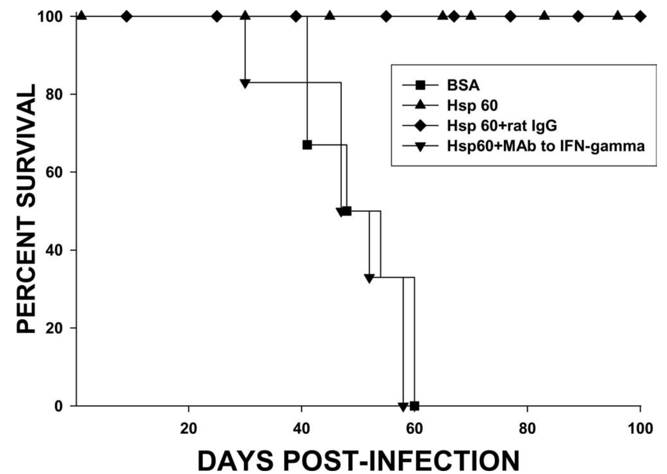

FIG. 5. Protective efficacy of PbHSP60 in animals infected with 3×10^7 yeast cells i.n. The mice were vaccinated with PbHSP60 or with BSA twice, and three weeks after the second injection of antigen, the mice were challenged with 3×10^7 yeast cells i.n. Separate groups of PbHSP60-vaccinated mice received rat IgG or MAb to IFN-γ after infection. Survival was followed for 100 days.

tigen could confer protective immunity in BALB/c mice. Groups of six mice were injected twice with 10 μg of PbHSP60 or with an equal amount of BSA emulsified in RIBI adjuvant. The injections were separated by 2 weeks. As an additional control, a group of mice was left unimmunized. Two weeks after immunization, the mice were challenged with 1×10^6 or 1×10^7 yeast cells intranasally, and we analyzed the fungal burden in the lungs following immunization. The fungal burden was assessed for 12 weeks in mice that were infected with the smaller amount of inoculum and for 6 weeks in mice that received the larger amount of inoculum (Fig. 4). In mice that received 1×10^6 yeasts, there was a significant decrease in the fungal burden ($P < 0.01$) at weeks 2 and 4 (Fig. 4A). At weeks 8 and 12, the fungal burdens in the lungs were not different (Fig. 4A). With the higher inoculum, a modest to moderate effect, albeit significant ($P < 0.01$), was observed at weeks 2, 4, and 6 (Fig. 4B).

The above experiments were performed with lower levels of inocula of *P. brasiliensis* in which controls eventually clear the infection. We subsequently examined whether PbHSP60 would protect against a lethal inoculum of yeast cells. We infected mice immunized with BSA or with PbHSP60 with 3×10^7 yeast cells intranasally (i.n.) and followed their survival. The mice immunized with PbHSP60 survived for 100 days, whereas all of the controls died (Fig. 5). In parallel, groups of PbHSP60 mice received rat IgG or MAb to IFN-γ and were examined for survival. The neutralization of IFN-γ eliminated the protective effect of the vaccination. The survivors were checked for the presence of fungi, but none were detected in the lungs, livers, or spleens. Thus, PbHSP60 protected against a lethal challenge.

Inflammatory response of vaccinated animals. We infected PbHSP60-vaccinated mice and controls with 3×10^7 yeast cells i.n. and at 30 days postinfection examined the lung inflammatory response by flow cytometry and by histopathology. We selected this inoculum because it was lethal to controls, and it most likely would produce the greatest difference between the groups. The lungs of infected control mice manifested fewer $CD4^+$ cells but more B cells and inflammatory macrophages that are Mac-3^+ $CD62L^+$ and Mac-3^+ Ly-$6C^+$ (Table 1).

Histopathologically, the control lungs exhibited moderate to severe multifocal macrophage infiltration of the alveoli with perivascular and peribronchial lymphocyte cuffing and fibrosis. Numerous neutrophil plugs were observed in the lumen of bronchioles. In the lungs of mice immunized with PbHSP60, the lungs contained mild macrophage infiltration and focal perivascular lymphocyte cuffing. Neutrophils were scant (data not shown).

Requirement for $CD4^+$ or $CD8^+$ cells in vaccine-mediated protection. Groups of mice were depleted of either $CD4^+$ or $CD8^+$ or both before immunization with PbHSP60. As a control, groups of vaccinated mice were given rat IgG. After the second immunization with antigen, treatment with MAb was discontinued, and the cells were allowed to repopulate over a period of 6 weeks. Subsequently, the mice were challenged with 1.0×10^6 yeast cells i.n. The depletion of $CD4^+$ cells or both $CD4^+$ and $CD8^+$ cells during the inductive phase of immunization effectively abolished the protective efficacy of PbHSP60 as observed at 2 weeks (Fig. 6A). At 4 weeks, the effect of $CD4^+$ cell depletion was not as dramatic as at 2 weeks. However, the elimination of both subsets eliminated the effect

TABLE 1. Number of leukocytes in the lungs of *P. brasiliensis*-infected mice injected with BSA or HSP60[a]

Injection	Mean (± SEM) no. of leukocytes (10^4)[b]							
	$CD3^+$ $CD4^+$	$CD3^+$ $CD8^+$	Ly-$6G^+$ (PMN)	Mac-3^+ Ly-$6C^+$ (Mϕ)	Mac-3^+ $CD62L^+$ (Mϕ)	$CD11c^-$ $B220^+$ (B cells)	$CD11c^+$ Ly-$6C^+$ (DC)	$CD11c^+$ $CD205^+$ (DC)
BSA	4.81 ± 1.3	1.0 ± 0.24	3.92 ± 3.61	1.97 ± 0.13	2.69 ± 0.63	22.95 ± 1.23	0.44 ± 0.05	1.36 ± 0.08
HSP60	6.23 ± 0.9[c]	0.9 ± 0.16	1.95 ± 2.00[d]	1.05 ± 0.14[c]	2.02 ± 0.48[c]	10.23 ± 2.3[d]	0.31 ± 0.05	1.40 ± 0.15

[a] Analysis performed at day 30 of infection. Six to eight samples were analyzed in two separate experiments.
[b] PMN, neutrophils; Mϕ, macrophage; DC, dendritic cells.
[c] *P* of <0.05 compared to BSA.
[d] *P* of <0.01 compared to BSA.

of PbHSP60. The animals depleted of $CD8^+$ were protected by the vaccine and were able to control the fungal burden as well as the controls.

Subsequently, we examined the role of $CD4^+$ and/or $CD8^+$ cells during the expressive phase of immunization. In this experimental design, the animals were vaccinated with PbHSP60 while their T-cell subpopulations were intact. Two weeks after vaccination, the mice were administered MAb to CD4, CD8, or both, while other groups received rat IgG as a control. Infection with 10^6 yeast cells was initiated by i.n. inoculation 1 week after the cell depletion. The depletion of $CD4^+$ or $CD4^+$ plus $CD8^+$ cells altered the efficacy of the vaccination at week 2 (Fig. 6B). At 4 weeks, however, only the vaccinated mice depleted of the $CD4^+$ and $CD8^+$ subsets manifested higher fungal burdens (*P* of <0.05 compared to PbHSP60-immunized mice given rat IgG, MAb to CD4, MAb to CD8, or both). By day 60, all vaccinated mice depleted of $CD4^+$ and $CD8^+$ cells died, whereas the other groups survived.

DISCUSSION

PCM is classified as one of the deep endemic mycoses, sharing several clinical and pathological characteristics with histoplasmosis, blastomycosis, and coccidioidomycosis. At the histological level, *P. brasiliensis* infection is characterized by a chronic mononuclear and lymphocytic infiltrate, the formation of granulomas, caseating necrosis, and fibrosis. These features may also be observed in other deep mycoses and infections with facultative intracellular pathogens such as mycobacteria. From the clinical point of view, PCM may present as a chronic, disseminated infection with episodes of latency and relapses that is characteristically seen in infections with facultative intracellular parasites (1, 8, 30). Epidemiologically, PCM may often affect otherwise immunocompetent individuals, but severe, invasive, and lethal clinical forms are more prevalent in patients with defects in cellular immune responses, strongly suggesting that T-cell-mediated immunity plays a central role in resistance against *P. brasiliensis* (28, 46).

Distinct subpopulations of helper T cells are known to be involved in divergent pathways of immune responses (20). Immunity to *P. brasiliensis* may be regulated by Th1 or Th2 subsets that would ultimately determine the outcome of the infection (24). There are only few reports describing the role of *P. brasiliensis* antigens in the induction of protective immunity. Much of the work with antigens has focused on the humoral response specifically for serodiagnosis. An exocellular molecule termed gp43, a candidate serodiagnostic antigen from *P. brasiliensis*, has also been shown to be protective. Vaccination with the recombinant gp43 protein or the DNA encoding gp43 protects susceptible mice from infection (48). The success of the vaccination with either the entire protein or the peptide is dependent on eliciting a Th1 response. The P10 domain of gp43 also stimulates cellular immunity and is protective against intratracheal infection by a virulent strain of *P. brasiliensis* (47, 48). The protective effect of the peptide is correlated with the induction of an IFN-γ-secreting Th1 lymphocyte population.

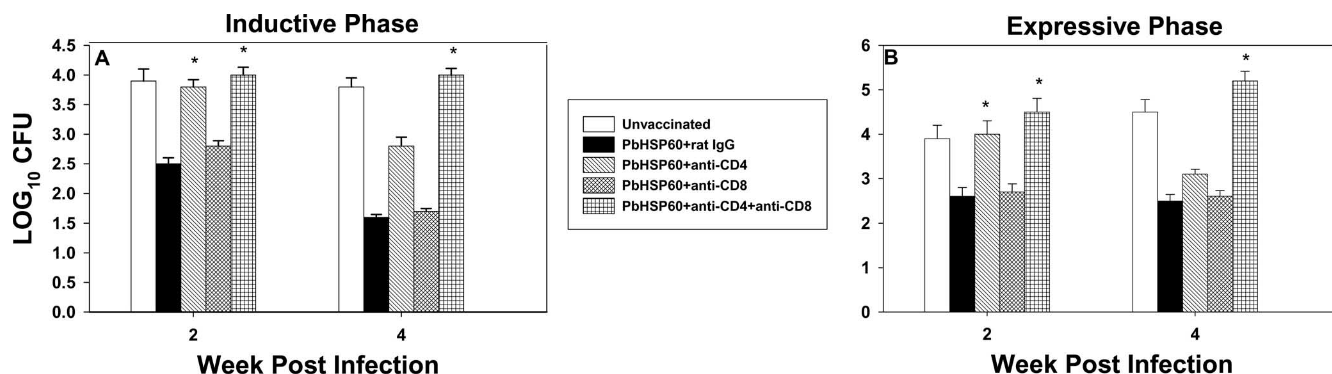

FIG. 6. CFU in the lungs of T-cell-depleted and PbHSP60-vaccinated mice. (A) Mice ($n = 6$) were immunized, concomitantly depleted of $CD4^+$ or $CD8^+$ cells or both, and challenged with 1×10^6 yeast cells. A group of immunized mice was administered rat IgG. (B) In the expressive phase, groups of mice ($n = 6$) were vaccinated and then depleted of $CD4^+$ or $CD8^+$ cells or both and challenged with 1×10^6 yeast cells. Immunized controls received rat IgG. CFU were enumerated at weeks 2 and 4 of infection. The data represent the mean $\log_{10}$ CFU ± SEM. The results of one of two experiments are shown. *, *P* of ≤0.05 compared to the immunized controls.

Other *P. brasiliensis* antigens fractioned by anion-exchange chromatography have been examined for protective activity. Two fractions, termed F0 and FII, have been demonstrated to confer protection in mice, whereas another fraction, FIII, exacerbates diseases (22). The immunization of mice with F0 and FII, predominantly surface-localized antigens, elicited a protective effect against highly infective yeast forms of *P. brasiliensis*. Although the FIII fraction induces IFN-γ, this property did not result in protection against the challenge infection (21, 23).

Previously, we had identified *H. capsulatum* HSP60 as a prominent target of T-cell-mediated immunity and a protective vaccine in experimental pulmonary histoplasmosis (18, 44). Given the high level of evolutionary conservation among the HSP family, and particularly among the *H. capsulatum* and *P. brasiliensis* HSP60 gene and protein sequences, we postulated that PbHSP60 would share some of the immunobiologic properties of *H. capsulatum* HSP60. We utilized a model of respiratory *P. brasiliensis* infection in BALB/c mice. This strain has been used previously by several groups for analysis of pulmonary infection with *P. brasiliensis* (3, 16, 21, 25, 33, 41). Mice inoculated with 1×10^6 or 1×10^7 *P. brasiliensis* yeasts developed a predictable course of infection, in which the infectious agent could be recovered and quantified up to 12 weeks after the initial challenge. Mice given 3×10^7 yeasts succumbed to infection.

When we immunized animals with recombinant PbHSP60, we detected a vigorous splenocyte proliferative response in association with the secretion of significant levels of IFN-γ but not IL-4, IL-10, or TNF-α. In terms of proliferation, we noticed a distinct dose response in which 10 μg/ml was optimal and 100 μg was suboptimal. This finding was not attributed to toxicity by the larger amount of antigen. PbHSP60 elicited vigorous IL-12 release during the vaccination period. Taken together, the results indicate that PbHSP60 immunization elicited a highly biased Th1 immune response. Our previous studies in vaccination with *H. capsulatum* Hsp60 also demonstrated a dominant Th1 response as manifested by IFN-γ release (18). In the absence of this cytokine, vaccine efficacy is abrogated and there is a major shift in the clonal phenotype and function of T cells (44). Differences did emerge between *H. capsulatum* HSP60 and PbHSP60. With the former, IL-10 was elevated and found to be essential for vaccine efficacy during the inductive phase (18) but was inhibitory of natural immunity (19). On the other hand, IL-10 was not released by PbHSP60-exposed splenocytes, strongly suggesting that it does not play a role in vaccine-associated immunity.

It has been well established that IFN-γ plays a pivotal role in protection against various pathogens through the enhancement of the killing activity of macrophages (13). Low levels or the absence of IFN-γ secretion have been associated with severe forms of PCM. Experimental findings show that different mouse strains previously infected with anti-IFN-γ MAb prevent the clearance of yeast cells from the lungs and promote their dissemination to the liver and to the spleen (13, 28). We demonstrated that the effect of PbHSP60 to mediate protective immunity was clearly dependent on the presence of IFN-γ. These findings extend observations of the role of this cytokine in protective immunity during vaccination.

To provide an important link between innate and acquired immunity, many groups of investigators have tried to elucidate the role of IL-12, the proinflammatory cytokine mainly produced by phagocytic cells (50). IL-12 is required for the development of Th1 responses against fungal infections. The treatment of mice with IL-12 at the onset of an intravenous or intratracheal infection with *Cryptococcus neoformans*, *H. capsulatum*, or *Coccidioides immitis* provides protection to the animals (27, 29, 52). The in vivo neutralization of IL-12 aggravates the pulmonary PCM of both susceptible and resistant mice. We were able to demonstrate that IL-12 was stimulated by PbHSP60 during the inductive phase of vaccination but that it was not released by cells 2 weeks after the vaccine was complete (R. Soares and G. Deepe, unpublished observations).

In murine PCM, both CD4 and CD8 cells participate in the clearance of infection (4, 13, 28, 46). Herein, we demonstrated that $CD4^+$ cells were important in the afferent phase of vaccination but that in the efferent phase, the elimination of either subset or both subsets dampened protection. These findings are similar to that found for HSP60 from *H. capsulatum* (18) but differ from that when live yeasts are used as an antigen (51). In that scenario, $CD4^+$ cells are dispensable and $CD8^+$ cells compensate for their absence in mediating protective immunity. These results clearly demonstrate that immunization with a viable microbe does activate both subsets of T cells, whereas a protein appears to generate only a single protective T-cell population. Modifying a protein to traffic in both the class I and class II major histocompatibility complex pathways might be of great value in generating protective $CD4^+$ and $CD8^+$ cells.

We also assessed the inflammatory response in the control and vaccinated mice. Significantly more $CD4^+$ cells were detected in the lungs of HSP60-vaccinated mice, but there were fewer neutrophils, B cells, and inflammatory macrophages. Moreover, the inflammatory response was milder in the vaccinated animals. The higher number of neutrophils and macrophages in the control mice would suggest a more vigorous but less organized response to infection. The greater number of B cells might be a compensatory mechanism to combat the infection.

In summary, we have demonstrated that HSP60 from *P. brasiliensis* can confer a protective immune response in BALB/c mice. This work extends the number of antigens that might be considered for vaccine development against this fungal pathogen and increases the number of pathogens that can be protected with HSP60.

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REFERENCES

1. **Anderton, S. M., R. van der Zee, A. Noordzij, and W. van Eden.** 1994. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. J. Immunol. **152:**3656–3664.
2. **Arango, M., and L. Yarzabal.** 1982. T-cell disfunction and hyperimmunoglobulinemia E in paracoccidioidomycosis. Mycopathologia **79:**115–123.
3. **Aristizabal, B. H., K. V. Clemons, A. M. Cock, A. Restrepo, and D. A.**

Stevens. 2002. Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses. Med. Mycol. **40:**169–178.
4. **Arruda, C., M. F. Franco, S. S. Kashino, F. R. Nascimento, F. R. A. Fazioli, C. A. C. Vaz, M. Russo, and V. L. G. Calich.** 2002. Interleukin-12 protects mice against disseminated infection caused by *Paracoccidioides brasiliensis* but enhances pulmonary inflammation. Clin. Immunol. **103:**185–195.
5. **Bava, A. J., A. S. Mistchenko, M. F. Palacios, M. E. Estevez, N. I. Tiraboschi, L. Sen, R. Negroni, and R. A. Diez.** 1991. Lymphocyte subpopulations and cytokine production in paracoccidioidomycosis patients. Microbiol. Immunol. **35:**167–174.
6. **Benard, G., M. J. Mendes-Giannini, M. Juvenale, E. T. Miranda, and A. J. Duarte.** 1997. Immunosuppression in paracoccidioidomycosis: T cell hyporesponsiveness to two *Paracoccidioides brasiliensis* glycoproteins that elicit strong humoral immune response. J. Infect. Dis. **175:**1263–1267.
7. **Biagione, L., M. J. Souza, L. G. Chamma, R. P. Mendes, S. A. Marques, N. G. S. Mota, and M. F. Franco.** 1984. Serology of paracoccidioidomycosis. Part II. Correlation between class-specific antibodies and clinical forms of the diseases. Trans. R. Soc. Trop. Med. Hyg. **78:**617–621.
8. **Blander, S. J., and M. A. Horwitz.** 1993. Major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen and member of the hsp 60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. J. Clin. Investig. **91:**717–723.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
10. **Brummer, E., E. Castaneda, and A. Restrepo.** 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. **6:**89–117.
11. **Calich, V. L. G., L. M. Singer-Vermes, A. M. Siqueira, and E. Burger.** 1985. Susceptibility and resistance of inbred mice to *Paracoccidioides brasiliensis*. Br. J. Exp. Pathol. **66:**585–594.
12. **Camargo, Z. P., and L. E. Cano.** 1994. Humoral immunity, p. 187–201. *In* M. F. Franco, C. S. Lacaz, A. Restrepo, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Boca Raton, FL.
13. **Cano, L. E., S. S. Kashino, C. Arruda, D. Andre, C. F. Xidieh, L. M. Singer-Vermes, C. A. C. Vaz, E. Burger, and V. L. G. Calich.** 1998. Protective role of gamma interferon in experimental pulmonary paracoccidioidomycosis. Infect. Immun. **66:**800–806.
14. **Castaneda, E., E. Brummer, D. Pappagianis, and D. A. Stevens.** 1987. Chronic pulmonary and disseminated paracoccidioidomycosis in mice: quantitation of progression and chronicity. J. Med. Vet. Mycol. **25:**377–387.
15. **Castaneda, E., E. Brummer, D. Pappagianis, and D. A. Stevens.** 1988. Impairment of cellular but not humoral immune response in chronic pulmonary and disseminated paracoccidioidomycosis in mice. Infect. Immun. **56:**1771–1777.
16. **Cock, A. M., L. E. Cano, D. Velez, B. H. Aristizabal, J. Trujillo, and A. Restrepo.** 2000. Fibrotic sequelae in pulmonary paracoccidioidomycosis: histopathological aspects in BALB/c mice infected with viable and non-viable *Paracoccidioides brasiliensis* propagules. Rev. Inst. Med. Trop. Sao Paulo **42:**59–66.
17. **Cunha, D. A., R. M. Zancopé-Oliveira, M. S. S. Felipe, S. M. Salem-Izacc, G. S. Deepe, Jr., and C. M. A. Soares.** 2002. Heterologous expression, purification, and immunological reactivity of a recombinant HSP60 from *Paracoccidioides brasiliensis*. Clin. Diagn. Lab. Immunol. **9:**374–377.
18. **Deepe, G. S., Jr., and R. S. Gibbons.** 2002. Cellular and molecular regulation of vaccination with heat shock protein 60 from *Histoplasma capsulatum*. Infect. Immun. **70:**3759–3767.
19. **Deepe, G. S., Jr., and R. S. Gibbons.** 2003. Protective and memory immunity to *Histoplasma capsulatum* in the absence of IL-10. J. Immunol. **171:**5353–5362.
20. **Deepe, G. S., Jr., R. S. Gibbons, G. D. Brunner, and F. J. Gomez.** 1996. A protective domain of heat-shock protein 60 from *Histoplasma capsulatum*. J. Infect. Dis. **174:**828–834.
21. **Diniz, S. N., B. S. Reis, T. S. Goes, C. S. Zouain, M. F. Leite, and A. M. Goes.** 2004. Protective immunity induced in mice by F0 and FII antigens purified from *Paracoccidioides brasiliensis*. Vaccine **22:**485–492.
22. **Diniz, S. N., P. S. Cisalpino, M. C. Koury, G. M. Andrade, M. G. Nogueira, and A. M. Goes.** 1999. *In vitro* human immune reactivity of fast protein liquid chromatography fractionated *Paracoccidioides brasiliensis* soluble antigens. Microbes Infect. **1:**353–360.
23. **Goes, T. S., V. S. Goes, E. Kalapothakis, M. F. Leite, and A. M. Goes.** 2005. Identification of immunogenic proteins from Paracoccidioides brasiliensis antigenic fractions F0, FII and FIII. Immunol. Lett. **101:**24–31.
24. **Gomez, F. J., R. Allendoerfer, and G. S. Deepe, Jr.** 1995. Vaccination with recombinant heat shock protein 60 from *Histoplasma capsulatum* protects mice against pulmonary histoplasmosis. Infect. Immun. **63:**2587–2595.
25. **Gonzalez, A., H. L. Lenzi, E. M. Motta, L. Caputo, J. H. Sahaza, A. M. Cock, A. C. Ruiz, A. Restrepo, and L. E. Cano.** 2005. Expression of adhesion molecules in lungs of mice infected with *Paracoccidioides brasiliensis* conidia. Microbes Infect. **7:**666–673.
26. **Izacc, S. M. S., F. J. Gomez, R. S. A. Jesuino, C. A. Fonseca, M. S. S. Felipe, G. S. Deepe, Jr., and C. M. A. Soares.** 2001. Molecular cloning characterization and expression of the heat shock protein 60 gene from the human pathogenic fungus *Paracoccidioides brasiliensis*. Med. Mycol. **39:**445–455.
27. **Jiang, C., D. M. Magee, and R. Cox.** 1999. Coadministration of interleukin-12 expression vector with antigen 2 cDNA enhances induction of protective immunity against *Coccidioides immitis*. Infect. Immun. **67:**5848–5853.
28. **Kashino, S. S., R. A. Fazioli, C. Cafalli-Favati, L. H. Meloni-Bruner, C. A. C. Vaz, E. Burger, L. M. Singer, and V. L. G. Calich.** 2000. Resistance to *Paracoccidioides brasiliensis* infection is linked to a preferential Th1 immune response, whereas susceptibility is associated with absence of IFN-γ production. J. Interferon Cytokine Res. **20:**89–97.
29. **Kawakami, K., M. Tohyama, Q. Xie, and A. Saito.** 1996. Interleukin-12 protects mice against pulmonary and disseminated infection caused by *Cryptococcus neoformans*. Clin. Exp. Immunol. **104:**208–214.
30. **LaVerda, D., M. V. Kalayoglu, and G. I. Byrne.** 1999. Chlamydial heat shock proteins and disease pathology: new paradigms for old problems? Infect. Dis. Obstet. Gynecol. **7:**64–71.
31. **Lima, K. M., S. A. Santos, V. M. Lima, A. A. Coelho-Castelo, J. M. Rodrigues, Jr., and C. L. Silva.** 2003. Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM-loaded PLGA microspheres protects mice against a virulent strain of *Mycobacterium tuberculosis*. Gene Ther. **10:**678–685.
32. **Long, K. H., F. J. Gomez, R. E. Morris, and S. L. Newman.** 2003. Identification of heat shock protein 60 as the ligand on *Histoplasma capsulatum* that mediates binding to CD18 receptors on human macrophages. J. Immunol. **170:**487–494.
33. **Marques, A. F., M. B. da Silva, M. A. Juliano, L. R. Travassos, and C. P. Taborda.** 2006. Peptide immunization as an adjuvant to chemotherapy in mice challenged intratracheally with virulent yeast cells of *Paracoccidioides brasiliensis*. Antimicrob. Agents Chemother. **50:**2814–2819.
34. **Mendes, R. P.** 1994. The gamut of clinical manifestations, p. 233–258. *In* M. F. Franco, C. S. Lacaz, A. Restrepo, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Boca Raton, FL.
35. **Milan, R., R. Alois, C. Josef, B. Jana, and W. Evzen.** 2004. Recombinant protein and DNA vaccines derived from hsp60 *Trichophyton mentagrophytes* control the clinical course of trichophytosis in bovine species and guinea-pigs. Mycoses **47:**407–417.
36. **Mota, N. G. S., M. T. Rezkallah-Iwasso, M. T. S. Peracoli, R. C. Audi, R. P. Mendes, J. Marcondes, S. A. Marques, N. L. Dillon, and M. F. Franco.** 1985. Correlation between cell-mediated immunity and clinical forms of paracoccidioidomycosis. Trans. R. Soc. Trop. Med. Hyg. **79:**765–772.
37. **Mota, N. G. S., M. T. S. Peracoli, R. P. Mendes, C. R. Gattass, S. A. Marques, A. M. V. C. Soares, I. C. Izato, and M. T. Rezkallah-Iwasso.** 1988. Mononuclear cell subsets in patients with different clinical forms of paracoccidioidomycosis. J. Med. Vet. Mycol. **26:**105–111.
38. **Musatti, C. C., M. T. Rezkallah, E. Mendes, and N. F. Mendes.** 1976. *In vivo* and *in vitro* evaluation of cell-mediated immunity in patients with paracoccidioidomycosis. Cell Immunol. **24:**365–378.
39. **Noll, A., and I. B. Autenrieth.** 1996. Immunity against *Yersinia enterocolitica* by vaccination with *Yersinia* HSP60 immunostimulating complexes or *Yersinia* HSP60 plus interleukin-12. Infect. Immun. **64:**2955–2961.
40. **Penttila, T., J. M. Vuola, V. Puurula, M. Anttila, M. Sarvas, N. Rautonen, P. H. Makela, and M. Puolakkainen.** 2000. Immunity to *Chlamydia pneumoniae* induced by vaccination with DNA vectors expressing a cytoplasmic protein (Hsp60) or outer membrane proteins (MOMP and Omp2). Vaccine **19:**1256–1265.
41. **Pinto, A. R., R. Puccia, S. N. Diniz, M. F. Franco, and L. R. Travassos.** 2000. DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *Paracoccidioides brasiliensis*. Vaccine **18:**3050–3058.
42. **Quintana, F. J., P. Carmi, F. Mor, and I. R. Cohen.** 2003. DNA fragments of the human 60-kDa heat shock protein (HSP60) vaccinate against adjuvant arthritis: identification of a regulatory HSP60 peptide. J. Immunol. **171:**3533–3541.
43. **Restrepo-Moreno, A.** 2003. Paracoccidioidomycosis, p. 328–345. *In* W. E. Dismukes, P. G. Pappas, and J. D. Sobel (ed.), Clinical mycology. Oxford University Press, New York, NY.
44. **Scheckelhoff, M., and G. S. Deepe, Jr.** 2005. A deficiency in gamma interferon or interleukin-10 modulates T-cell-dependent responses to heat shock protein 60 from *Histoplasma capsulatum*. Infect. Immun. **73:**2129–2134.
45. **Singer-Vermes, L. M., C. B. Caldeira, E. Burger, and V. L. G. Calich.** 1993. Experimental murine paracoccidioidomycosis: relationship among dissemination of the infection, humoral and cellular immune responses. Clin. Exp. Immunol. **94:**75–79.
46. **Souto, J. T., F. Figueiredo, A. Furlanetto, K. Pfeffer, M. A. Rossi, and J. S. Silva.** 2000. Interferon-gamma and tumor necrosis factor-alpha determine resistance to *Paracoccidioides brasiliensis* infection in mice. Am. J. Pathol. **156:**1811–1820.
47. **Taborda, C. P., C. R. Nakaie, E. M. Cilli, E. G. Rodrigues, L. S. Silva, M. F. Franco, and L. R. Travassos.** 2004. Synthesis and immunological activity of a branched peptide carrying the T-cell epitope of gp43, the major exocellular antigen of *Paracoccidioides brasiliensis*. Scand. J. Immunol. **59:**58–65.
48. **Taborda, C. P., M. A. Juliano, R. Puccia, M. Franco, and L. R. Travassos.** 1998. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein

of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. Infect. Immun. **66:**786–793.

49. **Thomas, P. W., E. E. Wyckoff, E. J. Pishko, J. J. Yu, T. N. Kirkland, and G. T. Cole.** 1997. The hsp60 gene of the human pathogenic fungus *Coccidioides immitis* encodes a T cell reactive protein. Gene **199:**83–91.
50. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
51. **Wuthrich, M., H. I. Filutowicz, T. Warner, G. S. Deepe, Jr., and B. S. Klein.** 2003. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: implications for vaccine development in immune-deficient hosts. J. Exp. Med. **197:**1405–1416.
52. **Zhou, P., M. C. Sieve, R. P. Tewari, and R. A. Seder.** 1997. Interleukin-12 modulates the protective immune response to SCID mice infected with *Histoplasma capsulatum*. Infect. Immun. **65:**936–942.

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