



Cellular and humoral immune responses during intrathoracic paracoccidioidomycosis in BALB/c mice

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Accepted 15 July 2008

Abstract

Paracoccidioidomycosis is a chronic infection that primarily affects the lungs. Here we investigated cellular and humoral immune responses after intrathoracic *Paracoccidioides brasiliensis* infection in BALB/c mice. *P. brasiliensis*-colony-forming units (CFUs), fungal DNA and granulomas in lungs increased progressively, peaking at day 90 postinfection (p.i.). IFN- γ production was highest on day 15 p.i., declining thereafter. The kinetics of the NO production was similar to that described for IFN- γ . In contrast, IL-10 increased from day 45 p.i. reaching a peak at day 90. Levels of serum IgG1 were higher than IgG2a between days 30 and 90 p.i. 30% of mice died by day 90 p.i. These data indicate that infection with *P. brasiliensis* by the intrathoracic route shows high IFN- γ and NO production at day 15 p.i., unable to control multiplication of fungi, which appears to be associated with a progressive increase in IL-10 and in the number and complexity of granulomas.

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Keywords: *Paracoccidioides brasiliensis*; Paracoccidioidomycosis; IFN- γ ; IL-10; Antibody response; Intrathoracic route

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Résumé

La paracoccidioïdomycose est une infection chronique qui affecte principalement les poumons. Nous avons réalisé une étude chez des souris BALB/c sur la réponse immunitaire cellulaire et humorale après une infection intrathoracique par *Paracoccidioides brasiliensis*. Nous avons constaté une augmentation progressive d'ADN de *P. brasiliensis* et de granulomes dans les poumons pour culminer au 90^e jour après l'infection. La production d'IFN- γ était plus élevée le 15^e jour après l'infection, pour décroître ensuite. La cinétique de la production de NO était similaire à celle décrite pour l'IFN- γ . En revanche, l'IL-10 a augmenté après le 45^e jour, pour atteindre un pic au 90^e jour après l'infection. Les niveaux d'IgG1 étaient supérieurs à IgG2a entre le 30^e et le 90^e jour après l'infection dans les sérum. 30% des souris sont mortes sur les 90 jours de l'expérimentation. Ces données indiquent que l'infection intrathoracique par *P. brasiliensis* montre une haute production d'IFN- γ et de NO après quinze jours, n'entraînant aucun contrôle de la multiplication fongique qui semble être associée à une augmentation progressive de IL-10, au nombre et à la complexité des granulomes.

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Mots clés : *Paracoccidioides brasiliensis* ; Paracoccidioïdomycose ; IFN- γ ; IL-10 ; Réponse anticorps ; Infection intrathoracique

1. Introduction

Paracoccidioïdomycosis, or South American blastomycosis, is a chronic granulomatous infection caused by the dimorphic fungus *Paracoccidioides brasiliensis* [1]. The lung is the organ of entry of *P. brasiliensis*, through the inhalation of fungal conidia, which can reach the pulmonary alveolar epithelium and differentiate into the pathogenic yeast form [2]. The spectrum of paracoccidioïdomycosis ranges from benign and localized to severe and disseminated forms. The acute form affects young patients of both sexes and involves mainly the reticuloendothelial system, whereas the chronic form is most prevalent in adult males and has a predominant pulmonary and/or mucocutaneous involvement that is often fatal [3].

Cellular immune response is markedly involved in host defense against *P. brasiliensis* infection, determining the severity of the disease and its clinical form [4]. High levels of specific antibodies are proved in the serum of infected individuals, but it has been almost definite that these antibodies have no direct connection with the defense mechanism of the host [3]. There are significant differences in susceptibility among inbred strains of mice, resistant mice exhibited low fungal load in many organs and the infection trends to resolution through a response mediated by a dominant T-helper 1 (Th1) cell phenotype with prominent IFN- γ production [5]. This cytokine appears to be a major mediator of resistance against *P. brasiliensis* infection in mice, as it promotes the antifungal activity of the macrophages in part through NO production. Although iNOS-derived NO appears to be essential for resistance to paracoccidioïdomycosis, the high and continued NO production was associated with susceptibility [6–8]. Susceptible mice develop a progressive disease, which seems to result mainly from T cell dysfunction and unbalanced activation of Th1 and Th2 cells with high IL-4 and IL-10 production [9] and [10]. The granuloma formation plays

a fundamental role in protection against paracoccidioidomycosis infection, and its highly influenced by the degree of T cell activation [11]. Patients with severe paracoccidioidomycosis present a pattern of diffuse inflammatory granulomatous response associated with T-cell suppression [12,13].

The route of infection can influence the immune response to pathogenic fungi [14]. The resistant patterns of mice infected by the intraperitoneal route, was similar to those observed when infection was done by either the intravenous route or the intratracheal route [15]. In general, the course of infection depends on the natural resistance of the host, immunological status and characteristics of the infecting agent, mainly its virulence [4,9,16].

The present work is the first study of paracoccidioidomycosis induced by the intrathoracic route. Intrathoracic infection with mycobacteria is characterized by a rapid evolution with high multiplication of bacteria in the lungs [17]. The aim of this study was to evaluate the progression of *P. brasiliensis* infection by the intrathoracic route in BALB/c mice, by counting colony-forming units (CFUs) and through the detection of *P. brasiliensis* DNA using the PCR method. Specific primers OL5 and ITS1 able to distinguish between DNA obtained from *P. brasiliensis* and other pathogenic fungi were used [18]. In addition, levels of IFN- γ , IL-10 and nitric oxide produced by intrathoracic cells and anti-*P. brasiliensis*-specific IgG1 and IgG2a antibodies in serum, were investigated searching for immune response patterns during the intrathoracic infection.

2. Materials and methods

2.1. Mice

Experiments were performed with 8–12-week-old male BALB/c mice. The animals were raised at the Biological Reproduction Center of the Federal University of Juiz de Fora, Brazil. The present study has the approval of the committee of ethics in animal research of the Federal University of Juiz de Fora, registered in the protocol 11/2003-CEA.

2.2. Obtaining and maintaining *P. brasiliensis*

The *P. brasiliensis* yeast isolate 69P from the University Hospital of the Federal University of Juiz de Fora was used in this study. The fungus was cultured at 35 °C and replicated every 20 days in tubes containing 9 ml of brain heart infusion (BHI) agar (Difco-BD, CA, USA).

2.3. Intrathoracic infection

The infection was induced by intrathoracic inoculation of 10^6 *P. brasiliensis* yeast forms diluted in 100 μ l of phosphate buffer saline (PBS) and injected in the right side of the thorax of mice approximately between the 8th and 10th ribs. Control mice received an equal volume of PBS, in accordance with the technique described previously [19] and adapted to the murine model [20].

2.4. Preparation of cells and lung tissue

All mice involved in the study ($n = 6/\text{group}$) were sacrificed by overdose of anesthesia (0.9% NaCl, 2% xylazine and 5% ketamine) at days 2, 7, 15, 30, 45, 60 or 90 p.i. The thorax was opened and the cavity washed with 1 ml of PBS. The washes were collected for cell culture. The right lung was used for counting *P. brasiliensis* CFU. Half of the left lung was fixed in formaldehyde for histopathologic evaluation, and half was immediately frozen for the PCR studies. For the histological study, the previously fixed lungs were submitted to the routine procedures for inclusion in paraffin, and cut to 5 μm -thickness, half-serried and stained with hematoxylin-eosin (HE). Slide images were captured and analyzed.

2.5. *P. brasiliensis* colony-forming units

The right lungs were macerated in 1 ml of sterile PBS. Hundred microliters was used for *P. brasiliensis* CFU determination and the remaining material used for antibody and cytokine detections. Serial dilutions of the mix were made and each sample was put in BHI agar enriched with 5% of horse serum and maintained at 36 °C for 15 days. At the end of the incubation period, the numbers of *P. brasiliensis* CFU were counted visually.

2.6. Detection of IgG1 and IgG2a *P. brasiliensis*-specific antibodies

Serum was used for the study of *P. brasiliensis*-specific IgG1 and IgG2a antibodies by direct ELISA. At days 30, 45, 60 and 90 p.i. sera were collected and subjected to antibody detections, following the manufacturer instructions (Pharmingen). Absorbance was measured at 492 nm using a microplate reader (SPECTRAMAX 190, Molecular Devices).

2.7. Polymerase chain reaction (PCR)

The samples of lungs from infected and non-infected mice and control yeast forms from *P. brasiliensis* cultures were put into microtubes that contained 900 μl of the extraction buffer (100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 2% cetyl trimethylammonium bromide (CTAB), 80 $\mu\text{g}/\text{ml}$ RNase, 1% polyvinylpyrrolidone (PVP), 10% dithiothreitol (DTT) and 20 ng/ml proteinase K) (Invitrogen, CA, USA) and incubated at 50 °C overnight. DNA was precipitated with frozen isopropanol (Invitrogen) for 48 h at -20 °C and then washed with ethanol 70%, and dissolved in 100 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA). The DNA concentration was evaluated by spectrophotometry at 280/260 nm (GeneQuant Pro, Amersham Biosciences, USA).

The ribosomal gene ITS was amplified using primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and OL-5 (5'-TGT GAC GAA GCC CCA TAC G-3') [18]. The reaction was made with 30 ng/ml DNA, 1 U Taq polymerase, 0.5 μM primers, 0.25 mM dNTPs and 1.5 mM MgCl_2 (Invitrogen) in a final volume of 25 μl . The amplification was made using a touchdown method with 95 °C/2 min, 10 cycles of 95 °C/1 min, 64 °C/1 min and 72 °C/1.5 min, 35 cycles of 95 °C/1 min, 54 °C/1 min and 72 °C/1.5 min and 72 °C/10 min. The amplification was done in the GeneAmp PCR System 9600 (Applied Biosystems, USA). The products of the amplification were carried in native gel 5% polyacrilamide (400 V,

1.5 h). The products were visualized by silver staining and the fragments were identified using a scale of 100-bp.

2.8. Cell culture

Cells of the intrathoracic washes were cultivated in RPMI medium (Gibco-Invitrogen, CA, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µl/ml non-essential aminoacids and 5% heat-inactivated fetal bovine serum (Gibco-Invitrogen, CA, USA) in 24-well culture plates in a concentration of 2×10^6 cells/ml in 500 µl for 48 h at 37 °C in 5% CO₂ atmosphere in the presence or absence of sonicated *P. brasiliensis* antigens (20 µg/ml). After incubation, the supernatants were collected and frozen at –20 °C for cytokine evaluation.

2.9. ELISA

The collected supernatants were submitted to the technique of indirect ELISA to determine the concentration of IFN-γ and IL-10 (according to manufacturer instructions, IFN-γ and IL-10 kit BD OptEIA, CA, USA). The reading was made in the microplate reader (SPECTRAMAX 190, Molecular Devices) at 450 nm. The amount of cytokines was calculated from the standard curve, for the different concentrations of the recombinant cytokines.

2.10. Nitric oxide production

Supernatants of pleural cells were analyzed for quantification of nitrites (NO₂[–]) through the Griess method [21]. Aliquots of supernatants were plated with 1% of sulfanilamide and 0.1% of *N*-(1-naphthyl)ethylenediamine. NO₂[–] production was quantified by comparing with a standard-curve, made with different concentrations of NaNO₂. The reading was made in the microplate reader (SPECTRAMAX 190, Molecular Devices) at 540 nm wavelength.

2.11. Statistical analyses

The Mann–Whitney test was used to determine the significant differences between the different groups of mice, with the level of significance $P < 0.05$ (MedCalc statistical software Version 5.00.020).

3. Results

3.1. Fungal load in lungs after intrathoracic infection

The infection of BALB/c by the intrathoracic route with 10⁶ yeast forms of *P. brasiliensis* was determined by a progressive multiplication of fungal cells in the site of infection, affecting the lungs (Fig. 1). The number of *P. brasiliensis* CFU counted in the

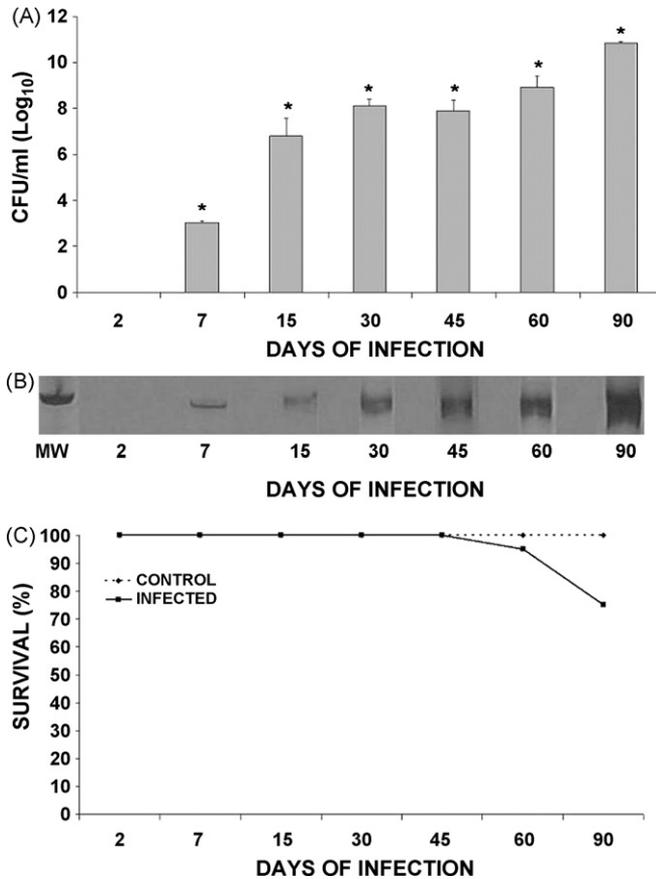


Fig. 1. Measurements of infection and survival of BALB/c mice infected with *P. brasiliensis* by the intrathoracic route. (A) *P. brasiliensis* CFU numbers in the lungs during progression of infection. Each bar represents the mean of six to eight animals. Range bars represent standard errors of the means. * $P < 0.05$ versus day 2 p.i. (B) DNA products obtained after digest of lungs. MW molecular weight (496-bp). GeneRuler 100-bp DNA ladder. (C) Survival percentages of BALB/c mice. (■) Infected with *P. brasiliensis*; (◆) healthy control. Data are from one study which was repeated with similar results.

lungs was significantly higher between days 7 p.i. and 90 p.i. in comparison with day 2 p.i. ($P < 0.05$). The CFU count grew progressively from days 7 to 90 p.i. The logarithmic increase of *P. brasiliensis* CFU numbers in the lungs after day 7 p.i. is parallel to the rising detection of *P. brasiliensis* DNA in the lung fragments (Fig. 1B). Survival studies show that the infected mice began to die from day 60 p.i. (Fig. 1C), having 75% survival at day 90 p.i.

3.2. Histopathological study

Examination of thin lung sections by HE staining showed an increase in the number, complexity and size of granulomas during the course of *P. brasiliensis* infection (Fig. 2).

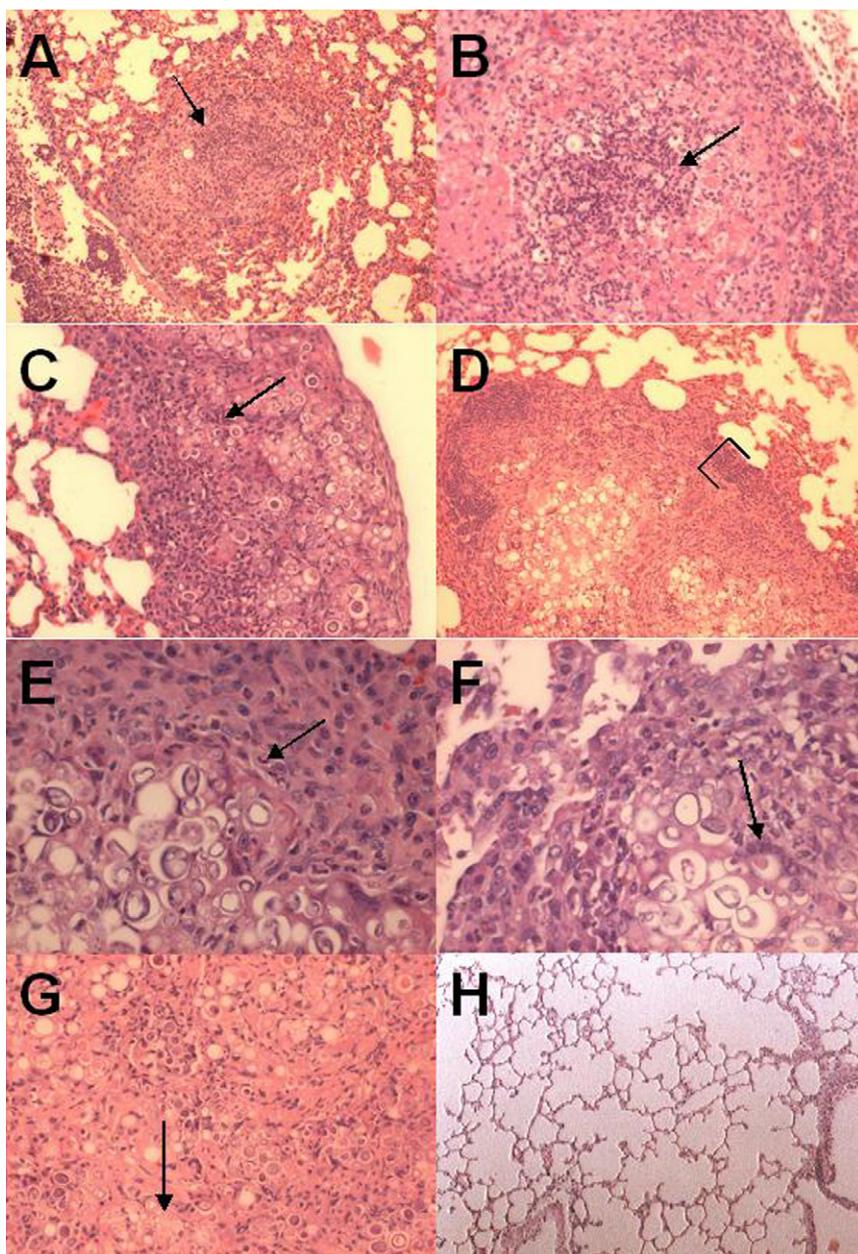


Fig. 2. Histopathological study of lung tissue from BALB/c mice infected with *P. brasiliensis*. Tissue sections were processed and stained as described in Materials and methods. (A) Day 2 p.i. (100 \times), arrow indicates initial granuloma; (B) day 7 p.i. (200 \times), arrow indicates PMN cell infiltrate; (C) day 15 p.i. (200 \times), arrow indicates giant cell; (D) day 30 p.i. (100 \times), bracket indicates large crown of monocytes; (E) day 45 p.i. (400 \times) arrow indicates PMN cells next to fungi; (F) day 60 p.i. (400 \times), arrow indicates giant cells with fungi; (G) day 90 p.i. (200 \times), arrow indicates fibrosis tissue; (H) lung histology of non-infected control (40 \times).

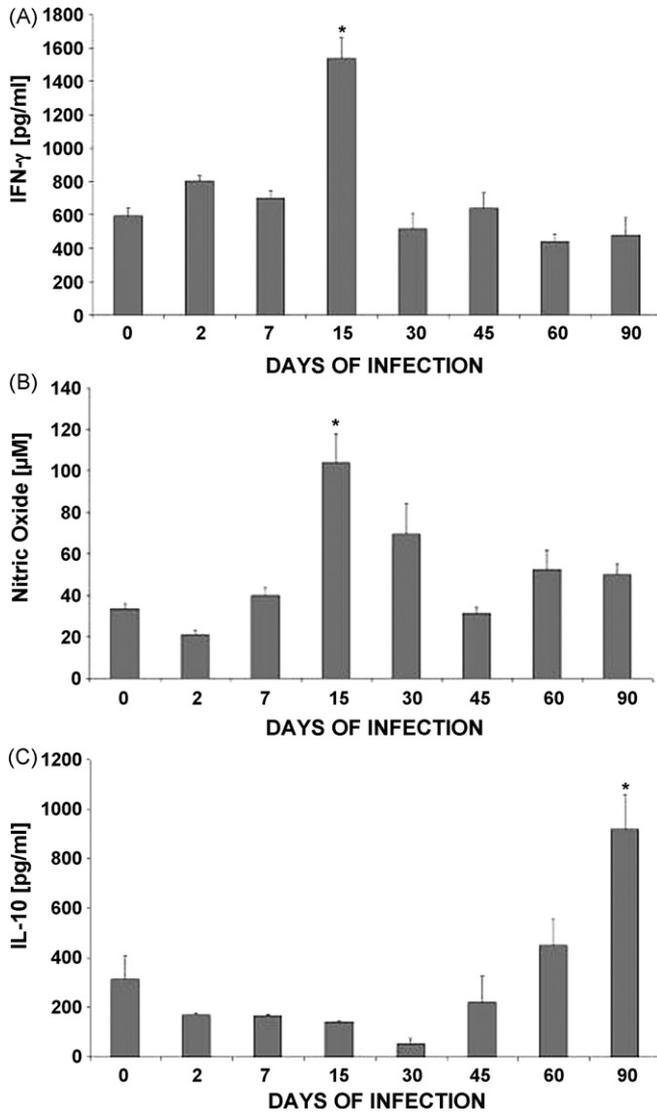


Fig. 3. Kinetics of IFN- γ , IL-10 and nitric oxide in supernatants of pleural cells. Measurements of (A) IFN- γ , (B) nitric oxide and (C) IL-10 from supernatants of cells of intrathoracic wash stimulated with *P. brasiliensis* antigen (20 μ g/ml) at different times. * $P < 0.05$ versus other time points. Each bar represents the mean of six to eight animals. Range bars represent standard errors of the means.

At day 2 p.i. a dense cellular inflammatory infiltrate, composed of PMN cells around the fungi, and an initial granuloma (Fig. 2A) were observed. At day 7 p.i., granulomas were observed near pleura containing predominant PMN infiltrates and fungi (Fig. 2B). At day 15 p.i., giant cells, many fungi and PMN cells in large granulomas were observed (Fig. 2C). At day 30 p.i., giant granulomas were observed surrounded by well-formed crowns

(lymphoid sheath) and many fungi inside the granulomas and PMN cells around yeast cells (Fig. 2D). At day 45 p.i., many fungi and PMN near to yeast cells were detected (Fig. 2E). At day 60 p.i., many fungi in granulomas, PMN and giant cells near to yeast cells were noted (Fig. 2F). At day 90 p.i., enormous diffuse inflammatory granulomatous responses with central fibrosis areas rich in *P. brasiliensis* yeast cells, many fungi, giant cells and few PMN were observed (Fig. 2G). The Fig. 2H shows a lung from a non-infected control mouse.

3.3. IFN- γ , IL-10 and NO production

IFN- γ , IL-10 and NO production were evaluated in supernatants of pleural cells cultures from *P. brasiliensis*-infected mice, stimulated *in vitro* with *P. brasiliensis* sonicated antigen. Fig. 3A shows a higher ($P < 0.05$) production of IFN- γ only at day 15 p.i. (Fig. 3A) which was declined thereafter. The kinetics of the NO production was similar to that described for the production of IFN- γ (Fig. 3A and B). In contrast, IL-10 production increased progressively from day 45 p.i. reaching a peak at day 90 p.i. ($P < 0.05$ versus day 0) (Fig. 3C).

3.4. IgG1 and IgG2a detected in serum

P. brasiliensis-specific IgG1 and IgG2a antibodies were determined from sera of BALB/c mice after intrathoracic infection. Sera levels of IgG1 were significantly higher ($P < 0.01$) at day 30, at day 45 and at day 90 p.i. when compared to day 0 (Fig. 4). Levels of IgG2a in sera were significantly higher at day 30 p.i. in comparison to day 0 ($P < 0.05$) but lower in comparison to IgG1 at days 30, 45 and 90 p.i. ($P < 0.05$).

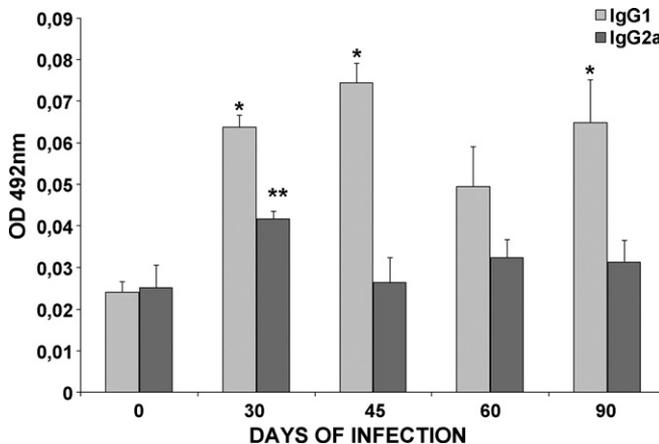


Fig. 4. Detection of anti-*P. brasiliensis* IgG1 and IgG2a in serum after intrathoracic infection. Levels of specific IgG1 and IgG2a anti-*P. brasiliensis* in serum of BALB/c mice infected or not with *P. brasiliensis* at different time points. OD: optical density at 492 nm. IgG2a bars $P < 0.05$ versus IgG1 bars at days 30, 45 and 90 p.i. * $P < 0.01$ versus non-infected control mice and ** $P < 0.05$ versus non-infected control mice. Each bar represents the mean of six to eight animals. Range bars represent standard errors of the means.

4. Discussion

The present work shows the pattern of cellular and humoral immune responses in BALB/c mice after intrathoracic infection with *P. brasiliensis*. The data indicate that infection with *P. brasiliensis* by the intrathoracic route shows high IFN- γ and NO production at day 15 p.i., which was unable to control multiplication of fungi. Aggravation of infection appears to be associated with a progressive increase in IL-10 and in the number and complexity of granulomas, aside an increase in serum IgG1 antibodies.

The progression of the infection was measured through the count of CFUs and by the detection of the fungus DNA in the lungs of the infected animals. The presence of fungi in the thoracic cavity was detected from day 7 p.i., and increased gradually thereafter leading to 30% death of the infected animals by day 90 p.i. The uncontrolled fungi proliferation can be related to the virulence of the fungus, the route of infection and to the susceptibility of the BALB/c lineage against *P. brasiliensis* [9] and [10]. The strain 69P used in this study appears to present a high virulence profile which deserves further investigation. The route of infection allows a rapid access to the lung, the pivotal site of multiplication of *P. brasiliensis*. Although some studies showed a 100% survival still after 200 days of intratracheal infection with the virulent *P. brasiliensis* (Pb18 strain) [10], our findings are more in agreement with other record [22] which showed a 30% survival of BALB/c mice at day 70 p.i. of intravenous *P. brasiliensis* (Pb IOC 3698 strain) infection.

Control of fungal growth depends on the organization of granulomas by cell-mediated immunity [23]. In this study, granulomas containing yeast forms were observed with a compact organization in the initial phase that loosened with the progression of infection. Granulomas gradually increased in size and complexity during infection. At day 90 p.i. the granulomas were unable to contain the spread of the fungus, losing their compact appearance and fusing with each other. Numerous yeast cells, in active multiplication, were detected outside the granuloma. This may be associated with the decrease in PMN cells in the granuloma, as well as an imbalance of molecules that may influence cellular immune responses, as shown by IFN- γ and IL-10 measurements [4] and [24]. Alterations of the chemokine and cytokine patterns influence the evolution of infection with *P. brasiliensis* [25].

Our results show high IFN- γ and NO levels at day 15 of *P. brasiliensis* infection, suggesting an initial host Th1 like response attempting to control fungal growth. An increase in the fungicidal activity of PMN was correlated to the presence of IFN- γ , leading to an increase in the capacity for fungal clearance [26–29]. With the progression of the infection, a reduction of IFN- γ and NO was accompanied by an increase of IL-10 that was inappropriate to detain fungal dissemination. It has been shown that low levels of IFN- γ associated with production of IL-10 characterize the progressive disease of susceptible animals [16]. IFN- γ primed alveolar macrophages from susceptible (B10.A) mice challenged with *P. brasiliensis* produced high levels of NO and low amounts of IL-10 [30]. IL-10 can significantly affect the activation of PMN cells, inhibiting its ability to kill *P. brasiliensis* [26]. NO production by PMNs may contribute to inhibition of T cell proliferation [31] and [32]. Dendritic regulatory cells may be involved in activation of TLR-2 and dectin-1 receptors promoting production of IL-10 and enhancing the mice susceptibility to *P. brasiliensis* infection [33]. Another study has shown that IL-23 is produced in response

to fungi in condition of high-threat inflammation in response to high yeast numbers through the TLR/MyD88 pathway. Moreover, IL-23 may contribute to the uncontrolled fungal growth and the resulting concomitant activation of nonprotective Th2 cells [34].

In this study, the specific humoral response to the *P. brasiliensis* shows prevalence of IgG1 and not of IgG2a isotypes, in serum of infected mice. In spite of the humoral response not playing a fundamental role in the defense of the host against *P. brasiliensis*, it may have an auxiliary function in the activation of the immune system, facilitating phagocytosis and activating the complement system [35,36].

Taken together, these results indicate that *P. brasiliensis* infection by the intrathoracic route is characterized by a strong Th1 like response in its initial phase that fails to control fungal growth. A Th2 like profile with high IL-10 and IgG1 predominates in the later phase of infection when numbers of *P. brasiliensis* in granulomas are markedly increased.

Acknowledgments

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). Caio C.S. Alves was recipient of an Ms Fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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