RESEARCH ARTICLE

Involvement of extracellular matrix proteins in the course of experimental paracoccidioidomycosis

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Paracoccidioides brasiliensis; adhesion; laminin; fibronectin; fibrinogen.

Abstract

We aimed at determining involvement of extracellular matrix proteins (ECMp) and an ECM-binding adhesin (32-kDa protein) from *Paracoccidioides brasiliensis*, in the course of experimental paracoccidioidomycosis. BALB/c mice were infected with *P. brasiliensis* conidia previously incubated with soluble laminin, fibronectin and fibrinogen or a mAb against the fungal adhesin. Inflammatory response, chitin levels and cytokine production at different postinfection periods were determined. Chitin was significantly decreased in lungs of mice infected with ECMp-treated conidia when compared with controls at week 8, especially with laminin and fibrinogen. Contrariwise, when animals were infected with mAb-treated conidia no differences in chitin content were found. The observed inflammatory reaction in lungs was equivalent in all cases. IFN-γ increased significantly in lungs from mice infected with soluble ECMp – (at day 4 and week 12) or mAb-treated conidia (at week 12) when compared with animals infected with untreated conidia. Significant increased levels of tumour necrosis factor-α were observed at 8 weeks in animals infected with ECMp-treated conidia while no differences were observed during the remaining periods. These findings point toward an inhibitory effect of ECMp on *P. brasiliensis* conidia infectivity and suggest that these proteins may interfere with conidia initial adhesion to host tissues probably modulating the immune response in paracoccidioidomycosis.

Introduction

Paracoccidioides brasiliensis is the causal agent of paracoccidioidomycosis (PCM), one of the most important deep systemic and endemic mycosis in Latin America (Restrepo & Tobón, 2005). The natural infection is assumed to be the inhalation of airborne propagules produced by the fungal mycelium form (conidia), which then change into the pathogenic yeast form in the lung (McEwen *et al.*, 1987). Subsequently, the disease can disseminate to the other organs and systems, forming secondary lesions, for example in the mucous membranes, skin, lymph nodes and adrenal glands (Restrepo & Tobón, 2005). The mycosis presents a wide range of clinical and immunological manifestations, varying from benign and localized to severe and disseminated forms (Restrepo & Tobón, 2005).

Clinical and experimental data indicate that cell-mediated immune response plays a significant role in host defence against *P. brasiliensis* infection, whereas high levels of specific antibodies and polyclonal activation of B cells are associated with the most severe forms of the mycosis (Arango & Yarzabal, 1982; Mota *et al.*, 1985; Singer-Vermes *et al.*, 1993). Various studies have demonstrated a Th1-biased immune response in the asymptomatic form of PCM, whereas a Th2 pattern has been associated with the severe disease (Karhawi *et al.*, 2000; Benard *et al.*, 2001; Oliveira *et al.*, 2002). In the pulmonary model, using different mouse strains that present resistance (A/Sn) or susceptibility (B10.A) to *P. brasiliensis* infection, it was found that both mouse strains secreted IFN-γ, IL-2, IL-4, IL-5 and IL-10 in the lungs, but these molecules appeared in larger amounts in B10. A mice than in A/Sn mice (Cano *et al.*, 2000).

In vitro, it has been shown that IFN-γ- or tumour necrosis factor (TNF)-α-activated macrophages exert an antifungal effect against *P. brasiliensis* conidia, and that these mechanisms are dependent or independent of nitric oxide production, respectively (González *et al.*, 2000, 2004). *In vivo*, the importance of these cytokines has already been demonstrated, as depletion or genetic deficiency of IFN-γ, as well as of TNF-α receptor, led to exacerbate disease (Cano *et al.*, 1998; Souto *et al.*, 2000).

The adherence of microorganisms to host tissues is a crucial step in the development of infection. Adherence implies that the pathogen recognizes carbohydrate or protein ligands on the surface of host cells, or a constituent of basement membranes underlying epithelium and endothelium, such as ECM proteins (ECMp) including fibrinogen, laminin, collagen or fibronectin (Patti *et al.*, 1994). The interaction of ECMp with various infectious agents has been reported, where these ECMp could serve as adherence substrates (Silva-Filho *et al.*, 1988; Furtado *et al.*, 1992; Li *et al.*, 1995; Gaur *et al.*, 1999; Wasylnka & Moore, 2000). Of particular interest in this context is the identification of ECM-binding proteins on the surface of several fungi of clinical importance such as *Candida albicans* (López-Ribot *et al.*, 1996; Gaur *et al.*, 1999), *Aspergillus fumigatus* (Coulot *et al.*, 1994; Gil *et al.*, 1996; Wasylnka & Moore, 2000), *Histoplasma capsulatum* (McMahon *et al.*, 1995), *Cryptococcus neoformans* (Rodrigues *et al.*, 2003), *Pneumocystis carinii* (Narasimhan *et al.*, 1994), *Sporothrix schenckii* (Lima *et al.*, 2001) and *Penicillium marneffei* (Hamilton *et al.*, 1998, 1999).

A 43-kDa surface glycoprotein known as the main antigenic component in *P. brasiliensis* has been recognized by 100% of PCM patients' sera (Travassos *et al.*, 1995); this glycoprotein has been shown to bind laminin (Vicentini *et al.*, 1994). In addition, Vicentini *et al.* (1994) have previously shown that laminin-coated yeasts have increased the ability of *P. brasiliensis* to invade and destroy tissues (Vicentini *et al.*, 1994). In contrast, André *et al.* (2004) studied the influence of previous treatment of yeast cells with laminin on the course of experimental PCM of resistant and susceptible mice infected with high- and low-virulence *P. brasiliensis* isolates, and they observed a less intense inflammatory reaction in the lungs of the laminin-treated groups (Andre *et al.*, 2004). Furthermore, laminin treatment of low-virulence isolate resulted in a less severe infection as revealed by the lower fungal loads recovered from the lungs (Andre *et al.*, 2004).

Recently, three adhesins with molecular masses of 19, 30 and 32 kDa from *P. brasiliensis* and their capacity to bind ECMp have been described, although only two of them have so far been purified and partially characterized (Andreotti *et al.*, 2005; González *et al.*, 2005a). The 30- and 32-kDa adhesins were able to bind laminin, fibronectin and fibrinogen, as well as to bind to epithelial cells (Andreotti *et al.*, 2005; González *et al.*, 2005a). In addition, these purified proteins or a monoclonal antibody raised against the 32-kDa protein inhibited in a significant form the adherence of conidia and yeasts forms of *P. brasiliensis* to immobilized ECMp and to epithelial cells, respectively (Andreotti *et al.*, 2005; González *et al.*, 2005a). However, the role played by specific adhesins or by other ECMp involved in the adherence of *P. brasiliensis* conidia, in the course of experimental PCM, has not yet been explored.

In the present study, we studied the participation of ECMp such as laminin, fibronectin and fibrinogen, as well as a mAb raised against the 32-kDa adhesin in the course of experimental PCM.

Materials and methods

Fungus culture and conidia production

Paracoccidioides brasiliensis isolate ATCC 60855, previously known to sporulate freely on special media, was used (Restrepo *et al.*, 1986). The techniques used to grow the mycelial form, collect and dislodge conidia have been reported previously (Restrepo *et al.*, 1986). Briefly, the stock mycelial culture was grown in a liquid synthetic medium, the modified Mc Veigh-Morton broth (Restrepo & Jiménez, 1980), at 18 °C (± 4 °C) with shaking. Growth was homogenized and inoculated into agar plates; the latter were incubated at 18 °C (± 4 °C) for 3 months. After this time, sterile physiological saline solution containing 0.01% Tween-20, plus 100 U penicillin and 100 μg streptomycin mL^{-1} was used to flood the culture surface. Growth was removed with a bacteriological loop and the resulting suspension pipetted into an Erlenmeyer flask containing glass beads. This was then shaken in a reciprocating shaker at 250 r.p.m. for 45 min at 18 °C. The homogeneous suspension was filtered through a syringe packed with sterile glass wool (Pyrex fiber glass, 8 μm, Corning Glasswork, Corning, NY). The filtrate was collected in a polycarbonate centrifuge tube and centrifuged for 30 min at 1300×***g***; the pelleted conidia were washed, counted with a hemacytometer, and their viability assessed by the ethidium bromide–fluorescein diacetate technique (Calich *et al.*, 1978). For the experiments, only inocula with a conidial viability > 90% were used.

All assays were performed under conditions designed to minimize endotoxin contamination.

Yeast suspensions

Paracoccdioides brasiliensis yeast cells (ATCC No. 60855) were grown for 4–5 days in liquid brain–heart infusion (BHI) plus glucose (1%) media at 36 °C with shaking. Yeast cells were harvested by centrifugation, washed in distilled

water and then stored at –20 °C. All fungal suspensions were adjusted to 1×10^6 cells mL^{-1}.

Treatment of conidia with ECMp or a mAb against the 32-kDa protein (adhesin)

A total of 4×10^6 *P. brasiliensis* conidia were treated with 100 μg mL^{-1} of laminin (derived from Engelbreth–Holm Swarm mouse sarcoma; Sigma, St. Louis, MO), human fibronectin (Sigma) or bovine fibrinogen (Sigma) in a final volume of 100 μL of phosphate-buffered saline (PBS) for 2 h at 37 °C before infection. Control *P. brasiliensis* conidia were incubated with PBS alone. In addition, a monoclonal antibody (mAb2G4) against the 32-kDa protein from *P. brasiliensis* produced as previously described (González *et al.*, 2005a) was used to treat the conidia suspension, as described above, and an isotype IgG1 from mouse were used as control at concentrations of 100 μg mL^{-1}.

Animals

Isogenic 6-week-old BALB/c male mice, obtained from the breeding colony of the Corporación para Investigaciones Biológicas (CIB), Medellín-Colombia, were used in all experiments and were kept and fed under the conditions previously indicated (Brummer *et al.*, 1984). Mice were supplied with sterilized commercial food pellets, sterilized bedding and fresh acidified water; their care took into consideration the recommendations given by the Colombian Government (Law 84 of 1983, Rs No. 8430 of 1993) and the regulations of the European Communities and Canadian Council of Animal Care (1998).

Experimental infection

Animals were anesthetized by intramuscular injection of 50 μL of a solution containing a mixture of ketamine hydrocloride (100 mg) (Park-Davies & Co. Bogotá, Colombia) and xylazine (20 mg) (Bayer S.A., Brazil). When deep anesthesia was obtained, 4×10^6 conidia suspended in a 60 μL inoculum divided in two portions were instilled intranasally within a 10 min period. The abdomen was compressed and droplets were deposited on the nares (Cock *et al.*, 2000; González *et al.*, 2003, 2005b). Control mice received an intranasal inoculum of 60 μL of PBS.

Animals were sacrificed at the following time intervals 0 (2 h postinoculation), 2, 4 days; and 1, 4, 8 and 12 weeks. At each period, six to eight mice from each experimental group, as well as 4–6 noninfected control animals, were sacrificed by the intraperitoneal injection of 1.0 mL of 2.5% sodium penthotal (Abbott Laboratories, Chicago, IL; E.U.A). Different mouse groups were used for histology and to determine the cytokine and chitin levels.

Chitin assay

We used an assay for chitin as a parameter to measure the burden of *P. brasiliensis* in lungs, liver and spleen. Chitin, a component of the fungal cell wall, is absent from mammalian cells. The assay was adapted from a previously described method (Lehmann & White, 1975; Mehrad *et al.*, 1999). Organs were homogenized in 2 mL of distilled water and centrifuged (1500×***g***, 5 min, 20 °C). The supernatants were discarded, and pellets were resuspended in 4 mL of sodium lauryl sulfate (3% w/v) and heated at 100 °C for 15 min. Samples were then centrifuged (1500×***g***, 5 min, 20 °C), and pellets were washed with distilled water and resuspended in 3 mL of KOH (120% w/v). Samples were heated at 130 °C for 60 min. After cooling, 8 mL of ice-cold ethanol (75% v/v) was added to each sample, and tubes were shaken until ethanol and KOH made one phase. The tubes were kept in ice for 15 min, and 0.3 mL of Celite suspension (supernatant of 1 g of Celite mixed with 100 mL of 75% ethanol and allowed to stand for 2 min) was added to each. Samples were centrifuged (1500×***g***, 5 min, 4 °C) and supernatants were discarded. Pellets were washed once with ice-cold ethanol (40% v/v) and twice with ice-cold distilled water, and resuspended in 0.5 mL of distilled water. Standards consisting of 0.2 mL of distilled water and 0.2 mL of glucosamine (10 μg mL^{-1}) were made up. $NaNO_2$ (0.2 mL; 5% w/v) and $KHSO_4$ (0.2 mL; 5% w/v) were added to each standard; 0.5 mL of each solution was added to the samples. All tubes were gently mixed three times during 15 min and then centrifuged (1500×***g***, 2 min, 4 °C). Two 0.6 mL aliquots of supernatant from each tissue prep were transferred to separate tubes. 0.2 mL of ammoniun sulfamate (12.5% w/v) was added to each tube, and all tubes were shaken vigorously for 5 min. A fresh solution of 3-methyl-2-thiazolone hydrazone HCl monohydrate (50 mg in 10 mL of distilled water) was made, and 0.2 mL was added to each tube. Samples were then heated at 100 °C for 3 min and cooled. After cooling, 0.2 mL of $FeCl_3 \cdot 6H_2O$ (0.83% w/v) was added to each, and OD was measured at 650 nm after 25 min. Chitin content, measured in glucosamine equivalent, was measured by the following formula: chitin content = [5 × (OD of organ − OD of control organ)]/(OD of glucosamine − OD of water).

Assessment of pulmonary tissue cytokine concentration

Lungs were removed and homogenized in 2 mL PBS with tissue grinders (Tissue Tearor, model 985-370; Biospec Products, Racine, WI). The homogenates were kept in ice and then centrifuged at 4 °C; the supernatants were kept at − 70 °C until their use. Lung homogenates were thawed only once immediately before performing the assays. Proinflammatory cytokines such as IL-6 and TNF-α, and also

IFN-γ (Th1) and IL-4 (Th2) were quantified using an enzyme-linked immunosorbent assay (ELISA) kit from PharMingen (OptEIA set; San Diego, CA). The ELISA procedures were performed according to the manufacturer's protocol. The concentrations of cytokines were determined with reference to a standard curve for serial twofold dilutions of murine recombinant cytokines. The lower limit of detection of each recombinant's standard curve was 15.6, 31.3, 15.6 and 7.8 pg mL^{-1} for IL-6, IFN-γ, TNF-α and IL-4, respectively.

Histopathologic analysis

The animals were sacrificed as described above using an i.p. injection of 1.0 mL of 2.5% sodium penthotal. After sacrifice the animals' thoracic cavity was opened and the right auricle sectioned; 10 mL of PBS were then injected directly on the heart in order to insuflate the lungs and withdraw any remaining blood. Lungs were excised and then fixed in 10% neutral formaldehyde in PBS, embedded in paraffin and cut into 5-μm-thick sections, then stained with hematoxylin and eosin. The tissue sections corresponding to areas from both lungs of 4 or 6 mice were read in a blinded fashion by a pathologist and scored for the degrees of inflammation as shown in Table 1.

Statistical analysis

Data were analyzed using GRAPHPAD PRISM version 3.02 for Windows, GraphPad Software, San Diego, CA (www.graphpad.com). The results were expressed as the mean ± standard error of the mean (SEM) and compared using *t* test or ANOVA. The *P* values were considered statistically significant if they were < 0.05.

Results

Influence of ECMp- or mAb-treated conidia on fungal burden

In order to verify the reliability of chitin assays, various volumes of a suspension of yeast grown *in vitro* were tested; this assay showed a linear relationship between the volume of yeast suspension and the chitin content ($r = 0.98$; data not shown).

The progression of disease in BALB/c mice infected with ECMp or mAb2G4-treated *P. brasiliensis* conidia was monitored by determining chitin content in the lungs, liver and spleen at different postinfection periods. As shown in Fig. 1a, when conidia were treated with ECMp there was a significant decrease in chitin content in the lungs at week 8, especially with laminin and fibrinogen ($P < 0.03$ and $P < 0.01$, respectively) when compared with the animals infected with untreated conidia; the remaining periods studied did not shown difference in the lungs chitin content. Moreover, the chitin content in liver and spleen had the same behavior as that observed in the lungs (Table 2). In contrast, when *P. brasiliensis* conidia were treated with the mAb2G4 no differences in chitin content were found in any of the organs tested at the different periods studied when compared with organs from mice infected with untreated conidia or treated with an isotype control (Fig. 1b, Table 2).

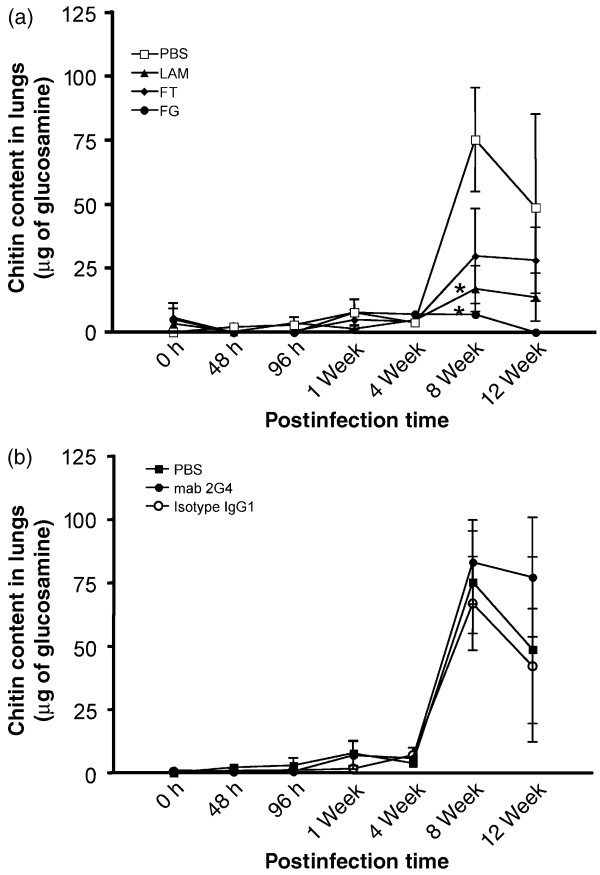

Fig. 1. Chitin content in lungs of mice infected with *Paracoccidioides brasiliensis* conidia. (a) Mice were infected with 4×10^6 *P. brasiliensis* conidia previously treated with various ECMp such as laminin (LAM), fibronectin (FT), and fibrinogen (FG) (all at 100 μg mL^{-1}) or with untreated conidia (PBS). (b) Lungs of mice infected with 4×10^6 *P. brasiliensis* conidia previously treated with mAb2G4 and an isotype (IgG1) as a control (at 100 μg mL^{-1}). The values represent the mean ± SEM of chitin content expressed as μg of glucosamine. *Significant difference ($P < 0.05$) when compared with the control (mice infected with untreated conidia).

Table 1. Pulmonary histologic scoring criteria

Extent of pulmonary inflammation	Score
No inflammation present	0
Up to 33% infiltration of lung	1
Between 33 and 66% infiltration of lung	2
Between 66 and 100% infiltration of lung	3

Table 2. Chitin content in liver and spleen of mice infected with treated or untreated *Paracoccidioides brasiliensis* conidia* at different post-infection periods

Organ	Post-infection time (weeks)	Untreated conidia	LAM	FT	FG	mAb2G4	Isotype (IgG1)
Liver	1	5.8 (2.0)	1.5 (1.8)	2.2 (2.6)	2.5 (1.4)	5.0 (6.4)	4.2 (1.8)
	4	1.5 (2.0)	1.8 (2.2)	2.0 (1.6)	1.1 (1.6)	2.2 (0.9)	1.3 (1.4)
	8	10.3 (6.7)	1.0 (0.2)†	26.1 (53.7)	1.3 (1.2)†	9.2 (1.3)	2.5 (2.5)
	12	1.8 (2.4)	0.2 (0.5)	1.3 (1.5)	1.2 (1.7)	41.1 (58.4)	6.9 (6.4)
Spleen	1	1.8 (1.6)	1.9 (3.5)	1.9 (2.3)	1.7 (1.8)	2.6 (3.8)	0.9 (1.9)
	4	1.6 (2.6)	1.9 (1.8)	2.6 (2.2)	2.2 (2.1)	3.8 (5.6)	1.1 (1.5)
	8	8.6 (6.3)	1.3 (1.2)†	19.2 (38.3)	1.0 (0.6)†	2.7 (2.0)	0.8 (1.2)
	12	1.7 (1.9)	3.3 (6.6)	0.1 (0.3)	1.1 (1.0)	84.5 (143.6)	27.6 (51.8)

*Liver and spleen from infected mice were collected and disrupted in 2 mL of PBS, and processed for chitin assays as described in 'Materials and methods.' Numbers indicate the mean (standard deviation) for five mice tested at each time point.
†Difference statistically significant; $P < 0.03$ when compared to results obtained from mice inoculated with untreated *P. brasiliensis* conidia (PBS).

Histopathologic findings in mice infected with ECMp- or mAb2G4-treated and untreated *P. brasiliensis* conidia

The extent of pulmonary inflammation was analyzed semi-quantitatively and scored as described in Table 1. As shown in Figs 2, 3 and 4, when mice were infected with ECMp- or mAb2G4-treated conidia, no changes in the inflammatory response were observed when compared with the controls. During the first 2 days there was an increase in the number of neutrophils and alveolar macrophages, which appeared dispersed in the inflammatory infiltrate (not shown). On day 4 postinfection the cellular inflammatory reaction consisted of neutrophils and macrophages located inside alveolar spaces and surrounding the peribronchial vessels (Figs 3 and 4). From week 4 postinfection and on, mice presented a chronic inflammatory response composed by granulomas of various sizes, which contained a large amount of fungal cells circumscribed by macrophages, lymphocytes, plasma cells and occasional giant cells were observed.

Effect of ECMp- or mAb-treated conidia on pulmonary cytokine production

Levels of some pulmonary cytokines such as IL-6, TNF-α, IFN-γ and IL-4 were measured in lung homogenates in order to examine if the treatment of *P. brasiliensis* conidia with different ECMp or with a mAb directed against the 32 kDa adhesin present on fungal surface had some effect on the host inflammatory immune response to infection. When mice were infected with *P. brasiliensis* conidia treated with the various ECM proteins or with the mAb2G4, they presented significant high levels of proinflammatory cytokines (IL-6 and TNF-α) during the first 4 days postinfection when compared with noninfected animals ($P < 0.01$, data not shown), but no differences were found when compared with animals infected with untreated conidia (Figs 5 and 6). Nonetheless, a low but significant increase of TNF-α was observed at week 8 in infected mice with ECM proteins-treated conidia when compared with animals infected with untreated propagules ($P < 0.05$; Fig. 5c).

IFN-γ levels were significantly increased in the lung homogenates at day 4 postinfection when the animals were infected with treated or untreated *P. brasiliensis* conidia when compared with noninfected animals ($P < 0.02$, data not shown). In addition, higher levels of this Th1 cytokine were observed at day 4 and/or week 12 postinfection in lung homogenates of mice infected with *P. brasiliensis* conidia treated with laminin ($P < 0.05$), fibronectin ($P < 0.01$), fibrinogen ($P < 0.05$) (Fig. 5b and d) and with the mAb2G4 ($P < 0.001$) (Fig. 6b) when compared with the animals infected with untreated conidia.

IL-4 did not shown any differences when the various treatments were used and at different postinfection periods (Figs 5 and 6).

Discussion

This work showed that ECMp participate in the course of experimental PCM. Treatment of *P. brasiliensis* conidia with soluble ECMp before animal infection had the capacity to decrease the fungal burden as observed by diminishing of chitin content in the lungs, liver and spleen at week 8 postinfection, especially when propagules were treated with laminin and fibrinogen. Previous studies had characterized some ECM compounds involved in host–*P. brasiliensis* interaction (Vicentini *et al.*, 1994, 1997; Hanna *et al.*, 2000; Mendes-Giannini *et al.*, 2000), and had shown gp43, the major immunodiagnostic component of *P. brasiliensis*, to be an important molecule that confers binding capacity to laminin. These studies also described the enhanced pathogenicity of laminin-coated yeast cells in a hamster model of testicle infection (Vicentini *et al.*, 1994). In addition, these findings were further explored using a mAb that recognizes a laminin-binding epitope of a protein from *Staphylococcus*

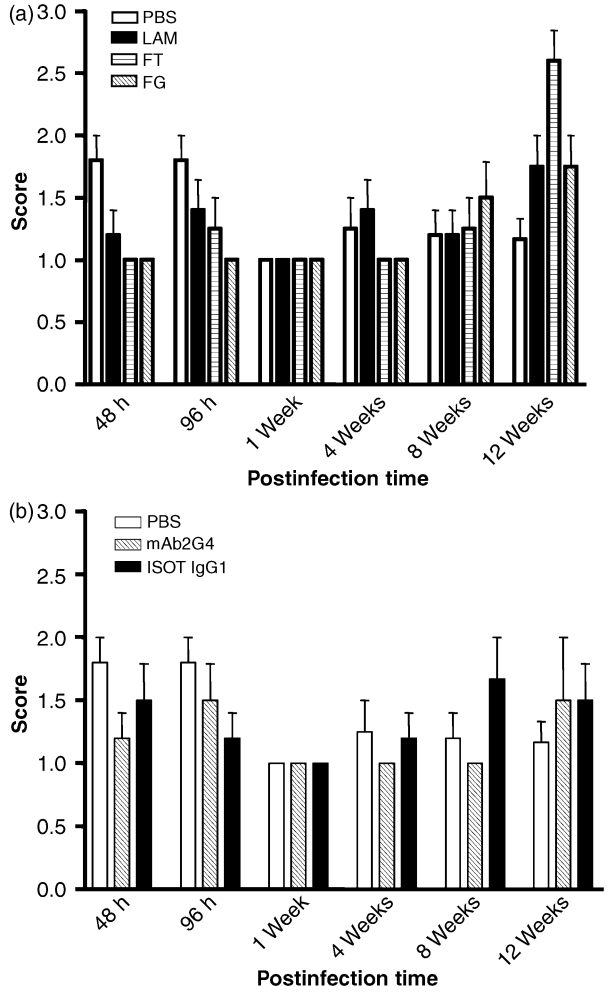

Fig. 2. Histopathological analysis of lungs from mice infected with treated or untreated *Paracoccidioides brasiliensis* conidia. (a) Lungs of mice infected with conidia previously treated with various ECMp such as laminin (LAM), fibronectin (FT), and fibrinogen (FG) (all at 100 μg mL^{-1}) or with untreated conidia (PBS). (b) Lungs of mice infected with conidia previously treated with mAb2G4 or an isotype (IgG1) as a control (at 100 μg mL^{-1}). The values represent the mean ± SEM of the score of the extent of pulmonary inflammation as described in 'Materials and methods.'

aureus, which had the capacity to inhibit the laminin-mediated adhesion of *P. brasiliensis* yeast to epithelial cells in culture, as well as, to diminish the severe *P. brasiliensis* infection in the hamster model induced by laminin-coated yeast (Vicentini *et al.*, 1997). In contrast, different results were obtained by Andre *et al.* (2004), who studied the influence of previous treatment of yeast cells with laminin on the course of the intratracheal infection of resistant and susceptible mice using high-virulence (Pb18) and low-virulence (Pb265) *P. brasiliensis* isolates, and showed that previous treatment with this ECMp did not enhance *P. brasiliensis* pathogenicity in a pulmonary model of infection, even when low infecting doses of virulent yeast or a low-virulent isolated were used. On the contrary, treatment with laminin led a to less severe pathology as revealed by histopathological analysis of the Pb18-infected group and by diminished CFU counts in the lungs of mice infected with the low-virulence isolate (Andre *et al.*, 2004). Similar results were observed in the present study, where treatment of *P. brasiliensis* conidia with laminin and fibrinogen decreased significantly the chitin content in the different organs analyzed at week 8.

Despite the reduction in chitin contents, the inflammatory response was not modified by conidia treatments. We can suppose that this behavior could be owed to the levels of TNF-α detected locally in the lungs able to attract inflammatory cells (Tracey & Cerami, 1993).

Identification of the fungal surface molecules that mediate the interaction with host cell receptors or compounds of the ECM proteins is certainly important for an understanding of the host–invader interplay. Recently, we demonstrated the presence of two proteins of 19 and 32 kDa in the surface of *P. brasiliensis*, which interacted with laminin, fibronectin and fibrinogen. Moreover, a mAb raised against the 32-kDa protein or the purified protein inhibited the adherence of *P. brasiliensis* conidia to these immobilized ECMp, indicating that such interaction is mediated by this adhesin (González *et al.*, 2005a). In addition, we had studied the interaction of *P. brasiliensis* conidia and human type II alveolar cells (A549), where the treatment of *P. brasiliensis* conidia with soluble laminin, fibronectin and fibrinogen or the mAb2G4, as well as, the treatment of A549 cells with antibodies against these ECMp or the purified 32-kDa protein inhibited significantly the adherence of fungal propagules to these epithelial cells, suggesting the importance of the ECM compounds and the 32-kDa protein in the adhesion of *P. brasiliensis* to host cells (unpublished data). However, *in vivo* the role of specific adhesins in the *P. brasiliensis* infection process is not yet explored. In the present study, we studied the participation of the adhesin of 32 kDa (González *et al.*, 2005a). To this intent, *P. brasiliensis* conidia were treated with a mAb raised against this adhesin before the animals were infected. Contrary to what we expected, this treatment did not affect the course of infection, suggesting that *in vivo* this adhesin does not participate in the infection process or that other different mechanisms are involved.

On the other hand, it is well documented that IFN-γ plays a pivotal role in host resistance against various pathogens by augmenting the killing activity of macrophages (Buchmeier & Schreiber, 1985; Denis, 1991; Mody *et al.*, 1991; Lucchiari *et al.*, 1992). *In vitro* it was shown that IFN-γ-activated macrophages exert an enhanced killing activity against *P. brasiliensis* conidia and yeast cells (Brummer *et al.*, 1988; González *et al.*, 2000), and that this fungicidal mechanism is

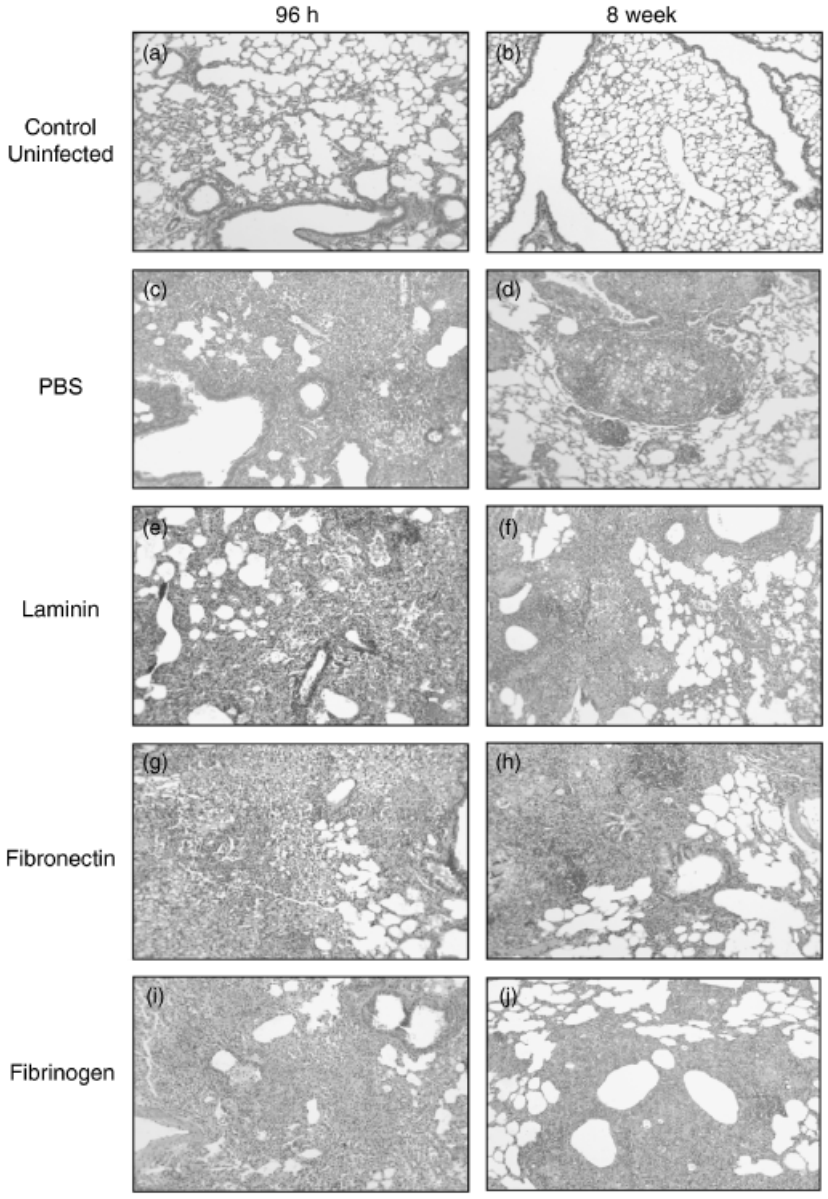

Fig. 3. Photomicrographs of pulmonary lesions from mice inoculated with PBS alone (a and b), with untreated *Paracoccidioides brasiliensis* conidia (c and d) and with conidia previously treated with laminin (e and f), fibronectin (g and h) or fibrinogen (i and j) at different postinfection periods: 96 h (a, c, e, g and i) and 8 weeks (b, d, f, h and j). Lungs sections were stained with H&E. Magnification 100 ×.

mediated by nitric oxide production (González *et al*., 2000). *In vivo*, the importance of this Th1 cytokine has been demonstrated, where both depletion and genetic deficiency of IFN-γ led to exacerbated disease (Cano *et al*., 1998; Souto *et al*., 2000). In the present work, we observed higher levels of IFN-γ in lung homogenates from infected mice when the *P. brasiliensis* conidia were treated with the various ECM proteins tested during early periods postinfection, especially at day 4. These results are not consistent with those obtained by Andre *et al*. (2004), who did not observe changes in the production of cytokines TNF-α, IL-12, IL-4 and IFN-γ under similar circumstances. However, it is clear that during the early period of infection the adaptive immune response is not yet well established, and we can speculate that the IFN-γ production was mainly due to the cytokine secretion by other cells of innate immunity such as natural killer cells (Trinchieri & Scott, 1995), which could be stimulated by IL12-secreted after macrophages are activated. However, when *P. brasiliensis* conidia were treated with the mAb2G4, the IFN-γ level was augmented at week 12 but the chitin content did not change when compared with infected animals with untreated conidia. This effect could be explained due to exposition of macrophages to classical activating signals in the presence of immunoglobulin G (IgG) immune complexes (in this case *P. brasiliensis* conidia-mAb2G4) induce the production of large amounts of IL-10, which plays an important role in dampening macrophage activation (Mosser, 2003).

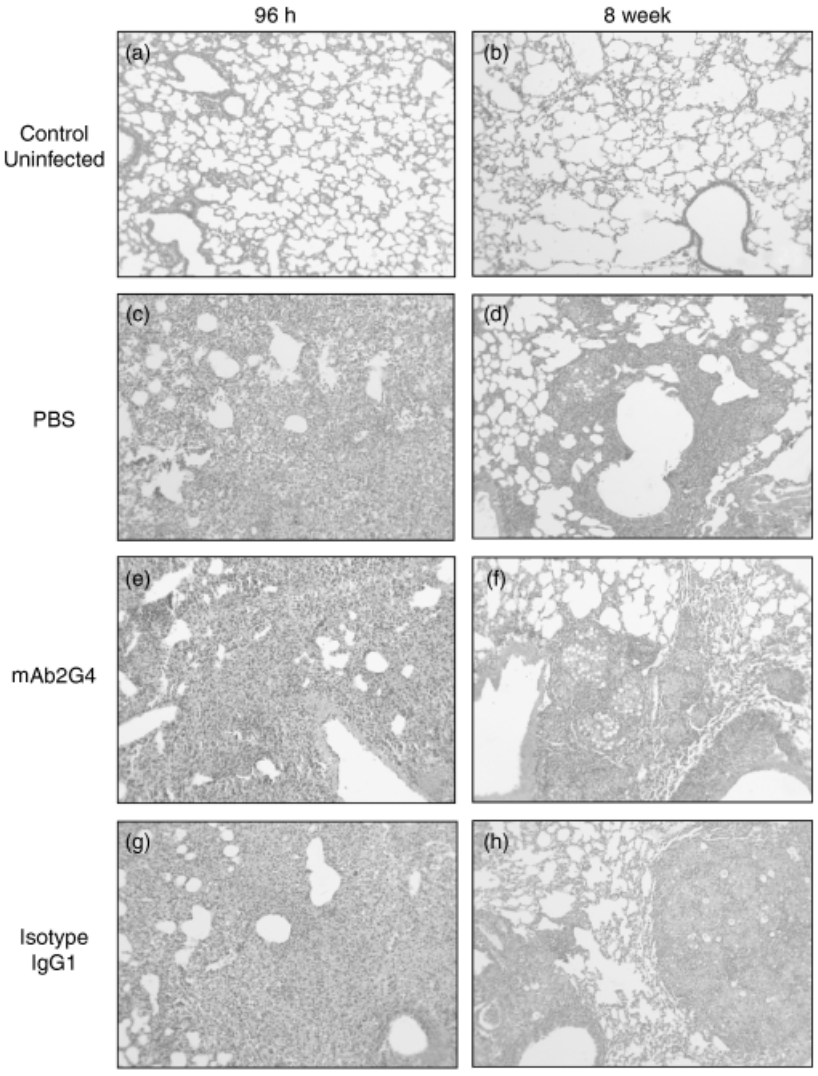

Fig. 4. Photomicrographs of pulmonary lesions from mice inoculated with PBS alone (a and b), with untreated *Paracoccidioides brasiliensis* conidia (c and d) and with conidia previously treated with mAb2G4 (e and f) or with control isotype (g and h) at different postinfection periods: at 96 h (a, c, e, and g) and at week 8 (b, d, f, and h). Lungs sections were stained with H&E. Magnification × 100.

Moreover, it was observed that although resistance of A/Sn mice to PCM was linked with the secretion of Th1 cytokines (IFN-γ and IL-2), susceptibility was no clearly associated with a Th2 profile, because IL-4 was not found in the supernatants of antigen-stimulated lymph node cells from infected susceptible B10.A mice (Calich & Kashino, 1998; Kashino *et al.*, 2000). Recently, the same research group described that IL-4-deficient mice showed a decreased pulmonary fungal loads at week 8 postinfection with low levels of IL-10 production; and else, they observed that IL-4 can have a protective or a disease-promoting effect in pulmonary PCM depending on the genetic background of the host (Arruda *et al.*, 2004; Pina *et al.*, 2004). In this study, we can suppose that the low levels of IL-4 observed could be sufficient to regulate the effect exerted by IFN-γ or that this cytokine could be replaced by another mediator, such as IL-13, whose activity is similar to that of IL-4.

The lower fungal burden observed at 8 weeks in infected mice with ECMp-treated conidia could also be associated with increased secretion of TNF-α detected locally in the lungs at this time. TNF-α appears to be involved in the immunological control of *P. brasiliensis* infection, because in previous studies with TNF-α receptor knockout mice, animals succumbed more rapidly than their TNF-α receptor competent counterparts (Souto *et al.*, 2000).

Chemokines also appear to play a major role in regulating the migration of specific leukocytes populations in both the acute and chronic inflammation processes in several diseases (Luster, 1998). Thus, recent studies have shown that IFN-γ modulates the production of high levels of various chemokines such as RANTES, MCP-1 and IP-10, and this production is associated with mononuclear cell recruitment into the lungs of *P. brasiliensis*-infected C57BL/6 mice (Souto *et al.*, 2003). Indeed, IFN-γ has been shown to up-regulate

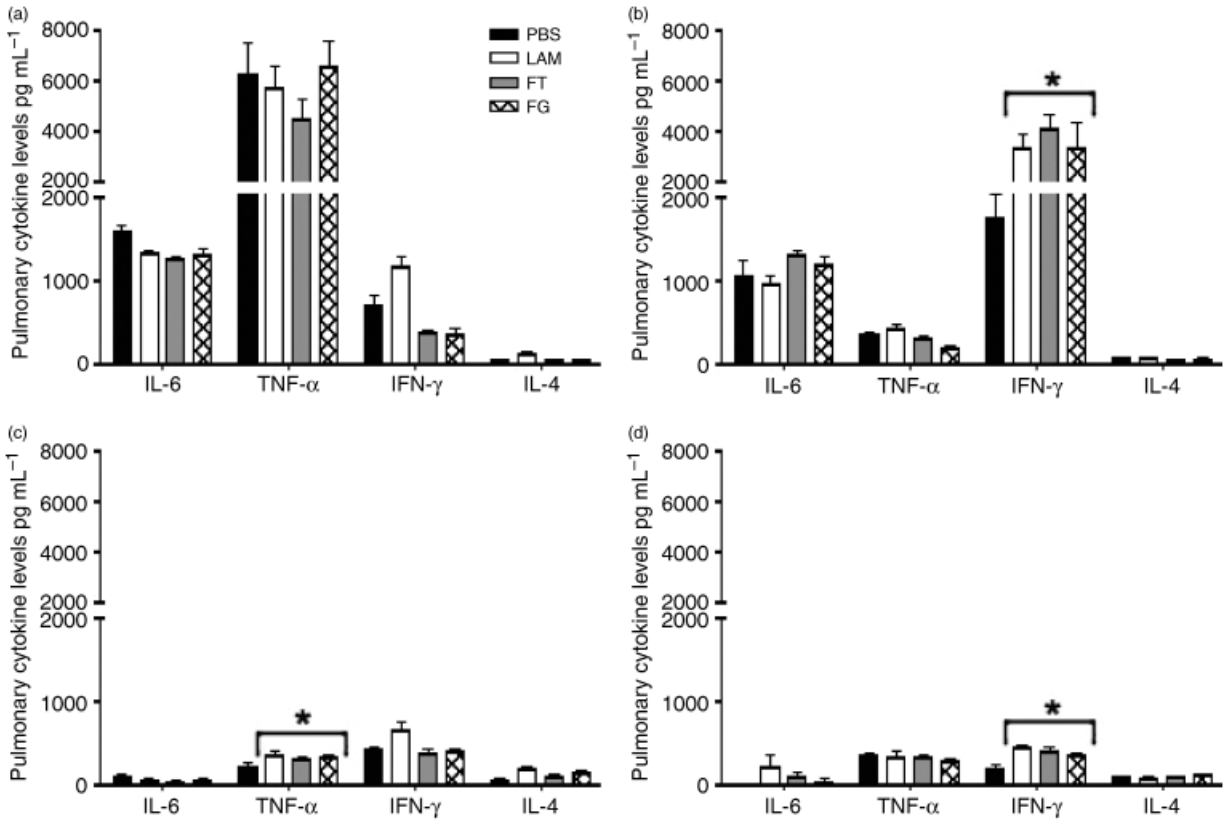

Fig. 5. Levels of pulmonary cytokines in lungs of mice infected with *Paracoccidioides brasiliensis* conidia previously treated with various ECMp such as laminin (LAM), fibronectin (FT), and fibrinogen (FG) (all at 100 μg mL^{-1}), or with untreated conidia (PBS). Levels of IL-6, TNF-α, IFN-γ and IL-4 were measured in lung homogenates by ELISA at 48 h (a), 96 h (b), 8 weeks (c) and 12 weeks (d) postinfection periods. The values represent the mean ± SEM of cytokine levels (pg mL^{-1}). *Significant difference ($P < 0.05$) when compared with mice infected with untreated *P. brasiliensis* conidia (PBS).

ICAM-1 on endothelial cells promoting lymphocyte-endothelial cell binding (Pober *et al.*, 1986). We demonstrated that during early PCM infection periods, there are higher levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, as well as, the chemokine MIP-2 in the lungs of infected animals, and this was associated with the recruitment of leukocytes (González *et al.*, 2003). Recently, the expression of adhesion molecules such as ICAM-1, VCAM-1, CD18, LFA-1 and Mac-1 were up-regulated during the first 4 days in the lungs of mice infected with *P. brasiliensis* conidia, and their expression was associated with the recruitment of leukocytes into the lungs as well as with the significant decrease of CFU after the first 2 days postchallenge (González *et al.*, 2005b).

Toll-like receptors (TLRs) play an important role in the activaction of innate immunity by recognizing specific pattern of microbial components; although, mechanisms involving TLRs were not yet described for murine PCM. However, some TLRs are apparently able to mediate response to host molecules including breakdown products of ECM proteins (Okamura *et al.*, 2001; Smiley *et al.*, 2001; Kuhns *et al.*, 2007); and therefore, we can speculate that the recognition by TLRs of the ECM proteins covering the *P. brasiliensis* conidia could contribute to the observed effect.

In conclusion, our results demonstrated that ECMp, especially laminin, fibrinogen and to a lesser extent fibronectin are involved in the fungal infection of the lungs. These ECMp appear to be important in the initial events of pulmonary PCM, and have shown their capacity to block the adherence to lessening the pathogenic effect of *P. brasiliensis* infection by covering ECM-binding epitopes and/or by modulating the inflammatory response. Awareness of these types of interaction could be important for the understanding of early events of infection and may be of interest for developing vaccines or receptor-blocking therapies based on adhesins present on the *P. brasiliensis* fungal surface.

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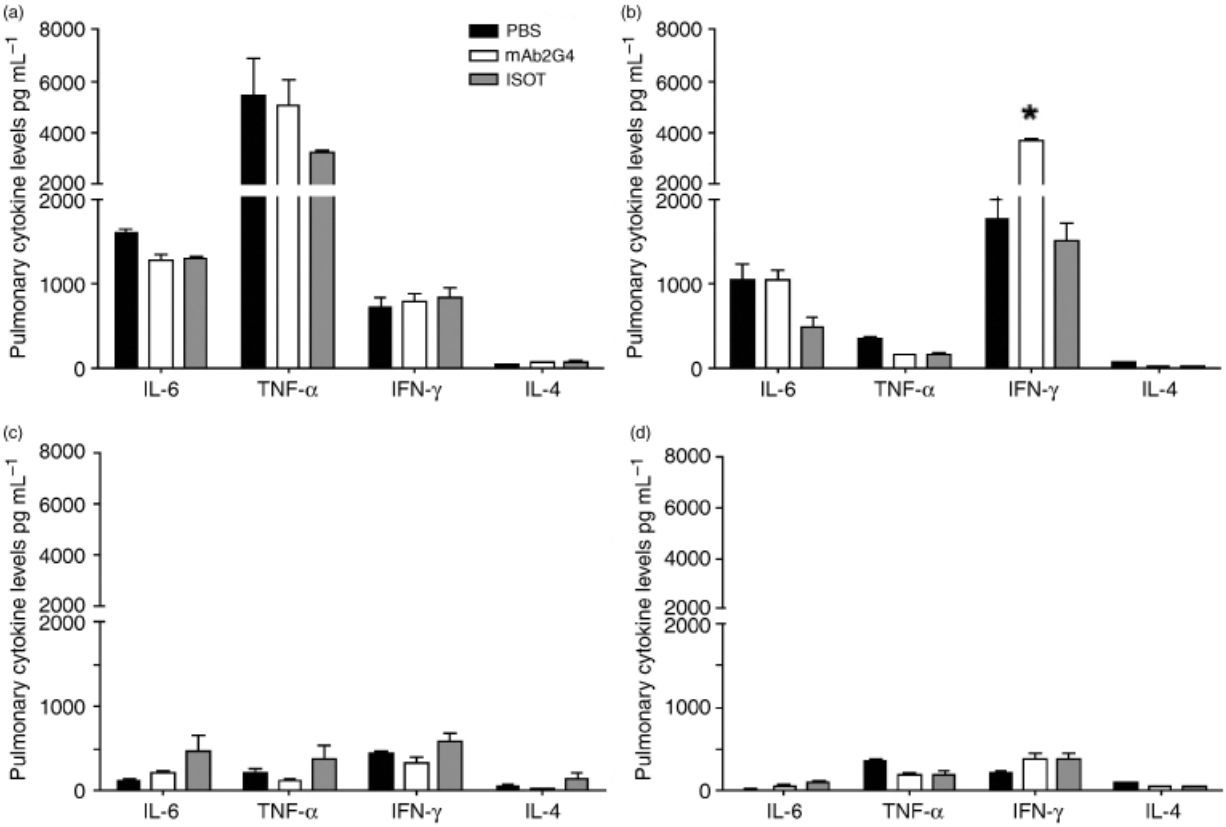

Fig. 6. Levels of pulmonary cytokines in lungs of mice infected with *Paracoccidioides brasiliensis* conidia previously treated with mAb2G4 and an isotype (IgG1) as a control (at 100 μg mL^{-1}) or with untreated conidia (PBS). Levels of IL-6, TNF-α, IFN-γ and IL-4 were measured in lung homogenates by ELISA at 48 h (a), 96 h (b), 8 weeks (c) and 12 weeks (d) postinfection periods. The values represent the mean ± SEM of cytokine levels (pg mL^{-1}). *Significant difference ($P < 0.05$) when compared with mice infected with untreated *P. brasiliensis* conidia (PBS).

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