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B Cell-Deficient Mice Display Enhanced Susceptibility to *Paracoccidioides brasiliensis* Infection

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Abstract Paracoccidioidomycosis (PCM) is a chronic granulomatous disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. T helper 1 (Th1)-mediated immunity is primarily responsible for acquired resistance during *P. brasiliensis* infection. On the contrary, the susceptibility is associated with occurrence of type-2 immunity (Th2), which is characterized by IL-4 release, B cell activation, and production of antibodies. Although antibodies are frequently associated with severe PCM, it is not clear whether they contribute to susceptibility or merely constitute a marker of infection stage. Here, we assessed the function of B cells during experimental *P. brasiliensis* infection in mice, and our results showed that B cell-knockout (B^{KO}) mice are more susceptible than their wild-type littermate controls (C57BL/6, WT). The B^{KO} mice showed higher mortality rate, increased number of colony-forming units in the lungs, and larger granulomas than WT mice. In the absence of B cells, we observed high levels of IL-10, whereas IFN-γ, TNF-α, and IL-4 levels were similar between both groups. Finally, we showed that transference of WT immune serum to B^{KO} mice resulted in diminished infiltration of inflammatory cells and better organization of the pulmonary granulomas. Taken together, these data suggest that B cells are effectively involved in the control of *P. brasiliensis* growth and organization of the granulomatous lesions observed during the experimental PCM.

Keywords *Paracoccidioides brasiliensis* · B cells · Immunoprotection · Granuloma

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Introduction

Paracoccidioidomycosis (PCM), a chronic granulomatous disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, is one of the most important human systemic mycosis in Latin America [1]. The infection is acquired after inhalation of fungal conidia, propagules that convert into the invasive yeast form once in the lungs [2]. Next, the *P. brasiliensis* yeast cells may induce a chronic inflammatory reaction, which culminates with the formation of paracoccidioidal granulomas. Patients with severe disease show disorganized granulomas associated with increased number of viable yeast cells in the lesions, which can spread to multiple organs through lymphatic and circulatory systems, resulting in disseminated lesions throughout the body [3, 4].

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The antifungal host defense is regulated by a complex interaction between immunocompetent cells and a network of cytokines and chemokines [5, 6]. Several studies involving clinical and experimental data indicate that cell-mediated immunity is the main host defense during *P. brasiliensis* infection [7]. In fact, patients with a compromised cellular immunity are more susceptible to present a severe form of PCM [8]. Moreover, several studies have shown that different disease outcomes can be derived from the commitment of precursors to either T helper 1 (Th1) or Th2 lineage [9, 10]. Resistance to *P. brasiliensis* infection has been linked to the preferential production of the Th1-type cytokines. In fact, in a murine system, the depletion of IFN-γ using monoclonal antibodies against IFN-γ favored the exacerbation of pulmonary *P. brasiliensis* infection [11]. Moreover, the IFN-γ-deficient mice are highly susceptible to PCM, showing loose granulomas and pronounced loss of protective response due to a diminished production of nitric oxide [12].

The role of antibodies during infectious diseases is controversial. Some groups consider that the humoral immunity is crucial to the neutralization of toxins and has protective role against the most known viruses, bacteria, and fungal pathogens, as *Candida* sp. and *Cryptococcus neoformans* [13–17]. Conversely, there are evidences suggesting that antibodies can also be deleterious. For example, the production of high levels of anti-*P. brasiliensis* antibodies is usually associated with severe PCM in humans [18]. In addition, gp43, the main antigenic component of *P. brasiliensis*, was distinguishably presented by B cells in susceptible mice, which induced strong activation of Th2 subset [18]. Moreover, a subclass of B lymphocytes, the B-1 cells, has been associated with the progression of experimental PCM, leading to increased mortality of *P. brasiliensis* infected BALB/c mice [19].

In order to better understand the role of B cells during PCM, we used an experimental model of *P. brasiliensis* infection in B cell-deficient mice. Our results showed that B lymphocytes are effectively involved in the control of *P. brasiliensis* growth and in the organization of the granulomatous lesions in the lungs.

Materials and Methods

Ethics Statement

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Universidade de São Paulo, Ribeirão Preto, Brazil (process 061/2004). Accordingly, experiments were conducted adhering to both institutional and national guidelines for animal research, and all possible steps were taken to minimize animal suffering.

Animals

Male 6 to 8-week-old B cell-knockout mice (B^{KO}), originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and their counterpart C57BL/6 wild-type (WT) mice, were maintained under specific pathogen-free conditions in micro-isolator cages in the animal housing facility of the Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil. The animals were supplied with sterilized food and water ad libitum. Groups of 3 mice were used for each period of infection, and data are representative of 3 independent experiments.

Fungus and Mice Infection

Yeast cells of a highly virulent strain of *P. brasiliensis*, Pb18 [20], were used in this study. The isolate was maintained as yeast form at 37 °C in BHI culture medium for 7 days. Next, fungal cells were harvested, washed in phosphate-buffered saline (PBS, pH 7.2), and counted using hemocytometer. The concentration was adjusted to 1×10^7 cells per mL. The viability of fungal suspensions was determined by fluorescein diacetate-ethidium bromide staining as previously described [21]. The mice were anesthetized with 2,2,2-tribromoethanol (250 μg g^{-1}) and inoculated intravenously (i.v.) with 1×10^6 viable yeast cells in 100 μL of PBS. The lungs were harvested at days 7, 15, 30, and 60 post-infection (p.i.). Control mice received sterile PBS (100 μL, i.v.).

Survival Analysis

Male 6 to 8-week-old B^{KO} and WT mice were i.v. infected with 1×10^6 viable yeast cells. Survival of Pb18-infected mice (10 mice in each group) was verified daily for a period of 120 days.

Assay for Colony-Forming Units (CFU)

The number of viable yeast cells in the lungs from Pb18-infected mice was determined at 7, 15, 30, and

60 days p.i. by counting the CFU as previously described [22, 23]. Plates were incubated at 35–37 °C for 14 days, and the amount of CFU per gram of tissue was calculated.

Measurement of Cytokines in the Lung Homogenate

Lungs were collected at days 7, 15, 30, and 60 p.i., weighed and homogenized in 1 mL of PBS with protease inhibitor (Phenylmethylsulfonyl fluoride, Sigma; 1.6 mM) using a tissue homogenizer (Ika-Werke, GMB4 & Co. KG, Germany). After centrifugation (10,000*g* for 10 min), the supernatants were harvested and dispensed (0.05 mL) into a 96-well plate containing capture monoclonal antibodies. The levels of IFN-γ, TNF-α, IL-4, and IL-10 were measured by standard sandwich ELISA according to the manufacturer's protocol (BD Pharmingen, San Jose, CA, USA). Optical densities were measured at 492 nm using a microplate ELISA reader (EMAX; Molecular Devices).

Histopathology

Whole lungs were excised, fixed with 10 % formalin for 24 h and embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) for lesion analysis, or impregnated with silver to demonstrate reticulum fibers using standard protocols [24]. The images were captured in a common optical microscope and the data were obtained by a triplicate analysis of the sections.

Delayed-Type Hypersensitivity (DTH) Assay

Male 6 to 8-week-old B^{KO} and WT mice were i.v. infected with 1×10^6 viable yeast cells. The DTH reactions were evaluated at days 15, 30, and 60 p.i. by the footpad test as previously described [25]. Briefly, mice were challenged by injection of 25 µL (2 µg ml^{-1}) of exoantigen [26] in one hind footpad and the same volume of PBS in the other side. The footpad thickness was measured 24 h later with a dial caliper (precision 0.01 mm; Mitutoyo Corporation, Tokyo, Japan), and the swelling was expressed in millimeters. Non-infected mice subjected to the same footpad test were used as controls. After DTH measurement, the mice were immediately killed.

Passive Immunization of B^{KO} Mice

Male 6 to 8-week-old B^{KO} and WT mice were i.v. infected with 1×10^6 viable *P. brasiliensis* yeast cells. The serum was collected after 15, 30, and 60 days p.i. and mixed in a unique sample pool. Three different serum samples were obtained: B^{KO} immune, WT immune (Immune serum), and WT non-immune (Normal serum). Male 6 to 8-week-old B^{KO} mice were intraperitoneally injected with 500 µl of one serum sample 24 h before the *P. brasiliensis* infection. The treatment was repeated on days 4 and 12 p.i., and mice were killed after 15 or 30 days after Pb18-infection. Another group of B^{KO} mice received serum samples at days −1, 5, 15, and 25, and the killing was realized at day 30 p.i.

Statistical Analysis

Data are expressed as the mean ± standard error of the means (SEM). Statistical analyses were performed using ANOVA to compare multiple groups followed by the parametric Tukey–Kramer test for two group comparison. The Gehan–Breslow–Wilcoxon method was used to compare survival curves. All analyses were performed with Prism software (version 5.0, GraphPad, San Diego, CA, USA). A p value < 0.05 was considered to indicate statistical significance.

Results

B Cells are Important for Mice Survival and Fungal Control During *P. brasiliensis* Infection

To directly assess the role of B lymphocytes during PCM, we first investigated the mice resistance to the experimental *P. brasiliensis* infection. We observed that all WT mice survived until day 120 p.i. while more than 30 % of the B^{KO} animals succumbed until the day 85 of infection (Fig. 1a). Next, we determined the number of viable fungal cells recovered from the lungs of Pb18-infected animals. The B cell-deficient mice showed increased CFU counts ($p < 0.05$) in the lungs from day 15 until 60 p.i. (Fig. 1b). At 15, 30, and 60 days p.i., the amount of CFU recovered from B^{KO} mice were, respectively, 2.3, 1.78, and 4.01 times increased in the pulmonary tissue when compared with WT group at same period of infection. Moreover, B^{KO}-infected mice showed increased ($p < 0.05$) number of yeast cells in the liver

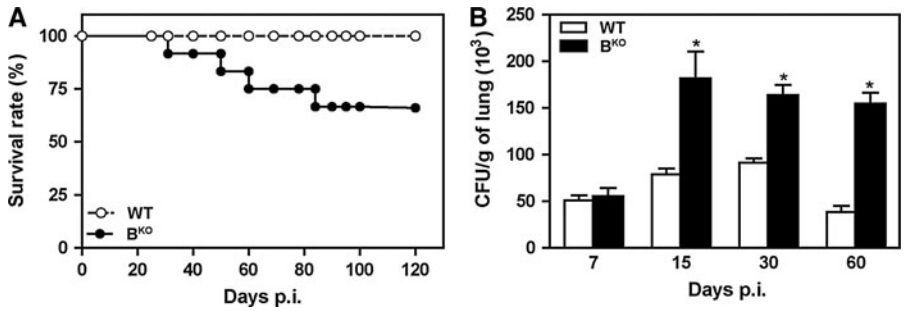


Fig. 1 B cells are necessary for increased host resistance during experimental *P. brasiliensis* infection. The WT and B^{KO} mice were intravenously infected with 1×10^6 viable Pb18 yeast cells. **a** The survival was observed daily during 120 days. **b** The number of colony-forming units (CFU) was determined in the lungs at days 7, 15, 30, and 60 post-infection (p.i.). The data represent the mean ± SEM of 3 mice and are representative of 3 independent experiments. *, $p < 0.05$ compared with WT mice at same period of infection

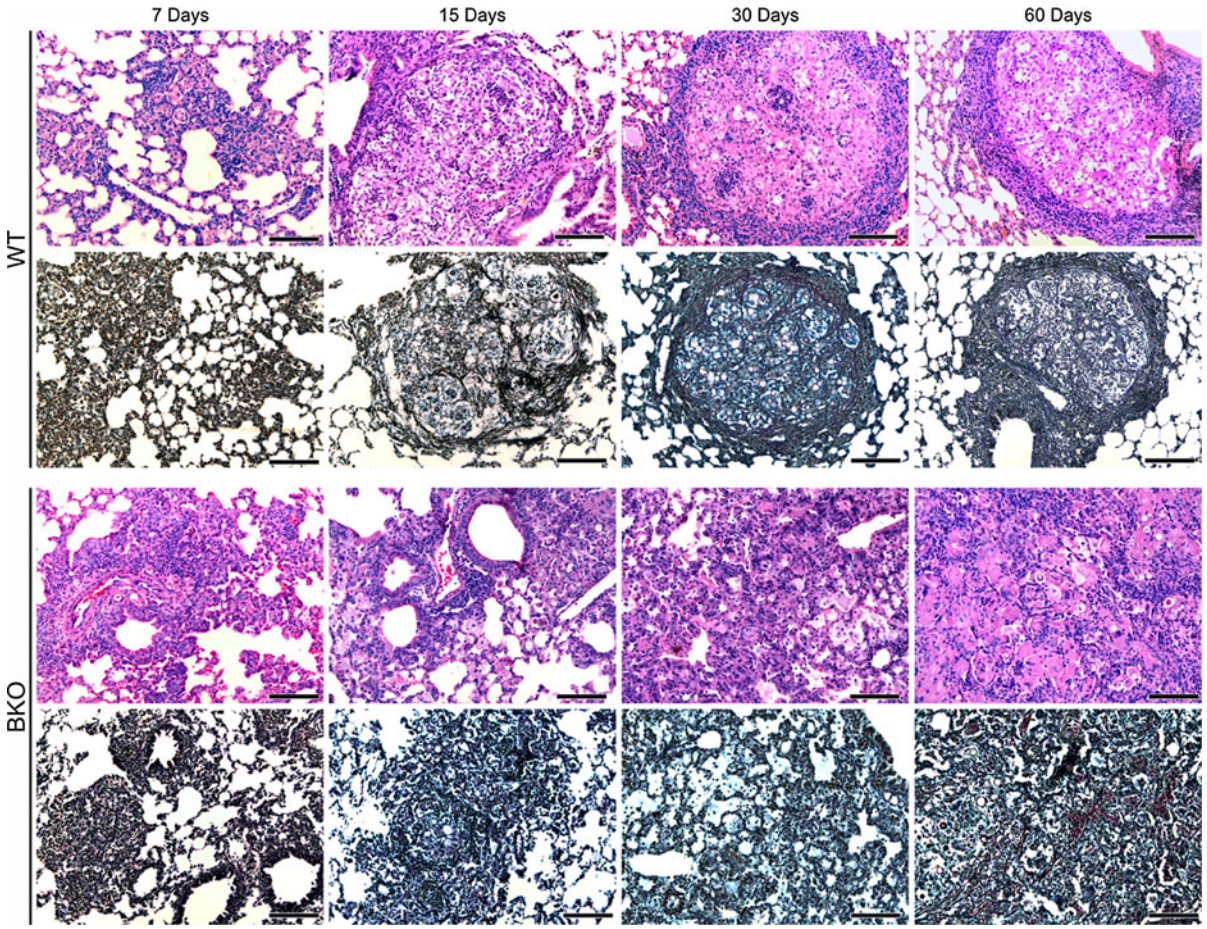


Fig. 2 B cells contribute to the development and organization of *P. brasiliensis*-induced granulomas. The WT and B^{KO} mice were intravenously infected with 1×10^6 viable Pb18 yeast cells. The lungs were collected at days 7, 15, 30, and 60 p.i., fixed in formalin, paraffin embedded, and cut into 5 μm sections. Next, tissue was stained with H&E for analysis of granulomatous lesions and pattern of inflammatory infiltrates (*lines 1* and *3*), or impregnated with silver for demonstration of reticulum fibers (*lines 2* and *4*). All slices were analyzed by light microscopy (magnification, ×10)

at day 60 p.i. and in the spleen after 15 days when compared with WT mice (data not shown). Overall, these data suggest that absence of B lymphocytes contribute to increased growth of *P. brasiliensis* yeast cells.

B Cells Modulate the Development and Organization of Granulomas in the Lungs from *P. brasiliensis*-Infected Mice

The lungs obtained from Pb18-infected WT and B^{KO} mice were histopathologically examined after 7, 15, 30, and 60 days p.i. As shown in Fig. 2, both groups showed similar pulmonary architecture at day 7 p.i. However, from day 15 until day 60 p.i., the lungs from WT mice showed more compact and organized granulomas, which were delimitated by a rim of lymphocytes. Unlike, we found in B^{KO} tissue an intense and disorganized inflammatory infiltrate composed by neutrophils, macrophages, and lymphocytes in all evaluated periods (Fig. 2). Taken together, these data suggest that B cells are associated with granuloma formation during experimental PCM.

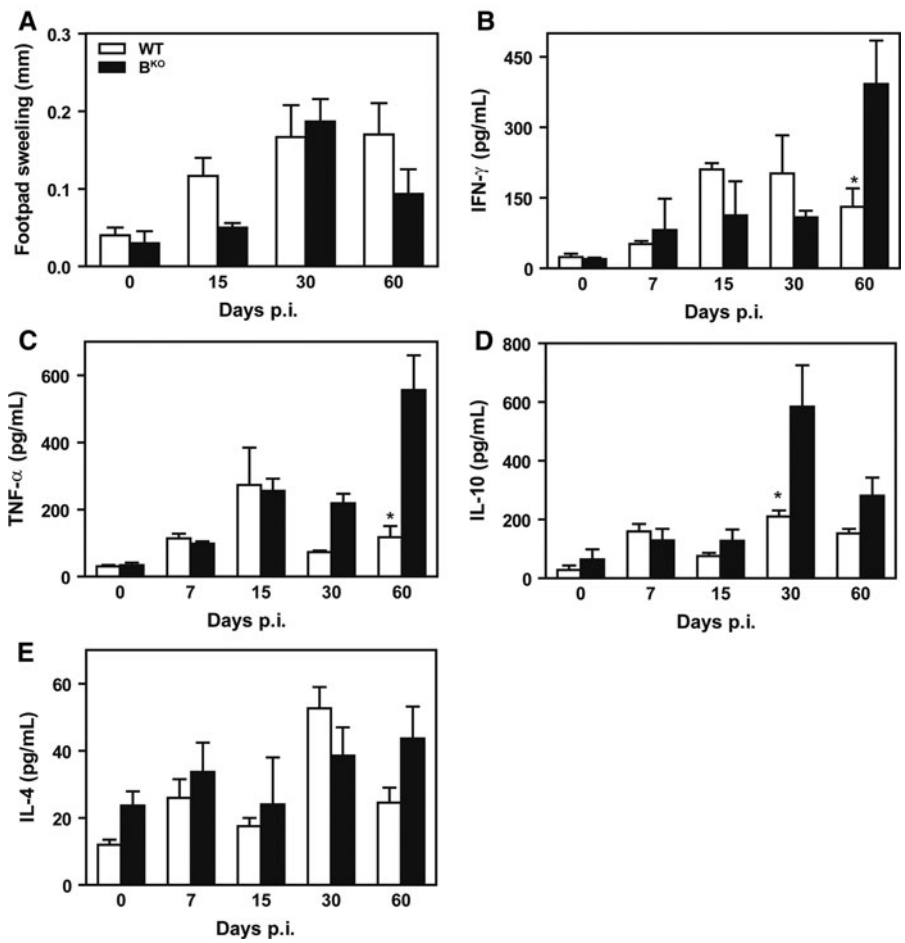


Fig. 3 B cells do not modulate the cellular immunity during experimental *P. brasiliensis* infection. The WT and B^{KO} mice were intravenously infected with 1×10^6 viable Pb18 yeast cells. **a** Mice were challenged by injections (2 μg/ml) of fungal antigens (*right footpad*) or PBS (*left footpad*) 24 h before measurement of footpad thickness. The DTH assay was performed on days 15, 30, and 60 p.i. The *bars* represent the mean ± SEM of the difference between the *right* and *left footpad* of 3 mice per group and are representative of 3 independent experiments. **b**–**e** The lungs were collected at days 7, 15, 30, and 60 p.i. The tissue was weighed and homogenized in sterile PBS, and the levels of IFN-γ (**b**), TNF-α (**c**), IL-10 (**d**), and IL-4 (**e**) were determined by ELISA. *Scale bars* represent the mean ± SEM of 3 mice and are representative of 3 independent experiments. *, $p < 0.05$ compared with B^{KO} mice at the same period of infection

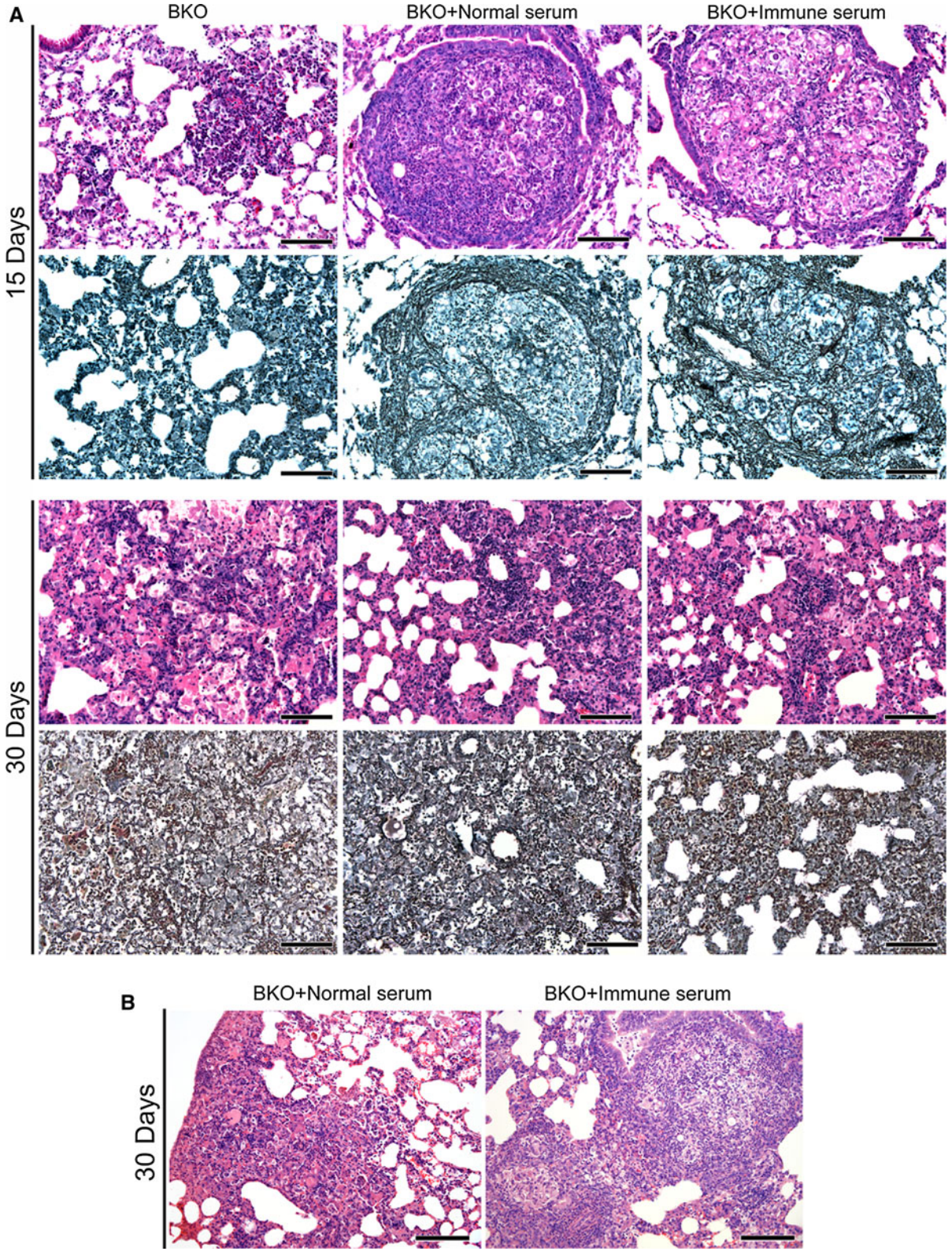
B Cells Do Not Modulate the Cellular Immunity During Experimental *P. brasiliensis* Infection

To verify whether B cells modulate the cellular immunity, we next performed the DTH test in both WT and B^{KO} mice infected with *P. brasiliensis*. As shown in Fig. 3a, the maximum footpad thickness was observed in both groups at day 30 p.i. In addition, both WT and B^{KO} mice showed similar DTH intensity in all evaluated periods, suggesting that B cells do not modulate the T cell response during Pb18-infection. The protective response against *P. brasiliensis* is characterized by the production of Th1-like cytokines, while a predominant Th2 response predisposes infected host to susceptibility [11, 12, 27, 28]. In order to verify if the absence of B cells is associated with the skew of the immune response toward a Th2 profile, we measured the levels of cytokines in the lung homogenate from WT and B^{KO} *P. brasiliensis*-infected mice. At 7, 15 and 30 days p.i., both groups showed similar IFN-γ and TNF-α production (Fig. 3b, c). However, at day 60 p.i., the synthesis of IFN-γ and TNF-α was 2.99 and 4.73 times increased ($p < 0.05$) in B^{KO} than WT mice, respectively (Fig. 3b, c). Both groups showed similar IL-10 release at days 7, 15, and 60 after *P. brasiliensis* infection. On the other hand, at 30 days p.i., the IL-10 production was increased in B^{KO} (584 ± 141.59) in comparison with WT mice (210 ± 20.79, $p < 0.05$) (Fig. 3d). Importantly, IL-10 level diminished at the same time that TNF-α began to increase in B^{KO} mice. Finally, we observed a similar IL-4 production in both groups in all evaluated periods (Fig. 3e). Taken together, these data indicate that, during experimental Pb18-infection, the cytokine production in the lungs is observed even in the absence of B cells.

Adaptive Transference of Immune Serum to B^{KO} Mice Leads to Granuloma Formation During *P. brasiliensis* Infection

To investigate whether immune serum is required to the inflammatory response and granuloma formation during experimental Pb18-infection, we used a serum transference protocol. The serum was collected from *P. brasiliensis* infected mice at days 15, 30 and 60 p.i. and pooled in a unique sample. Next, the B^{KO} mice were inoculated with WT immune (B^{KO} + immune serum), WT non-immune (B^{KO} + normal serum) or B^{KO} immune sera (B^{KO}) at days −1, 4 and 12 post-infection. As shown in Fig. 4a, the passive immunization of B^{KO} mice using WT normal serum or WT immune serum resulted in mild inflammatory infiltration and reasonably organized granuloma at day 15 p.i. However, when the histopathological analysis was performed at day 30 p.i., we unexpectedly observed a diffuse inflammatory infiltrate associated with interstitial edema, protein exudation and no granuloma formation in both groups treated with WT normal serum or WT immune serum (Fig. 4a). Interestingly, WT non-immune serum administration also induced the granuloma formation in the lungs from B^{KO}-infected mice; however, this protocol culminated in an intense inflammatory response when compared to WT immune serum group (Fig. 4a). On the other hand, B^{KO} mice that received B^{KO} serum showed an extensive diffuse inflammatory infiltrate with no granuloma formation at 15 and 30 days p.i. (Fig. 4a). Subsequently, we decided to investigate the lung histopathology of *P. brasiliensis*-infected B^{KO} mice after a prolonged passive serum immunization. For this, B^{KO} mice were inoculated with WT serum at days −1, 5, 15 and 25 of Pb18-infection. The histopathological analysis at day 30 p.i. showed that B^{KO} mice treated with WT immune serum presented more organized granulomas than B^{KO} animals that received the WT non-immune pool (Fig. 4b). Our results suggest that a continuous transference of WT immune serum to B^{KO} mice result in diminished infiltration of inflammatory cells and better organization of the granulomas in the lungs from *P. brasiliensis*-infected mice.

Fig. 4 Adaptive immunization induces granuloma formation in the lungs from B^{KO} mice during Pb-infection. The B^{KO} mice were intravenously infected with 1×10^6 viable Pb18 yeast cells. **a** Histopathological analysis of lung sections from Pb-infected B^{KO} mice intraperitoneally treated with 500 µl of B^{KO}, WT non-immune (normal serum) or WT immune serum at days −1, 5, and 12 p.i. The lungs were collected at days 15 and 30 p.i., fixed in formalin, embedded in paraffin, and cut into 5-µm sections. Next, tissue was stained with H&E for analysis of granulomatous lesions and pattern of inflammatory infiltrates (*lines 1* and *3*), or impregnated with silver for demonstration of reticulum fibers (*lines 2* and *4*). **b** Histopathological analysis (H&E staining) of lung sections collected at day 30 p.i. from B^{KO} mice treated with 500 µl of WT non-immune (normal serum) or WT immune serum at days −1, 5, 15, and 25 after Pb-infection. All slices were analyzed by light microscopy (magnification, ×10)


A
BKO
BKO+Normal serum
BKO+Immune serum
15 Days
30 Days
B
BKO+Normal serum
BKO+Immune serum
30 Days

Discussion

The cell-mediated immunity is the predominant host defense mechanism against fungal infections [29]. However, it is not well characterized how the specific antibodies can mediate this protection [30], but it appears that multiple and perhaps interdependent mechanisms may underlie the protective efficacy of antibodies.

Besides antibody production, the B cells are also involved in the antigen presentation and production of cytokines. Thus, we decided to investigate if B cells modulate the antifungal response toward protection or susceptibility during the experimental *P. brasiliensis* infection. We observed that the absence of B cells was associated with increased susceptibility and impaired control of fungal growth and dissemination. In addition, B^{KO} mice showed intense and sustained influx of mononuclear cells to the lungs, associated with disorganized granulomas and extended areas of edema. This pattern suggests that increased CFU counts in B^{KO} mice arose from the host inability to mount an organized granulomatous lesion during experimental PCM.

Up to day 60 p.i., the fungal burden was diminished in both WT and B^{KO} mice (data not shown). These data indicate that despite increased fungal load in B^{KO} mice, they are able to control the disease similarly to WT group, suggesting that B lymphocytes play a significant role in containing fungal growth at early stages of disease, but are not essential for the resolution of the infection.

The host susceptibility during *P. brasiliensis* infection can be associated with the low production of IFN-γ, a Th1-type mechanism of immune response. IFN-γ is involved in the activation of macrophages, which are the effectors of the antifungal immunity and protection against *P. brasiliensis* [3, 31], *Candida albicans* [32], and *C. neoformans* [33, 34]. Previous studies showed that B cells could drive to a Th2-like immune response [35]. Interestingly, we verified an improved IFN-γ production and concomitant CFU decrease in B^{KO} mice at day 60 p.i. Similarly, the TNF-α level raised in later periods of disease (60 days p.i.) in B^{KO} mice, what can probably be a compensative mechanism to contain the disease. In addition, we found increased IL-10 levels in B cell-deficient mice, corroborating previous studies which described that B lymphocytes stimulate IL-10-bearing T cells [18]. IL-4 is the major Th2 cytokine produced during PCM [36]; however, in our model, we did see a similar IL-4 production in both experimental groups.

Protective and non-protective results have been observed after antibody treatment during systemic fungal infectious diseases [37–39]. Seen that antibodies are mainly associated with susceptibility during *P. brasiliensis* infection, very few studies have been conducted on adaptive therapy during experimental PCM. It was experimentally demonstrated that antibodies anti-gp70, a circulating antigen detected during PCM, prevented the establishment of the disease in mice [40]. Here, we decided to investigate the effects of the administration of immune serum during PCM. Our results showed that the adaptive transference of WT immune or non-immune serum to B^{KO} mice is associated with better clinical features, including diminished infiltration of inflammatory cells and formation of organized granuloma. Conversely, mice that received B^{KO} serum did not show clinical improvements. Taken together, our data suggest that serum from B cell-competent mice contain key factors, as for example specific or non-specific antibodies, which can modulate the inflammatory response during *P. brasiliensis* infection. We believe that future studies based on the transference of B cells or purified immunoglobulins to B^{KO} mice during PCM could be very interesting and enlightening.

Previous works have been studying the role of B cells during granuloma formation. It was demonstrated that B cell function is required for the *Schistosoma japonicum* egg-induced liver granuloma pathology in early infection (5 weeks), but is no longer required in later periods (8 weeks) [41]. Similarly, during *M. tuberculosis* experimental infection, the lungs of mice with no B cells contain fewer granulomas than those of wild-type mice, and these granulomas are much smaller with little cellular infiltrate [42]. In addition, B cells are also important for non-infectious granuloma, such as the oil granuloma induced by intraperitoneal injection of pristane, a substance which has been broadly used to induce systemic lupus erythematosus (SLE), arthritis, and plasmacytoma in mice [43].

We noticed that a continuous adaptive immunization of B^{KO} mice did result in better clinical features. Mice that received 3 doses and were killed after 30 days post-infection did not show clinical improvements, whereas mice that were inoculated with 4 injections—the last one administered 5 days before the day of killing—

showed a consistent advance in the control of *P. brasiliensis* infection, indicating that during adaptive immunization, the serum must be continuously administered. Likewise, clinical recovery was demonstrated after adaptive immunization of mice infected with *P. aeruginosa,* which showed diminished bacterial burden and reduced pulmonary edema after treatment [44].

In conclusion, the present study shows that B cells are effectively involved in the control of *P. brasiliensis* growth and participate in the organization of the granulomatous lesion observed in the lungs from Pb18-infected mice.

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