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Original article

Therapeutic treatment with scFv–PLGA nanoparticles decreases pulmonary fungal load in a murine model of paracoccidioidomycosis

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Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis with lymphatic dissemination that is caused by *Paracoccidioides* species. Treatment of PCM consists of chemotherapeutics such as itraconazole, trimethoprim, sulfamethoxazole or amphotericin B. However, several studies are aiming to develop therapeutic alternatives for the treatment of fungal infection using new molecules as adjuvants. The single-chain variable fragments (scFv) from an antibody that mimics the main fungal component incorporated within poly(lactide-*co*-glycolic) acid (PLGA) nanoparticles helped treat the fungal disease. After expressing the scFv in *Picchia pastoris* (*P. pastoris*), the recombinant molecules were coupled with PLGA, and the BALB/c mice were immunized before or after infection with yeast *Paracoccidioides brasiliensis* (*P. brasiliensis*). Our results showed decreased disease progression and decreased fungal burden. Taken together, our results showed an increased of IFN-γ and IL-12 cytokine production and an increased number of macrophages and dendritic cells in the pulmonary tissue of BALB/c mice treated with a high concentration of our molecule. Our data further confirm that the scFv plays an important role in the treatment of experimental PCM.

Keywords: Paracoccidioidomycosis; *Paracoccidioides brasiliensis*; scFv; PLGA

1. Introduction

Paracoccidioides brasiliensis (*P. brasiliensis*) and *Paracoccidioides lutzii* (*P. lutzii*) are the etiological agents responsible for the most important mycosis in Latin America, paracoccidioidomycosis (PCM) [1,2]. PCM is responsible for the most mortality in endemic countries [3,4]. It is estimated that 1,500,000–2,000,000 people die of fungal infections each year, and some of these chronic diseases are difficult to treat [5].

Several drugs have been used in the treatment of PCM, including itraconazole, trimethoprim, sulfamethoxazole and amphotericin B [6,7]. Unfortunately, the amphotericin B which is the drug of choice for treatment of severe PCM, causes acute side effects following intravenous administration due to toxicity in kidneys [8–10]. Thus, it is crucial to improve the therapeutic options. Over the past decades,

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several methods have been developed for the treatment of fungal diseases [6,11]. Recombinant antibodies are being employed as therapeutic agents. Furthermore, widespread application of this methodology is predicted in varying technological areas of medicine, including vaccine development and nanotechnology [11,12].

Peptide antigens, such as P10 from *P. brasiliensis,* trapped within poly(lactic acid-*co*-glycolic) (PLGA) nanoparticles have been shown to induce an effective immune response against fungal infections when combined with antifungal drugs, thus increasing therapeutic efficacy against PCM [11]. PLGA is a biodegradable polymer that could be used as delivery systems for vaccines because of its ability to slowly and gradually control the release of an antigen [13]. Antigens encapsulated within polymers can be released at a controlled rate for a prolonged period of polymeric degradation [14].

Antibody fragments are currently the most variable proteins that can be employed as therapeutic, diagnostic and research tools, and they have the largest worldwide market among pharmaceutical proteins [15]. Single-chain variable fragments (scFv) are small heterodimers composed of antibody VH and VL chains connected by a peptide linker in such a way that the antigen-combining site is regenerated in a single protein [16,17].

Previous studies by our group [18] have suggested that the scFv that mimics Ab2-β 7.B12 Mab against gp43 in *P. brasiliensis* fungus may decrease the yeast cell burden in the lungs of infected mice when it is transfected into dendritic cells (DC-pMAC/PS-scFv). Furthermore, we have shown that pMAC/PS-scFv induces high expression of $CCR7^+/CD40^+$ molecules in DCs and that the scFv contains epitopes of both $CD4^+$ and $CD8^+$ T cells, which is an important aspect in the protection against PCM [19].

The present study reports that recombinant scFv trapped in PLGA nanoparticles and expressed in *Picchia pastoris* (*P. pastoris*) induces protection against experimental PCM and modulates cytokine production. Thus, this construction represents a promising alternative for the treatment of *P. brasiliensis* infection.

2. Materials and methods

2.1. scFv expression

To express the scFv obtained from Mab 7.B12 that mimics gp43, the main antigen of *P. brasiliensis* (gp43) [18] in *P. pastoris* (SMD1168 yeast strain) the scFv sequence was inserted into pPIgLE yeast expression vector. The plasmid was linearized to transform the *P. pastoris* SMD1168 strain by electroporation according to the manufacturer's instructions. His^+ clones were screened and cultured using previously described method [20]. The *P. pastoris* selected clone containing the insert was cultivated in 200 mL of BMGY medium (1%[wt/vol] yeast extract, 2% [wt/vol] peptone, 1.34% [wt/vol] yeast nitrogen base without amino acids, 4×10^{-5}% [wt/vol] biotin, 1% [wt/vol] glycerol, 0,1% M potassium phosphate [pH 6.0]) at 30 °C at 200 rpm until an OD_{600} of 2–6 was reached. After that, the cells were centrifuged and resuspended in 200 mL of BMMY medium, with 1% methanol plus 1 mM PMSF every 24 h. After shaking for 2 days at 20 °C, the supernatant was harvested and the cells were cultivated in 200 mL of BMMY medium for 24 or 48 h to analyze the optimal time for scFv expression. The supernatants of the cultures were harvested by centrifugation and added to 1 mL of Ni Sepharose 6 Fast Flow resin (GE Healthcare) for scFv purification.

2.2. scFv purification

After expression, scFv was eluted using binding buffer containing 500 mM imidazole. After the fractions were pooled and the buffer was exchanged with PBS and concentrated using centrifugal filtration devices. The purified proteins were analyzed by SDS–PAGE on 12% gels.

2.3. Dot-immunoblotting

After 24 or 48 h of expression, the scFv protein was collected and the analysis was performed using dot-immunoblotting as previously described by our group [18]. For this assay, 10 mL of the supernatants were adsorbed onto Hybond ECL nitrocellulose membrane (GE Healthcare) and blocked with 1% BSA in PBS. After washes with PBS-0.05% Tween 20, the membrane was incubated with a mouse anti-HIS antibody (1:2500) (Sigma–Aldrich) followed by the addition of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:5000). The membrane was subsequently reacted with diaminobenzidine. As a positive control, we used the antibody Ab1 from *P. brasiliensis*. Finally, the membrane was developed using the ECL Plus Kit (GE Healthcare).

2.4. Polymeric nanoparticles

The nanoparticles used to couple the purified scFv was done according to the previously described paper [11]. Poly(D, L-lactide-*co*-glycolide) 50:50 and the Poly(vinyl alcohol, PVA), MW 89 kDa and 99% hydrolyzed, were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals used for nanoparticles preparation were of reagent grade.

2.4.1. Preparation of nanoparticles containing the scFv

Polymeric nanoparticles containing varying concentrations of scFv peptide were prepared by water/oil/water double emulsion followed by solvent evaporation according to a modified methodology previously described [21]. Briefly, the aqueous phase containing the peptide was emulsified with the organic phase (120 mg/mL PLGA in dichloromethane) by sonication for 1 min with a sonicator (Branson Sonifier 250, Branson Ultrasonics Corporation) in an ice bath. The water/in/oil emulsion was formed by adding 1 mL of 3% w/w aqueous polyvinyl alcohol (PVA) and then vortexed for 1 min. Then, 5 mL of 3% w/w aqueous solution PVA was added, followed by sonication for 1 min in an ice bath. The suspension was stirred slowly until the solvent completely evaporated.

Thereafter, the suspension was washed three times by centrifugation at 14,000 rpm at 4 °C for 15 min. Then the supernatant was discarded, and the pellet was resuspended in 1 mL ultrapure water. The blank nanoparticles were prepared following the same procedure described except that the peptide was replaced by an equal volume of ultrapure water. The diameter (nm) and polydispersity index (PDI) for nanoparticles containing the scFv peptide were 205 nm and PDI 0.066, respectively. The values for unloaded nanoparticles were 229 nm and PDI 0.233, respectively.

2.5. Paracoccidioides brasiliensis strain

P. brasiliensis yeast cells strain 18 (Pb18) were grown on Sabouraud agar (Becton, Dickinson and Company, Le Pont de Claix, France). The fungus was used when the viability was greater than 90%. The viability was determined using 1% trypan blue.

2.6. Mice and ethics statement

For the murine model of PCM, male BALB/c mice (12 weeks old) were anesthetized using intraperitoneal ketamine (80–100 mg/kg) in combination with xylazine. The animals were housed under pathogen-free conditions at the animal laboratory facility of the University of São Paulo. The Committee on the Ethics of Animal Experiments of the University of São Paulo approved the use of mice (Permit Number: 447). When necessary, the mice were euthanized by carbon dioxide overdose.

2.7. Murine fungal infection

To analyze treatment after fungal infection with *P. brasiliensis*, mice were challenged with an intratracheal inoculation of 1×10^6 Pb18 yeast cells [22]. The mice were separated into four groups (5 per group) and after 7 and 14 days, they received therapeutic doses via intramuscular injection as follows: 1) PBS only (50 μL); 2) empty PLGA nanoparticles (20 ng/mL); 3) 20 ng/mL of scFv in PLGA nanoparticles and 4) 40 ng/mL of scFv in PLGA nanoparticles. To determine the types of cells activated in the lung, all animals were euthanized after 30 days of infection. The lungs were removed, and the dendritic and macrophage cell phenotypes were examined by flow cytometry. To determine the expression levels of the molecules of interest, we used labeled monoclonal antibodies against mouse PE CD11c (N418), APC MHC-II and F4/80 FITC (A-3-1) (all antibodies were obtained from BD Bioscience, San Jose, CA). DCs and macrophages cells were analyzed separated. First, we excluded F4/80 to examine DCs phenotypes. To study macrophages, we selected F4/80 positive cells. FlowJo was used for analyses of flow cytometry data, and FMO tubes were used as additional controls [23].

2.8. BALB/c vaccination

To analyze the prophylactic effect and potential therapeutic ability of our scFv molecules associated with nanoparticles, the mice were divided into four groups before receiving therapeutic doses of the following via intramuscular injection: 1) PBS only (50 μL) 2) empty PLGA nanoparticles (20 ng/mL) 3) 20 ng/mL of scFv in PLGA nanoparticles and 4) 40 ng/mL of scFv in PLGA nanoparticles. The mice were then challenged with an intratracheal inoculation of 1×10^6 Pb18 yeast cells one week later [22]. After 30 days of infection the mice were euthanized and their lungs were collected aseptically homogenized in PBS and plated in supplemented BHI agar. The colony forming units (CFU) were counted.

2.9. Measurement of cytokines

IFN-γ, IL-10, IL-12p70, IL-4 and IL-17 cytokine production was assayed. After 30 days of infection, the cells from homogenate of lungs were cultivated *in vitro* (RPMI medium plus 5% of serum fetal bovine) for 24 h and the cytokines were measured. The measurement was done using commercial kits in accordance with the BD manufacturer's instructions.

2.10. IgG production

IgG1 and IgG2a isotype products were analyzed 30 days after the fungal infection in mice, with exception of the naïve mice (PBS). After immunizations, serum samples were obtained via blood from the tail vein and stored at −20 °C until the analysis. The ELISA for IgG isotype anti-gp43 was performed as previously described, with minor modifications [18]. Briefly, polystyrene microplates were coated with gp43 (20 μg/mL), diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6), incubated at 37 °C for 1 h, and stored at 4 °C overnight. The plates were washed with PBS, blocked with a solution containing PBS, 0.05% gelatin, and 2% bovine serum albumin, and incubated for 1 h at 37 °C. After the plates were washed with PBS +0.1% Tween, 50 μL of varying mouse serum dilutions were added and incubated for 2 h at 37 °C. The plates were washed, and an anti-mouse peroxidase monoclonal antibody (Southern Biotechnology, Birmingham, AL) diluted 1:25 in PBS + 0.01% gelatin + 0.4% bovine serum albumin was added and incubated for 1 h at 37 °C. Horseradish peroxidase-streptavidin (1:1000, Genzyme, Cambridge, MA) was added for 30 min and incubated at 37 °C. The reaction was developed with 50 μL tetramethylbenzidine (TMB) (Organon Teknika, the Netherlands) diluted 1:1 with urea peroxide for 10 min. The reaction was stopped with 2 N H_2SO_4, and optical densities (OD) were measured at 450 nm with an ELISA reader (Bio-Rad, Hercules, CA).

2.11. Statistical analysis

Prism 5 (GraphPad Inc.) was used for all statistical analyses. The data were compared using either two-way ANOVA analysis of variance followed by Bonferroni's multiple comparison tests or ANOVA followed by the Tukey–Kramer test [24]. All data are represented as the mean and standard deviation (SD).

3. Results

3.1. scFv expression

After scFv expression in *P. pastoris*, we purified the molecules and analyzed the recombinant scFv using 12% SDS–PAGE. After 24 or 48 h of transfection, we could observe scFv (25–30 kDa) in the supernatant of *P. pastoris* cultures (Fig. 1A). In agreement with these results, we confirmed scFv secretion by dot-immunoblotting and observed the production of this molecule at the same timepoints, 24 and 48 h (Fig. 1B).

3.2. Fungal burden is decreased after treatment with scFv–PLGA nanoparticles

The BALB/c mice were infected with Pb18 and then treated twice with scFv in PBS or in PLGA nanoparticles (20 or 40 ng). The CFU results showed a decrease in fungal burden in the lungs after treatment with scFv. However, the potency of the scFv was augmented at least twice when this molecule was incorporated in PLGA nanoparticles at 20 and 40 ng (Fig. 2).

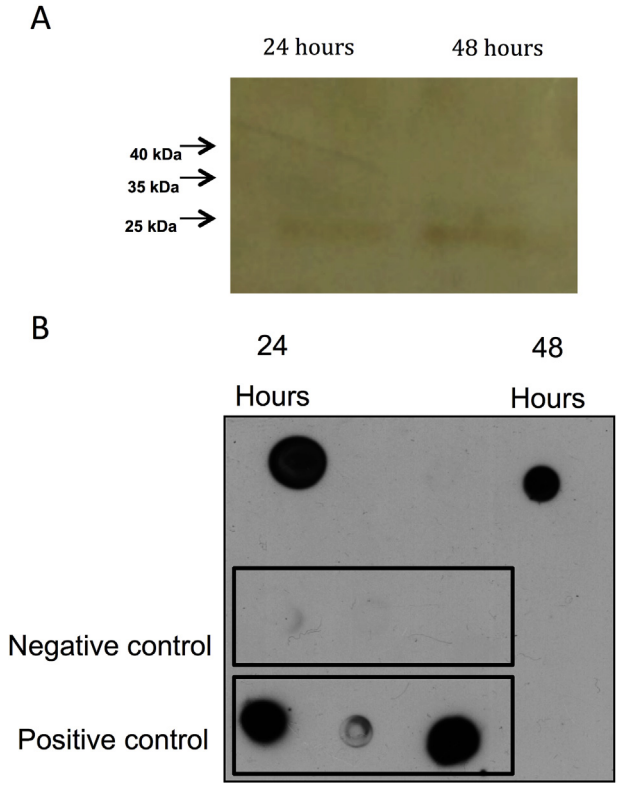

Fig. 1. scFv expression and production. After inserted the pPIgLEZ22-scFv in *P. pastoris*, we analyzed the scFv expression after 24 and 48 h (A). The analyses were done using SDS-PAGE 12%. The scFv molecules have 20–30 kDa. The yeast cells were transformed with pPIg16Z22-scFv or empty vector (negative control) and after 24 or 48 h, the supernatants were collected, and the secretion of scFv was measured by dot immunoblotting. As positive controls, we used Ab1 (5, 10 and 20 μg/mL) (B).

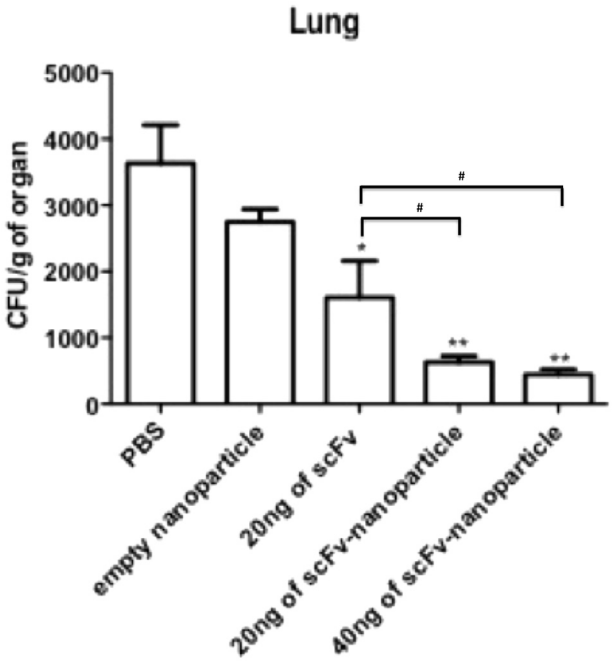

Fig. 2. Fungal burden in BALB/c therapy. The mice were infected with 1×10^6 yeast cells of Pb18. After 7 and 14 days of infection, the BALB/c mice were treated: Only PBS (50 μL), empty nanoparticle (20 ng/mL), 20 ng of scFv, 20 ng of scFv-nanoparticle or 40 ng of scFv-nanoparticle. After a week from the last immunization, the lungs were collected and we analyzed the UFC per g of organ. $*p < 0.01$ and $**p < 0.001$ compared with PBS. $\#p < 0.01$ when we compared scFv with scFv-nanoparticles. Results are representative of three independent experiments.

3.3. Cytokine production

The cytokines are important proteins in the modulation of immune response. We measured the IFN-γ, IL-12-p70, IL-17, IL-4 and IL-10 production after scFv (free or in PLGA nanoparticles) treatment in animals infected with virulent *P. brasiliensis* yeast cells. For this, the lung was collected, and the homogenized organ was used to analyze cytokine production. After 24 h of culture *in vitro* with organ homogenate, we observed a significant increase in IFN-γ and IL-12p70 production in BALB/c mice that were treated with scFv-nanoparticle when we compared with the scFv treatment (Fig. 3A, B). However, we did not observe a significant difference in IL-17 production when compared with the controls (empty PLGA nanoparticle or only PBS) (Fig. 3C). However, we showed decreased IL-4 and IL-10 production (Fig. 3D,E).

3.4. IgG induction

To study the humoral immune response in BALB/c mice against Pb18 infection, the animals were infected and treated as described above. After 7 days of the second immunization with scFv-nanoparticles, the serum was collected and IgG1 and IgG2a isotypes were measured against a specific fungal antigen (20 μg/mL of gp43). Fig. 4A and B shows a production of IgG1 and IgG2a. However, we did not observe a statistical difference between the groups.

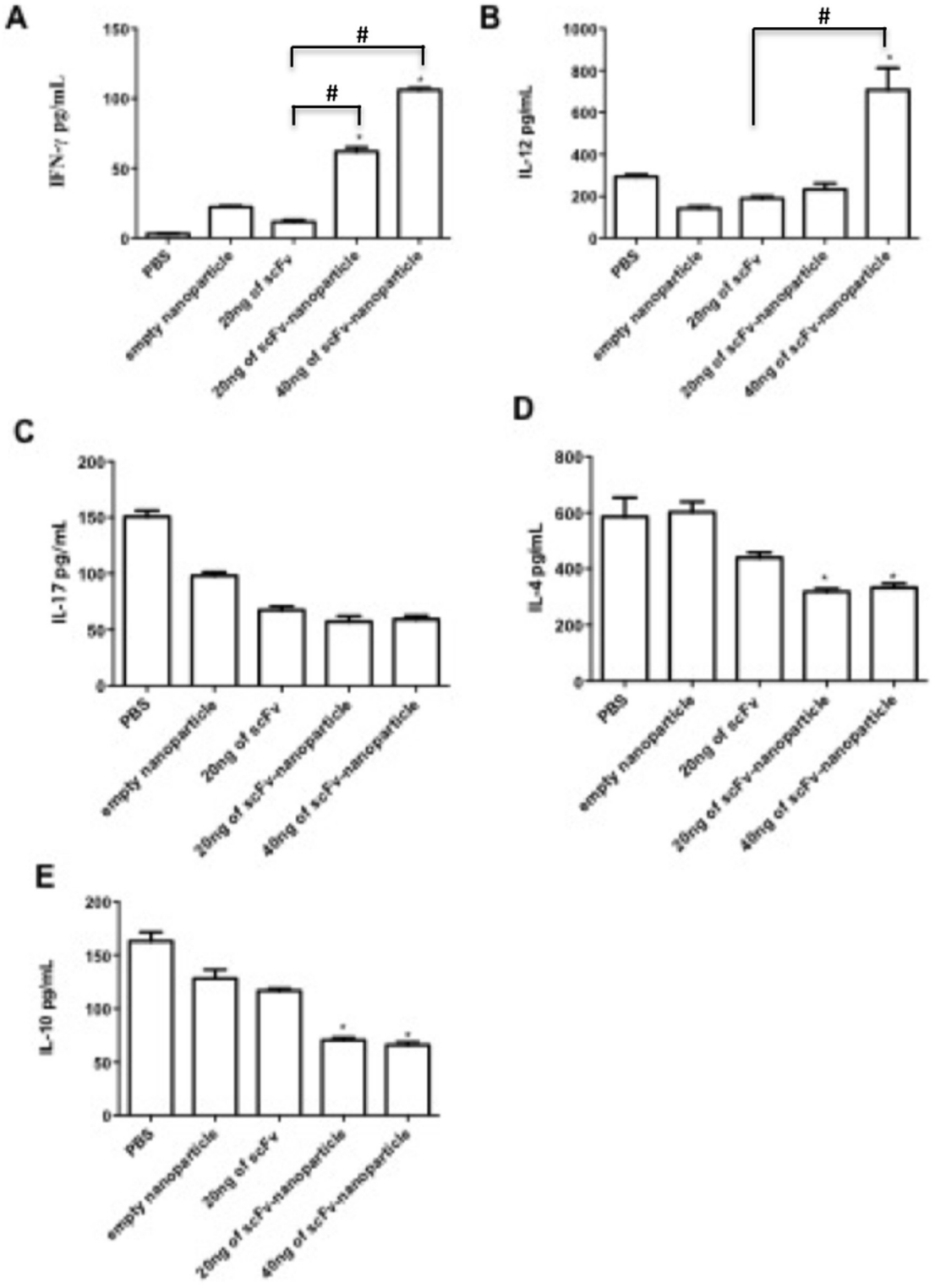

Fig. 3. Cytokine production after BALB/c therapy. The animals were infected with 1×10^6 yeast cells of Pb18 and after 7 and 14 days of infection, the BALB/c mice were treated: Only PBS (50 μL), empty nanoparticle (50 μL), 20 ng of scFv, 20 ng of scFv-nanoparticle or 40 ng of scFv-nanoparticle. After 30 days of infection, the supernatant of lungs homogenate were collected and IFN-γ (A), IL-12-p70 (B), IL-17 (C), IL-4 (D) and IL-10 (E) were measured by ELISA according the manufacturer (BD) $*p < 0.01$ compared with PBS. $\#p < 0.01$ when we compared scFv with scFv-nanoparticles. Results are representative of three independent experiments.

3.5. Macrophages and dendritic cells in the lung

Macrophages and dendritic cells are essential in the control of *P. brasiliensis* infection, mainly during the initial process of infection. The mice were infected with Pb18 yeast cells and after one week, the numbers of macrophages and dendritic cells in the lung were measured. As shown in Fig. 5A and B, we observed an increase of CD11c/MHC-II and F4/80 cell populations in animals that received scFv-nanoparticles (20 or 40 ng) when we compared with the controls.

3.6. Prophylactic assay using scFv purified

Previously, the scFv that mimics the main antigenic component of *P. brasiliensis* with nanoparticles was shown to be important in the control of experimental PCM. To analyze whether the same molecules (only scFv purified or scFv-nanoparticles) could prevent the infection, we performed a prophylaxis assay. BALB/c mice were vaccinated twice with scFv, scFv-nanoparticles or the controls (PBS and empty nanoparticle). After 7 days of the immunization, the mice were infected with Pb18 yeast cells. After 30 days, the lungs were collected, and the fungal burden was measured. Fig. 6 shows a significative decrease in fungal burden in the lungs of animals that were vaccinated with only scFv and compared with the PBS group. However, we did not observe any difference in fungal burden in the lungs of mice that received only empty nanoparticles. When we compared the mice that received scFv-nanoparticles with the BALB/c that received only scFv, we observed a statistical difference, a decreased of fungal burden.

4. Discussion

According to the Global Action Fund for Fungal Infection, globally, over 300 million people are afflicted with fungal infection and 25 million people are at risk of dying. In Latin America, PCM, a systemic mycosis caused by *P. brasiliensis* or *P. lutzii*, is responsible for one of the most common fungal diseases. The development of new molecules to be used as immunotherapeutic models is essential to improve the control of PCM. In 2011, we developed a scFv from Ab2-β of the gp43 protein in *P. brasiliensis* that confers protection against experimental PCM [18]. In 2015 [19], our research group showed that scFv modulates humoral and cellular immune responses and presents epitopes to $CD4^+$ and $CD8^+$ T cells. In addition, to improve our model, we decided to incorporate the scFv molecule into controlled-release systems, such as biodegradable polymers, that is an approach used to avoid degradation and promote their release at predetermined rates [13,25]. Accordingly, in this study, we expressed in *P. pastoris*, the scFv that is a molecule capable to activated T cell epitopes.

First, we analyzed the expression efficiency. The results showed that the scFv were expressed after 24 and 48 h. Similar results were observed when anti-LDL (−) scFv was expressed in the methylotrophic yeast *P. pastoris* [26]. The expression of scFv molecules is very important and could improve the development of the efficient treatment of fungal infection, such as experimental PCM. Therefore, the recombinant scFv from Ab2β that mimics gp43 from *P. brasiliensis* was coupled with or without varying concentrations of polymeric nanoparticles, and the resulting colony-forming units were then analyzed after the infected mice received the molecules. The increase of scFv-nanoparticles concentration induced a decrease in fungal burden relative to the controls. The PLGA delivery system (polymeric nanoparticles) showed efficiency and improved immunological protection provided by peptide P10 against murine PCM [11]. Considering that modulating the immune system is essential for the control of infection, the cytokine production was measured. Cytokine profiles detected

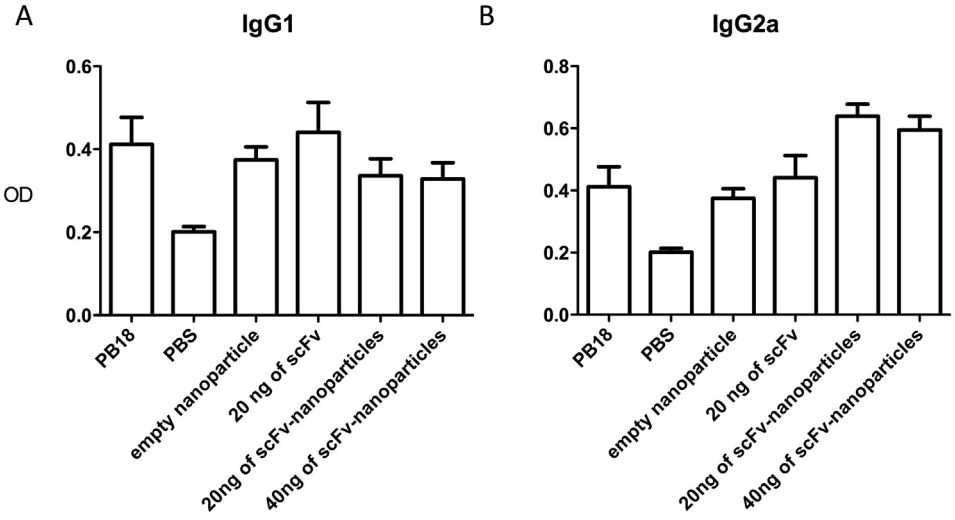

Fig. 4. Humoral immune response. The animals (six/group) were infected with 1×10^6 yeast cells of Pb18 by intratracheal route and after 7 and 14 days of infection, the BALB/c mice were treated: Only PBS (50 μL), empty nanoparticle (50 μL), 20 ng of scFv, 20 ng of scFv-nanoparticle or 40 ng of scFv-nanoparticle. After 7 days last treatment, the sera from mice were collected and IgG1 (A) and IgG2a (B) were analyzed. Statistical difference was not observed between the groups. Results are representative of three independent experiments.

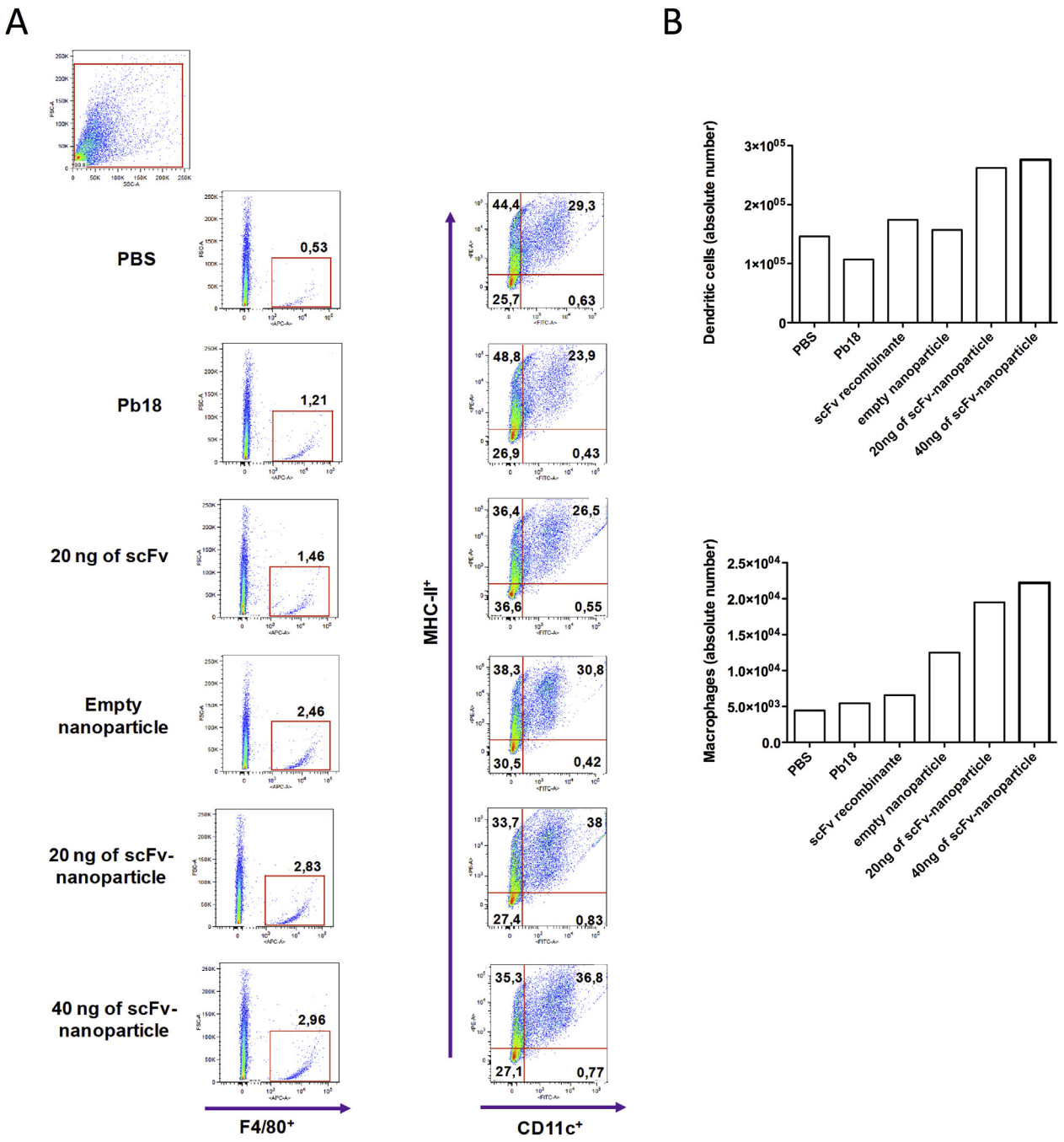

Fig. 5. Dendritic and macrophages cells in the lung. The BALB/c mice (six/group) were infected with 1×10^6 Pb18 by intratracheal route and after 7 and 14 days of infection, the BALB/c mice were treated: Only PBS (50 μL), empty nanoparticle (50 μL), 20 ng of scFv, 20 ng of scFv-nanoparticle or 40 ng of scFv-nanoparticle. After 30 days of infection, the lung cells were stained with anti-CD11c, MHC-II or anti- F4/80. The data was analyzed by flow cytometer. (A) Graph showing the frequency and (B) graph showing the absolute number of dendritic cells and macrophages. Flow graphs: results are representative of one independent experiment.

in the lung of the mice infected with yeast *P. brasiliensis* and treated with scFv-nanoparticles showed an increase of IFN-γ and IL-12 but a decrease of IL-4 and IL-10. In experimental PCM some studies showed the importance of these cytokines for control of the disease. The development of Th1 lymphocytes depends on stimulation by IL-12, a cytokine that is mainly derived from activated cells [27,28]. Mice treated with recombinant paracoccin exhibited lower pulmonary fungal burdens. These effects were associated with a high level of IL-12 and IFN-γ [29]. The gp43 elicits an IFN-γ-dependent Th-1 response that is protective against intratracheal infection by virulent yeast *P. brasiliensis* cells [6]. On the other hand, in experimental PCM, the Th-2 immune response increased the levels of IL-10 and IL-4 [6]. According to our results, that is,

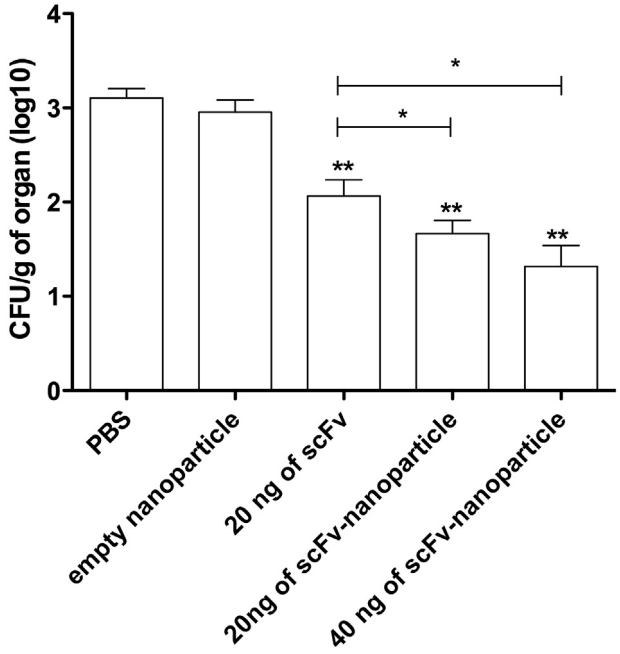

Fig. 6. Vaccine assay. The BALB/c mice (six/group) were immunized with: Only PBS (50 μL), empty nanoparticle (50 μL), 20 ng of scFv, 20 ng of scFv-nanoparticle or 40 ng of scFv-nanoparticle. After 7 days, the animals were infected with 1×10^6 Pb18 by intratracheal route. After 30 days of infection, we collected the lungs and counted the CFU. $^{**}p < 0.001$ compared with the control (PBS). $^{*}p < 0.01$ when we compared scFv with scFv-nanoparticles. Results are representative of three independent experiments.

that enhanced cytokines concentration decreases the fungal burden in the pulmonary tissue of animals that received treatment, we suggest a protective effect of the scFv-nanoparticles in our infection model.

Additionally, we analyzed IgG switch production. Our results demonstrated IgG2a and IgG1 production after treatment with scFv-nanoparticles or scFv. In accordance with our previous results [19], we also observed an increase of the same immunoglobulin switch after treatment using scFv transfected in dendritic cells. However, in our previously results, we observed more specific IgG2a levels. Although other studies have demonstrated that a higher production of IgG2a, compared with IgG1, have been reported after 90 days of *P. brasiliensis* infection [30], here, we suggested a modulation of humoral immune response after scFv or scFv-nanoparticle treatment with both IgG production. Together, we observed an increased number of macrophages and dendritic cells in BALB/c mice lungs after *in vivo* administration of scFv-nanoparticles. Recently, Silvana dos Santos and colleagues [33] showed that lung dendritic cells can phagocytose the yeast *P. brasiliensis* cells *in vivo*. Dendritic cells and macrophages play an important role in the induction of effector T cells in the control of *P. brasiliensis* infection. In this context, antigen-presenting cells are important to link and activate innate and adaptive immune responses against fungal infection. The production of IFN-γ and IL-10, both effector T cell products, appears to be involved in the resolution and dissemination of PCM, respectively [31,32]. Besides that, the IL-17 regulates the influx of neutrophils and together IFN-γ are responsible for the control of *P. brasiliensis* infection. In addition, they showed that lung dendritic cells migrate to lymph nodes, which could represent an important mechanism in PCM infection and murine macrophages activated by IFN-γ, incubated with different molecules, as antibodies against acidic GSLs, are more effectively phagocytosed and kill yeast forms of *P. brasiliensis* [34].

Here, we showed that our scFv-nanoparticles have emerged as an option for immunotherapy against experimental PCM. However, the role of this construct in prophylactic assays is not yet clear. When BALB/c mice were vaccinated with our molecule coupled in biodegradable polymers, we observed an important decrease of fungal burden in the lungs of the animals. Similar results were observed when the peptide P10 from gp43 of *P. brasiliensis* was administered in complete Freund's adjuvant and protection was observed using this prophylactic protocol [35].

In summary, the present work identifies a target novel molecule that can be used for protection against *P. brasiliensis* infection.

Conflict of interest

There are no conflicts of interest to disclose. The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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