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Efficacy of Chitosan as Vaccine Adjuvant Against Paracoccidioidomycosis in Mice

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Abstract

Paracoccidioidomycosis (PCM) is a systemic disease caused by thermo- dimorphic fungi of *Paracoccidioides genus* that is endemic in several Latin American countries. The treatment for this disease can be performed with different drugs. In general, it is a long duration treatment that aims the control of the clinical manifestations. Even after treatment, the patient usually shows relapse and incapacitating sequelae, such as fibrosis and pulmonary emphysema. So, drug therapy against PCM is considered inefficient. As an alternative to the therapeutic treatment, studies for the formulation of vaccines against *P. brasiliensis* have been performed, among them, recombinant Pb27 (rPb27) has demonstrated potential as an inducer of protective immune response against *P. brasiliensis* and in the reduction of disabling sequelae as a prophylactic vaccine. In this work, adjuvants that can be used in human vaccines were evaluated to modulate the immune response in an efficient manner and to produce lower adverse effects related to the post-vaccination inflammatory responses. The adjuvants chosen for this study were chitosan and CPG. Both adjuvants were associated with rPb27 in the prophylactic protocol and compared with the adjuvant used by our research group in previous studies, aluminum hydroxide associated with *Corynebacterium* parvum. We tested *in vitro* and prophylactic protocol. Chitosan adjuvant associated with rPb27 showed better results *in vitro* with high fungicidal capacity and IL-1β cytokine production. In addition, induced lower levels of IL-10 and IL-6. Moreover in the prophylactic assay, animals immunized with this adjuvant obtained high protective effect and satisfactory survival levels 60 days post infection.

Keywords: Chitosan, Pb27, Paracoccidioidomycosis, *Paracoccidioides, P. brasiliensis,* Vaccine, Adjuvant

Introduction

PCM is an endemic rural and suburban disease, prevalent in Latin America, with 80% of the cases described in Brazil [1]. The etiological agents of this mycosis are fungi of Paracoccidioides genus [2]. The treatment made against PCM is lengthy and usually allows the relapse of the disease [3,4]. In many cases, the disease usually leaves disabling sequelae, such as fibrosis and pulmonary emphysema. Because of this, drug therapy is considered inefficient [4,5]. As an alternative to the chemotherapeutic treatment, studies for the formulation of prophylactic and therapeutic vaccines have been performed, among them, rPb27 has demonstrated potential as an inducer of protective immune response against *P. brasiliensis,* and in the reduction of disabling sequelae of PCM as a prophylactic vaccine [6,7]. Although rPb27 has shown promising results, it is essential in a vaccine formulation to choose an adjuvant suitable for antigen-associated use [8-11].

The vaccination purpose is to induce a specific immune response to the pathogen leading to protection against infection and disease. Vaccines are an effective and cost-efficient method for preventing diseases caused by infectious pathogens. The development of potent vaccines for the prevention of diseases caused by infectious pathogens is based on the selection and use of suitable adjuvants [12]. The adjuvant term is derived from Latin *adjuvare*, which means "to help" [13]. Adjuvants have been defined as agents added to vaccine formulations which enhance antigens immunogenicity and induce protection against infection. Thus, adjuvants are immunopotentiating substances, and therefore, the use of adjuvants in vaccines is important, especially if the antigen has low immunogenicity [14]. They help the formation of an immune response of higher intensity, longer duration, faster and with a smaller amount of antigen, thus reducing costs in the production of vaccines [15,16].

Several mechanisms have been proposed to explain the adjuvants efficacy and how they can improve the vaccine's response to the antigen. Adjuvants may induce local inflammation by increasing antigen contact with additional cells that are attracted to the site; form an antigen deposit releasing it more slowly and thereby prolonging its interaction with the macrophage. Adjuvants can increase the speed and duration of the immune response. They can modulate the avidity, specificity, isotype, distribution of subclasses of antibodies, stimulate cell- mediated immunity; induce mucosal immunity and increase the immune response in immunologically immature or senile individuals [17-19].

Immunity to different infectious diseases requires distinct types of immune reactions. The most appropriate adjuvant for each vaccine depends largely on the type of immune response required for the protective immunity, since different adjuvants may stimulate different immune responses [19-22]. Different formulations have been tested and presented a robust adjuvant potential, such as emulsions, liposomes, microspheres, saponins, immunostimulant complexes, among others [23,24].

The adjuvant substances may be synthetic or natural, and many polymers have been investigated for vaccine delivery. Natural polymers available for the nanoparticles production include albumin, collagen, chitosan, dextran, among others. Examples of synthetic polymers include polyesters, polyanhydrides, and

polyamides. Also, bacterial components are often tested as potent activators of the immune system, for example, bacterial DNA with immunostimulatory motifs CpG is one of the most potent cellular adjuvants. The CpG immunostimulant consists of unmethylated cytosine-guanine dinucleotides found in bacterial DNA but absent in mammalian DNA [20,21].

In this study, we tested the efficacy of rPb27 associated with some adjuvants in a prophylactic assay against Paracoccidioidomycosis. The adjuvants were aluminum hydroxide associated with Corynebacterium parvum: used by our research group in previous studies; Chitosan; a natural non-toxic polysaccharide biopolymer consisting of D-glucosamine units; and CPG: a synthetic oligodeoxynucleotide containing unmethylated CPG sequences.

Methods

Adjuvants preparation

Hidróxido de alumínio e *C. parvum:* The antigen 50 μg rPb27 was emulsified with adjuvant composed of 1 mg of $Al(OH)_3$ (Pepsamar-Sanosi-synthelado, RJ) associated with 100 μg of *Corynebacterium parvum* per mouse (R.V. special manipulations, RJ).

Preparation of chitosan nanoparticles: Nanoparticles were prepared by mixing, while vortexing, equal volumes of a 0.1% (w/v) of chitosan in 5 mM CH_3COONa buffer, pH 5.5 and a solution containing 0.625% (w/v) of Na_2SO_4, both previously heated at 55 ºC for 10 min. Nanoparticles were allowed to form overnight under stirring. In the following day, the suspension was centrifuged at 3200 g for 20 min at 18 ºC. The particles were resuspended in about 1/8 of the volume with ultrapure water (Milli-Q, Millipore) [25]. The loading was done as described by Oliveira *et al.* [25]. Briefly, the solution of rPb27 protein was incubated with chitosan nanoparticles under mild agitation at 18 ºC. After 1 h of incubation, an aliquot of the particle suspension was centrifuged at 15700 g for 30 min, and the protein in the supernatant was quantified by BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 590 nm filter (Multiskan GO, Thermo Fisher Scientific). The absorbance reading value was corrected subtracting the average absorbance reading obtained in the BCA- protein assay from that one of the supernatants of unloaded nanoparticles prepared precisely in the same conditions.

Preparation of CPG adjuvant: CPG adjuvant ODN 2935 (invivogen) was solubilized in toxin-free water and vortexed to complete solubilization. The aliquots were stored in a freezer at -20 ºC. Each animal received 25 μg of the adjuvant which was associated with 50 μg of rPb27 in 0.15 M PBS.

Broncho-alveolar lavage (BAL) and cell count

BAL was performed at 60 days post-infection (DPI) to obtain leukocytes in the alveolar spaces. The trachea was briefly exposed, and a 1.7-mm outside- diameter polyethylene catheter was inserted. BAL was performed by twice instilling 1 ml aliquots of PBS, with 2 ml of fluid being retrieved per mouse, as previously described [26]. The total number of leukocytes was counted. Differential

counts were obtained from Cytospin preparations by evaluating the percentage of each leukocyte on a slide that was stained with panoptic dye. The total number of cells in the BAL fluid and the fraction of macrophages, neutrophils, and lymphocytes therein were calculated for each mouse. BAL was performed to obtain cell suspension for culture.

Cell culture

Healthy animal cells were collected in BAL as described above. Total BAL cells were cultured in 24 well plates, $5x10^3$ cells per well. The cells were incubated for 4 h at 37 °C in a 5% CO_2. After adhesion, non-adherent cells were removed. The cell culture was stimulated with adjuvants CHO (40 μl/mL), CPG (2.0 μl/ mL), ALOH-Cp (2.0 μl/mL).

Phagocytosis assay

Phagocytosis assay was performed with the stimulated cells as previously described. After stimuli, $1x10^5$ cells of Pb18 were added to each well, and the cultures were maintained at 37 °C for 6, 8 and 12 hours. Coverslips were subjected to immunofluorescence, and for a May-Grunwald-Giemsa stain for phagocytosis-index analysis. Each experiment was performed in triplicate and repeated 5 times. To determine the viability of fungal cells within the macrophages following stimulation, cells were lysed with SDS 0.05% and plated on a BHI medium to perform CFU counts. The phagocytic index (PI) was determined by multiplying the percentage of macrophages that had phagocytosed at least one yeast by the mean numbers of yeasts per infected macrophage [27,28].

Viability and cellular proliferation

The cell culture was stimulated with adjuvants solubilized into DMEM medium as previously described. To determine cell proliferation the number of cells were evaluated on days 1, 3, 6 and 8 after adjuvant stimulation. To determine whether the viability of the cells is affected during adjuvant treatment the Trypan Blue exclusion test was used (Thermo Fisher Scientific) at 0.04% final concentration on days 1, 3, 6 and 8 after adjuvant stimulation [29].

Confocal fluorescence microscopy

Cells obtained from BAL were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma) for 1 h at room temperature. The samples were blocked with 1% bovine serum albumin (BSA) and 5% goat serum in PBS solution and incubated for 1 h at room temperature. The samples were incubated with primary antibodies (anti-rPb27 and anti-F4/80) (at 1:100 dilutions in 1% BSA-PBS solution) overnight at 4 °C. The samples were washed with PBS twice. Alexa Fluor secondary antibodies (Invitrogen, U.S.A) were diluted (1:500) in the same solution as the primary antibodies and incubated with the samples in for 1 h at room temperature. The samples were washed with PBS twice, and the coverslips were sealed with Hydromount aqueous media (National Diagnostics). The images were collected using a Zeiss 5 Live confocal microscope.

Cytokine production

The cytokines TNF-α, IL-6, IL-1β, and IL-10 were evaluated (R&D Systems). The manufacturer's instructions were followed. Cytokine evaluation was performed on macrophage culture supernatant 18-21 hours after adjuvant stimulation [30].

P. brasiliensis strain

Virulent *P. brasiliensis*, strain Pb18, was maintained in yeast form in YPD agar medium [0.5% yeast extract, 0.5% peptone, 1.5% D-glucose, 1.5% agar, pH7.0] at 36 °C. The viability of fungal suspensions was determined by staining with Janus Green B vital dye method (Merck. Darmstadt, Germany) and was always higher than 90%. The virulence of the human isolate was checked in each experiment by infecting intratracheally BALB/c mice and recovering the yeast cells from their organs [11].

Ethics statement

This work was approved by the Committee on the Ethics of Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG) under permit number 363/2012 and was conducted in strict accordance with the regiment of the ethics commission on the use of animals. All surgery was performed under intraperitoneal anesthesia (ketamine, Dopalen, Vetbrands, Xylazine, Denpasar; *Laboratório Collier do Brazil LTDA*, Brazil), and all efforts were made to minimize suffering. Ethics committee specifically reviewed and approved the mortality aspects of the protocol.

Experimental groups

For the prophylactic assay, 8-week male BALB/c mice were divided into 8 groups composed of 8 mice each. The groups were divided into the following groups: Negative control (NC): composed of animals that did not undergo interventions. Positive control (PC): composed of mice infected only with the strain *P. brasiliensis* (Pb18). Adjuvant 1 (CHO): composed of mice inoculated with chitosan adjuvant only during the immunization period. Adjuvant 2 (CPG): composed of mice inoculated with CPG adjuvant only

during the immunization period Adjuvant 3 (ALOH-Cp) composed of mice inoculated with adjuvant alone (*C. parvum* and Al (OH)3) during the immunization period.

The adjuvant groups associated with the proteins were divided into the following groups: Adjuvant 1 +Pb27 (CHO*): composed of mice inoculated with chitosan adjuvant associated with rPb27 during the immunization period. Adjuvant 2 + Pb27 (CPG*): composed of mice inoculated with CPG adjuvant associated with the rPb27 during the immunization period. Adjuvant 3 + Pb27 (ALOH-Cp*) composed of mice inoculated with adjuvant *C. parvum* and Al (OH)3 associated with rPb27 during the immunization period. The groups are shown schematically in Table 1.

GROUP	ABREVIATION	IMMUNIZATION	INFECTION
Negative control	NC	NOT IMMUNIZED	NOT INFECTED
Positive control	PC	NOT IMMUNIZED	
ADJUVANT 1	CHO	Chitosan	
ADJUVANT 1+Pb27	CHO*	Chitosan + Pb27r	
ADJUVANT 2	CPG	CPG	1×10^6 células Pb18
ADJUVANT 2+Pb27	CPG*	CPG + Pb27r	
ADJUVANT 3	ALOH-CP	Aluminum hydroxide and *C. parvum*	
ADJUVANT 3+Pb27	ALOH-CP*	Aluminum hydroxide and *C. parvum* + rPb27	

Table 1: Experimental groups used in experimental PCM

Intratracheal Infection

The selected animals (male BALB / c mice) were inoculated intratracheally with 50μL of a fungal preparation containing 1×10^6 viable yeast cells of *P. brasiliensis* strain 18 (Pb18) suspended in sterile PBS. Mice were briefly anesthetized with a solution containing ketamine (10 mg/kg) and xylazine (80 mg/kg). After anesthesia, their necks were hyperextended, and the tracheas were exposed at the level of the thyroid and injected with yeast cells [11].

Protection assay

Immunized groups were boosted by the subcutaneous injection of preparation three times at fifteen-day intervals. Each immunized animal received 150 μl of the preparation containing 50 μg of rPb27(GenBank-NCBI accession number AAC49615) protein associated with the adjuvant. The animal sera were collected 7 days after each immunization. After 7 days of the last immunization challenge infection was made, using 1×10^6 yeast cells of Pb18. The analyzes were performed for 60 days after infection. During this time the survival of the animals was assessed. Animals dying during the experimental phase were counted, and the mortality/survival curve was defined. At euthanasia points animal sera and lungs were collected for further analysis. The assay timeline can be seen in Figure 4F.

CFU analysis

The mice were euthanized (anesthetic overdose), and the lungs were aseptically removed 30 and 60 days after infection. The number of viable cells was determined by plating crude and serially diluted homogenates onto brain- heart infusion agar (BHI, Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 4% fetal calf serum and 5% *P. brasiliensis* spent culture medium (Pb18) as growth factors. Gentamicin was added at a concentration of 40 mg/l. The CFUs were counted after 20 days of incubation. The results are expressed as the log10 per gram of tissue of CFUs of viable yeast cells of *P. brasiliensis* (log10/g).

Statistical analysis

The statistical analysis to determine the significance of the data was performed using Prism 5.0 software (GraphPad, CA, U.S.A.). The data were normally distributed, and the values obtained from the different groups of mice were compared using one-way analysis of variance (ANOVA) with Bonferroni post- tests. Data were considered statistically significant at $P < 0.05$.

Results

In vitro assays

Cell viability and proliferation analysis were performed at 1, 3, 6 and 8 days after stimulation with the adjuvants. The ALOH-Cp adjuvant induced higher proliferation rates in the initial phases of the analysis (days 1 and 3). The CHO group was the group that induced the lowest rates of cell proliferation (Figure1A). The remaining groups at the end of 8 days had similar levels of cell viability, with a percentage close to 100 (Figure 1B).
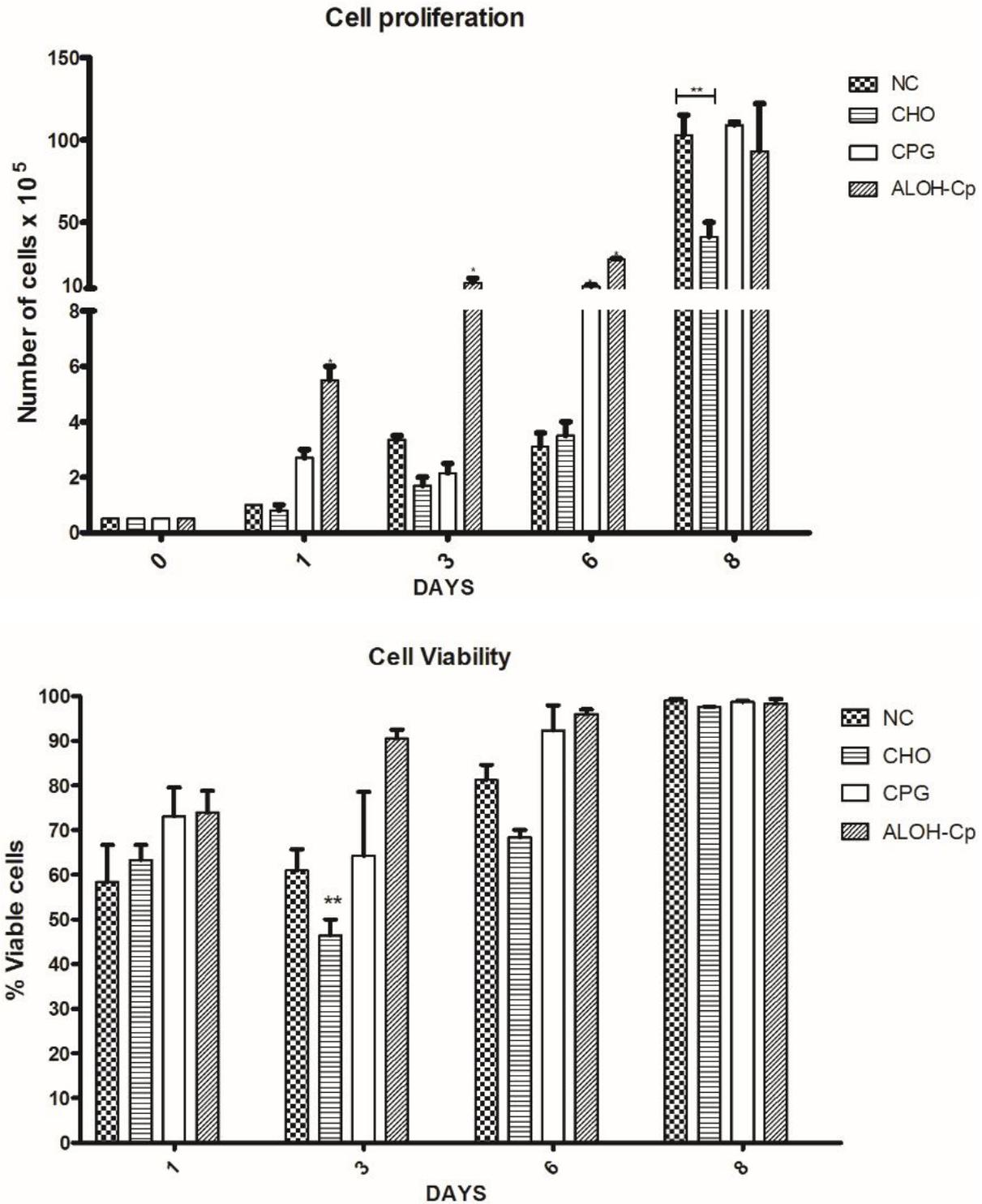
Figure 1: Cellular viability and proliferation

Graph showing (A) Cellular viability and (B) Proliferation of cell culture stimulated with adjuvants for 8 days using trypan blue exclusion test. The cell viability and proliferation were assessed at day 1, 3, 6 and 8. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ compared to the negative control. The groups represented in the figure are: (NC) negative control, not stimulated, (CHO) stimulated with chitosan adjuvant, (CPG) stimulated with CPG synthetic oligodeoxynucleotide and (ALOH-Cp) stimulated with aluminum hydroxide associated with *C parvum*

The phagocytosis assay was performed 6, 8 and 12 after stimulation with adjuvants. It was possible to observe that cells stimulated with adjuvants had greater phagocytic capacity compared to controls. The ALOH-Cp group had a lower statistical difference compared to the control group after 12 hours of stimulation. The groups that were stimulated with CPG presented increased phagocytic capacity in the times of 6 and 8 hours (Figure 2A and E).

A Phagocytic capacity

B Phagocytic index

C Yeast inside the phagocyte
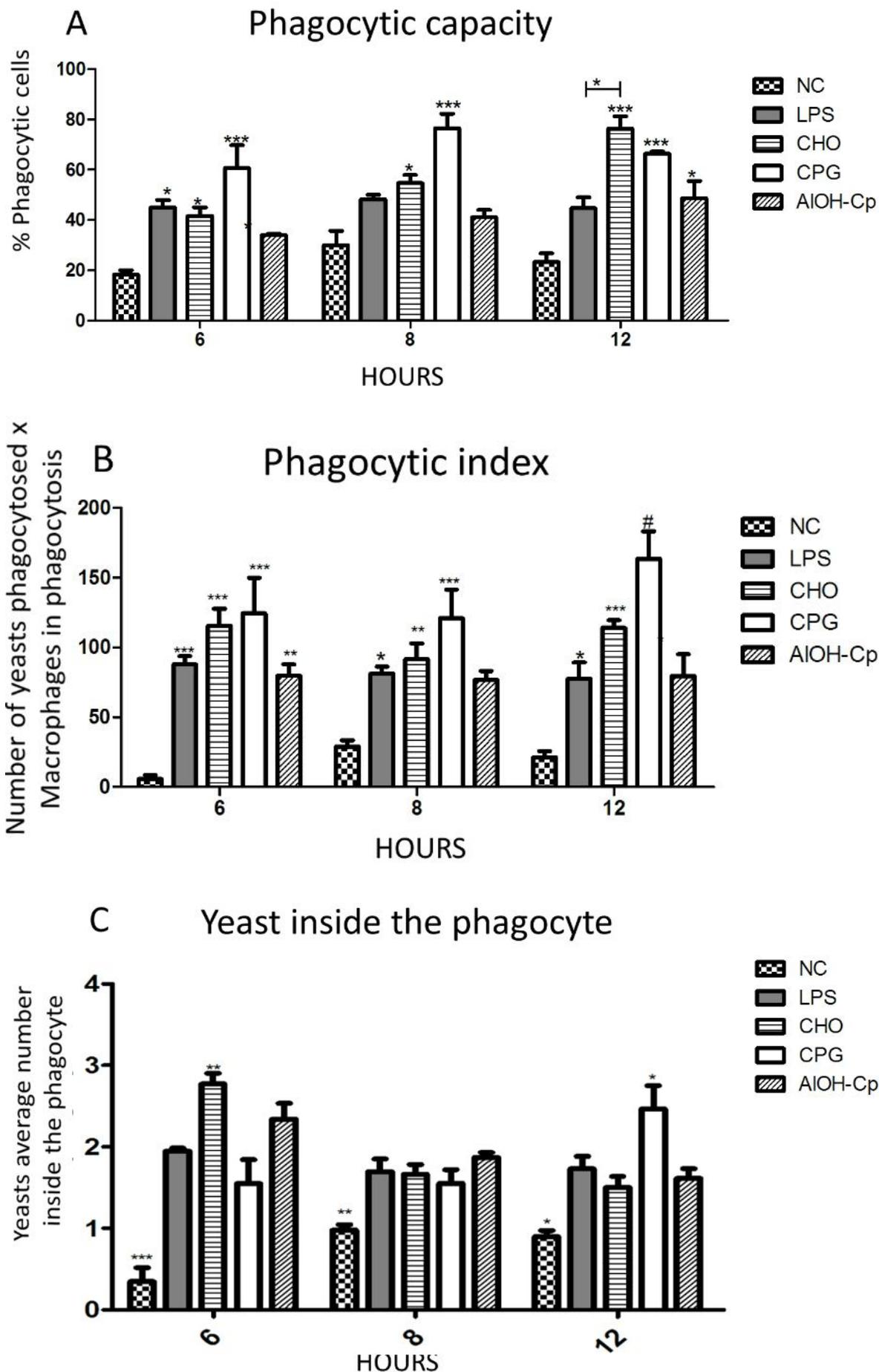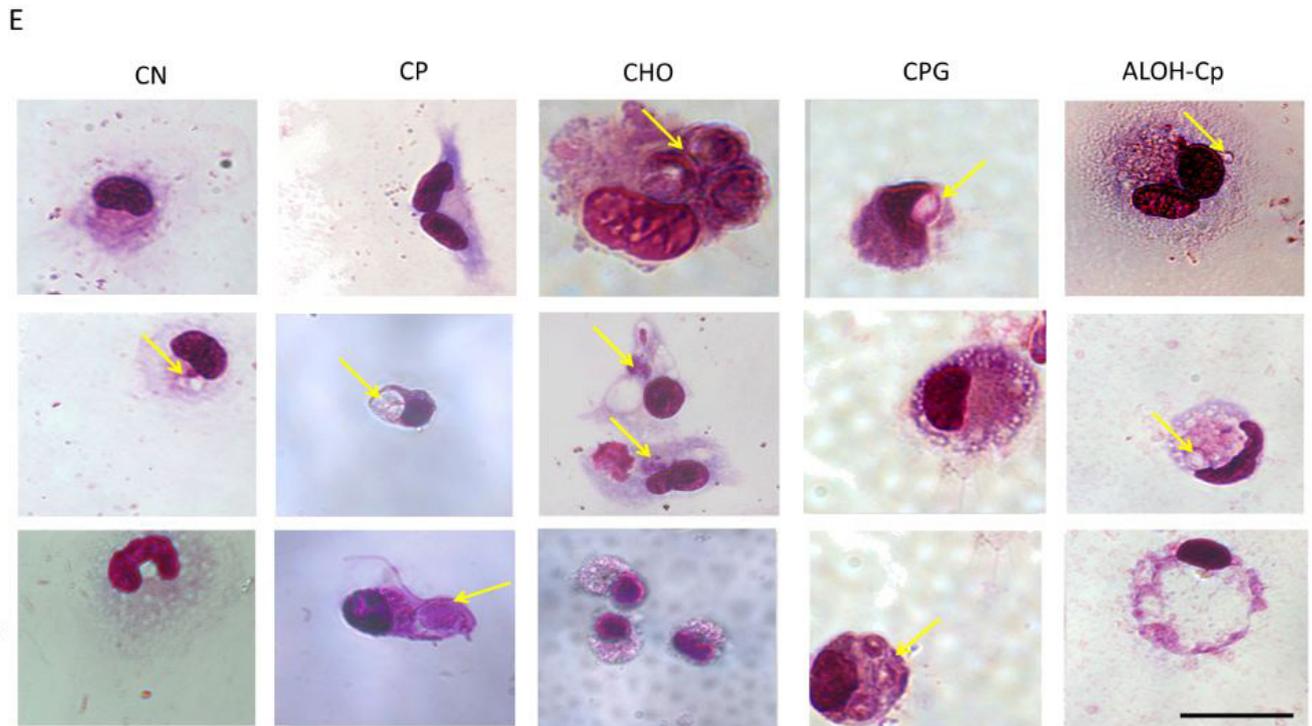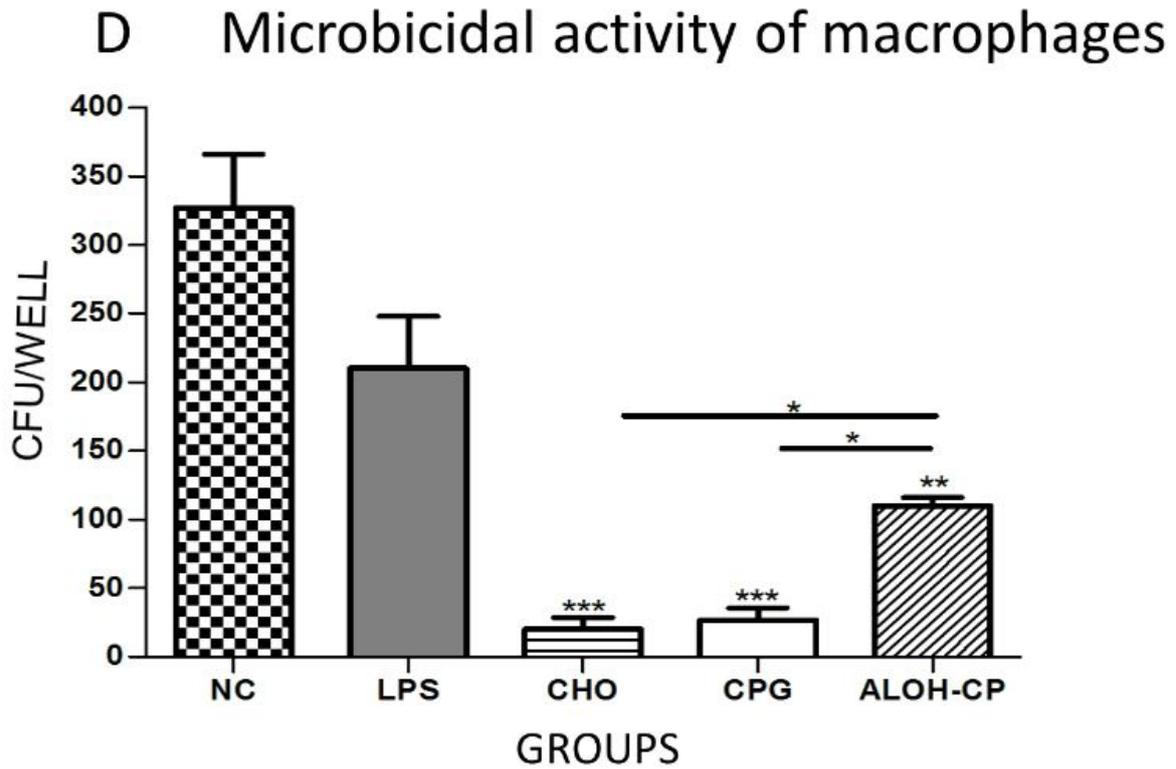
Figure 2: Phagocytosis assay of adjuvant stimulated cells
Phagocytosis assay analysis of adjuvant stimulated cells cultured with 1x105 cells of Pb18/well after for 6, 8 and 12 hours. Panel showing (A) Phagocytic capacity (B) Phagocytic index (C) Yeast inside the macrophage (D) Microbicidal activity of macrophages and (E) Representative May-Grunwald-Giemsa stain of each group. * indicates p < 0.05, ** indicates p < 0.01 and *** indicates p < 0.001 compared to the negative control. The groups represented in the figure are: (NC) negative control, (CHO) stimulated with chitosan adjuvant, (CPG) stimulated with (CPG) synthetic oligodeoxynucleotide and (ALOH-Cp) stimulated with aluminum hydroxide associated with *C parvum*

When we analyze the average yeast number within the macrophages, it was possible to observe a reduction in the number of fungal cells in the CHO and ALOH-Cp groups and an increase in the mean number of cells in the CPG group between 6 and 12 hours after stimuli (Figure 2B).

Phagocytic index was measured. Comparing the stimulated groups to the control it was possible to observe that all the adjuvant stimuli were able to increase the phagocytic activity of the macrophages. The CPG stimulated group presented statistical difference when compared to the other stimulated groups 12 hours after stimulation. The ALOH-Cp group had a lower phagocytic index compared with the other stimulated groups (Figure 2C).

The microbicidal activity of the macrophages was determined after 12 hours of stimulation, that is the viability of the fungi inside the phagocyte was assessed. It was possible to verify that all the groups stimulated by adjuvants had a greater microbicidal capacity compared to the control. Macrophages stimulated by the adjuvants CHO or CPG presented higher microbicidal activity versus the other groups. Immunofluorescence was also performed to identify the fungal cells within the phagocyte. It was not possible to identify full-label (whole fungal cells) in the CHO and CPG groups (Figure 2D and Supplementary data).

Cytokine evaluation was performed on macrophage culture supernatant 18-21 hours after adjuvant stimulation. TNF-α, IL-6, IL-1β and IL-10 cytokines were evaluated. The CHO group presented lower rates of IL-6 and IL-10 production however with elevated IL-1β levels. On the other hand, the CPG group presented high levels of TNF-α and IL-6. The ALOH-Cp except the TNF-α, presented elevated levels of the analyzed cytokines (Figure 3).

B   IL-1β

C   IL-6
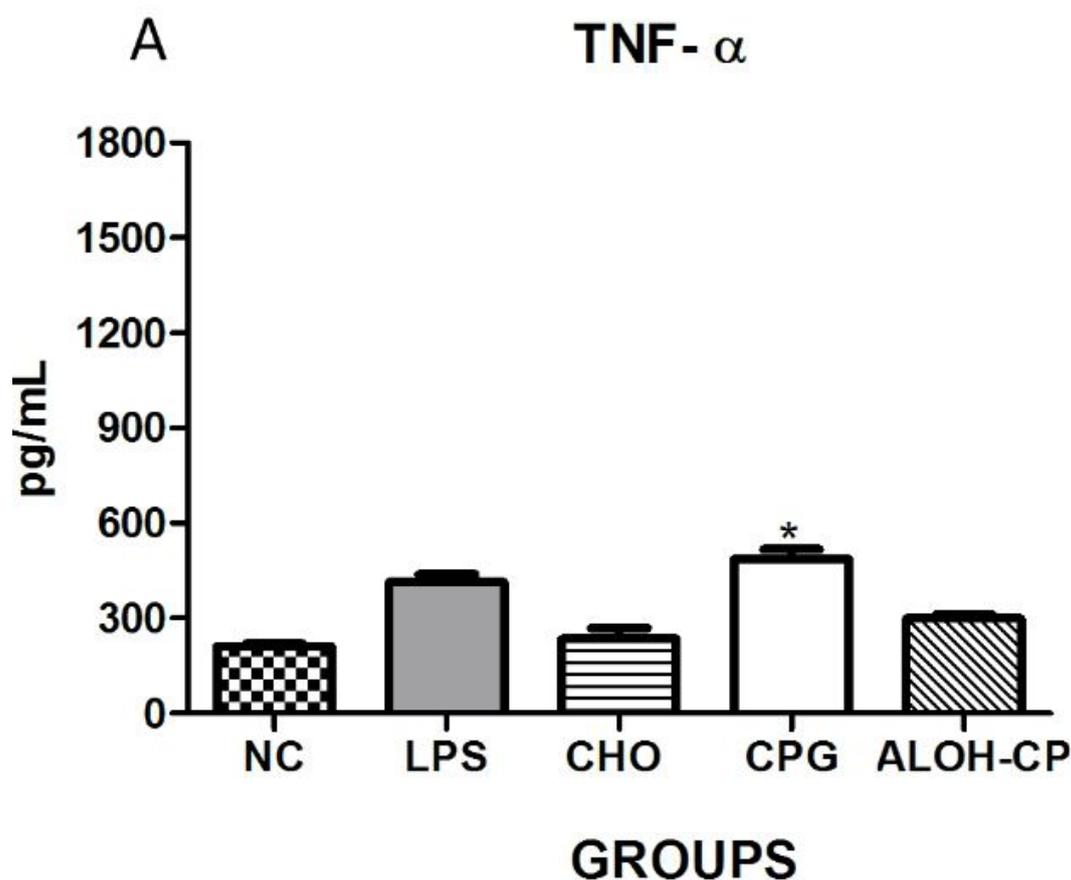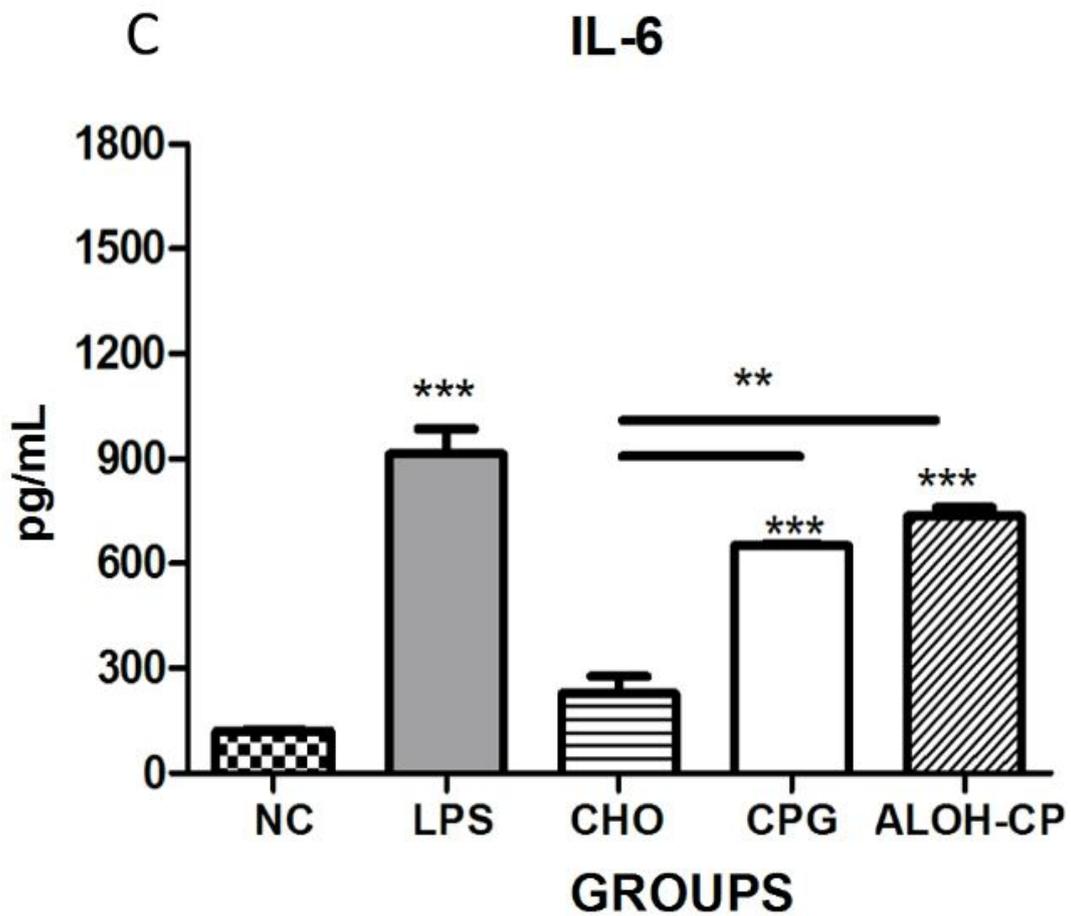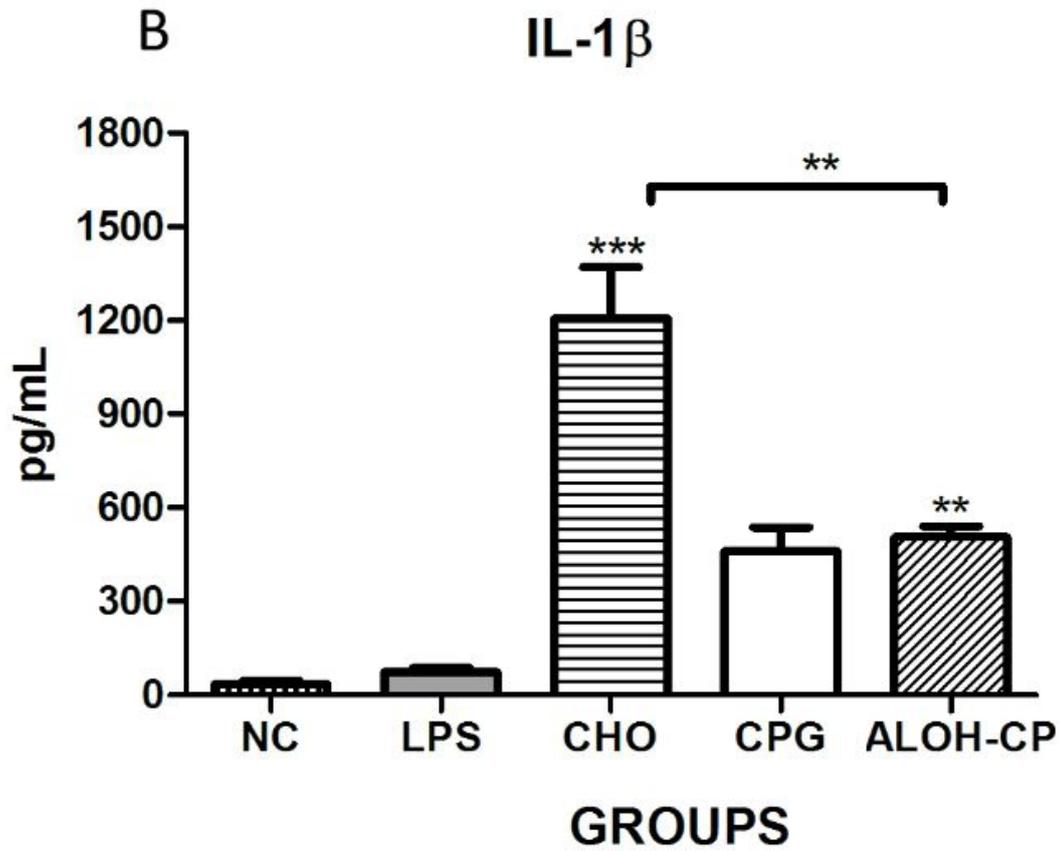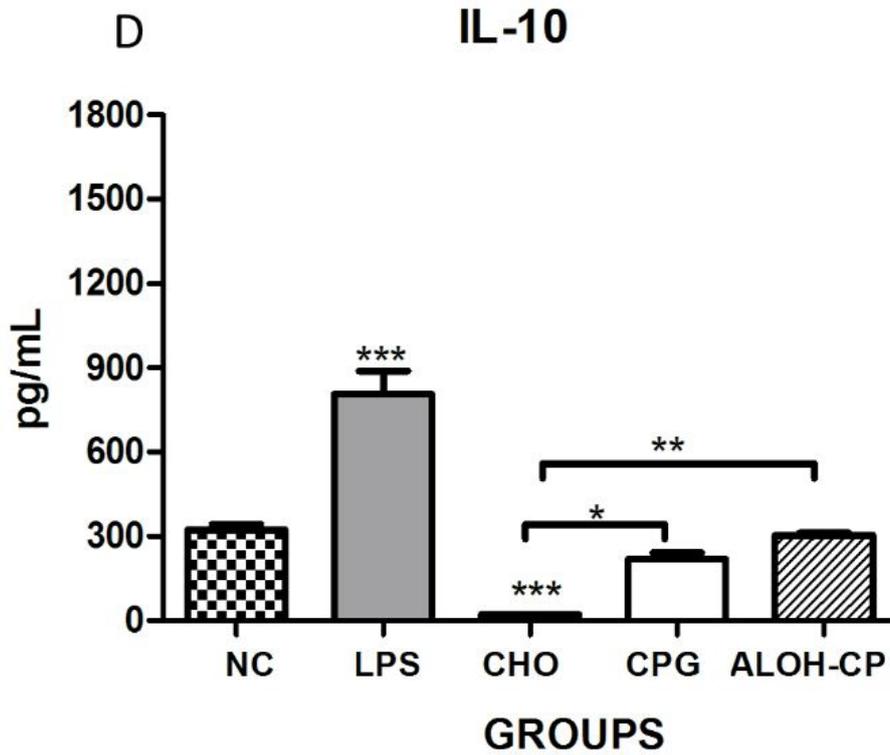
Figure 3: Cytokine expression

Cytokine production in cell culture supernatant after 18-21 hours after stimulation: (A) TNF-α (B) IL-1β, IL-6 (C), IL-10 (D). *Indicates significant differences at ($P < 0.05$), **at ($P < 0.01$) and ***at ($P < 0.001$) compared to the negative control. The groups were: (NC) negative control, (LPS) positive control, (CHO) stimulated with chitosan adjuvant, (CPG) stimulated with CPG synthetic oligodeoxynucleotide and (ALOH-Cp) stimulated with aluminum hydroxide associated with *C parvum*

Protection assay

The protection assay was developed following the prophylactic scheme used by our research group with the aluminum hydroxide adjuvant in previous works and was described in Figure 4F. Antibody levels were measured during and following immunizations for analysis of specific anti-Pb27r IgG. It is possible to observe that immunized animals showed an increase in the antibodies production after each immunization (Figure 4A). After the third immunization, all groups were able to induce production of specific IgG, with higher levels in the groups immunized with rPb27 (Figure 4B).
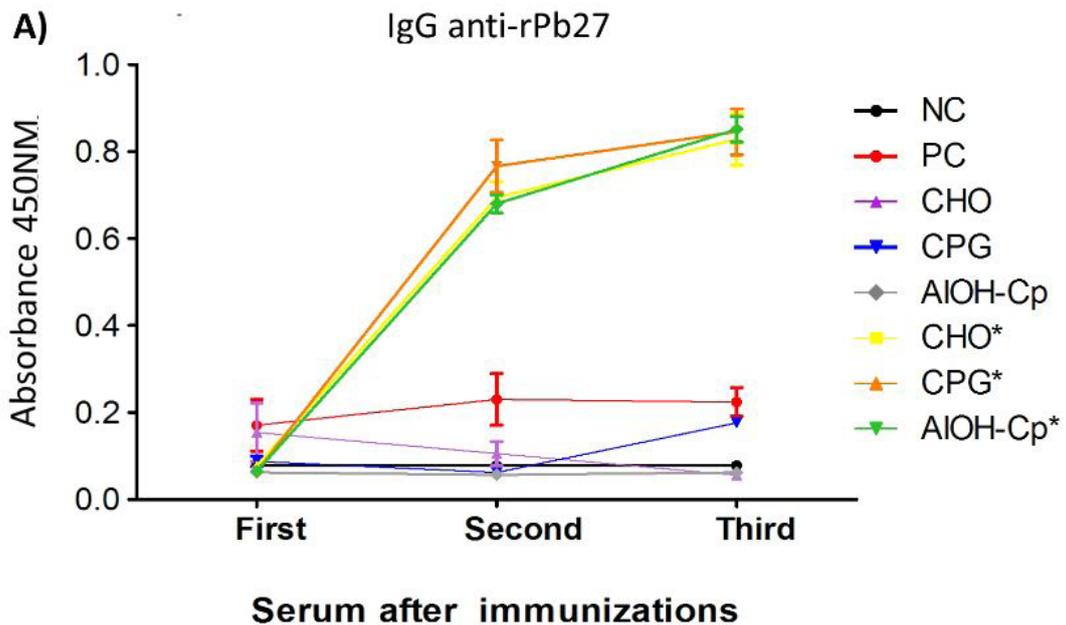
B) IgG anti-rPb27 after third immunization
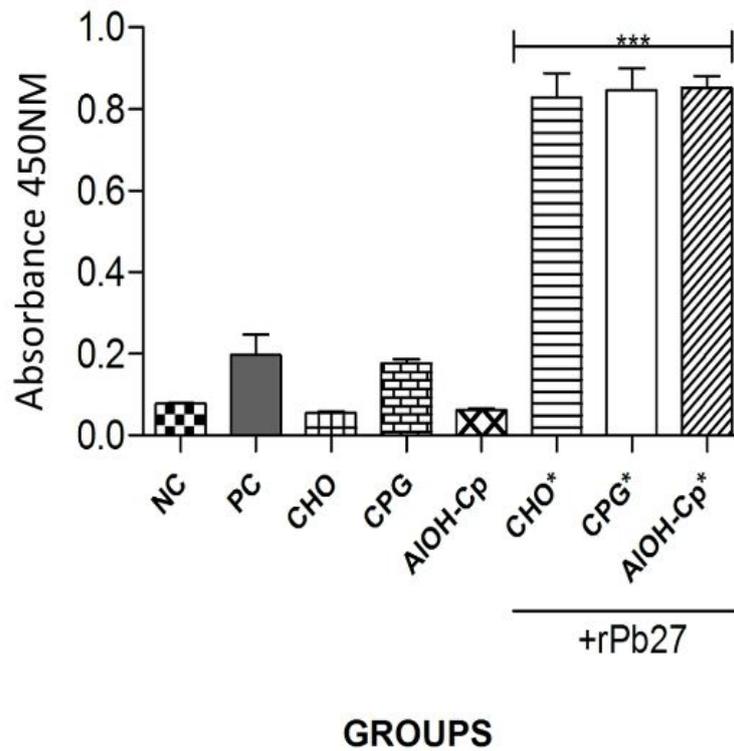
The survival of the animals during the experimental phase was measured. After 60 days of infection, all immunized groups presented higher survival rate (Figure 4C).
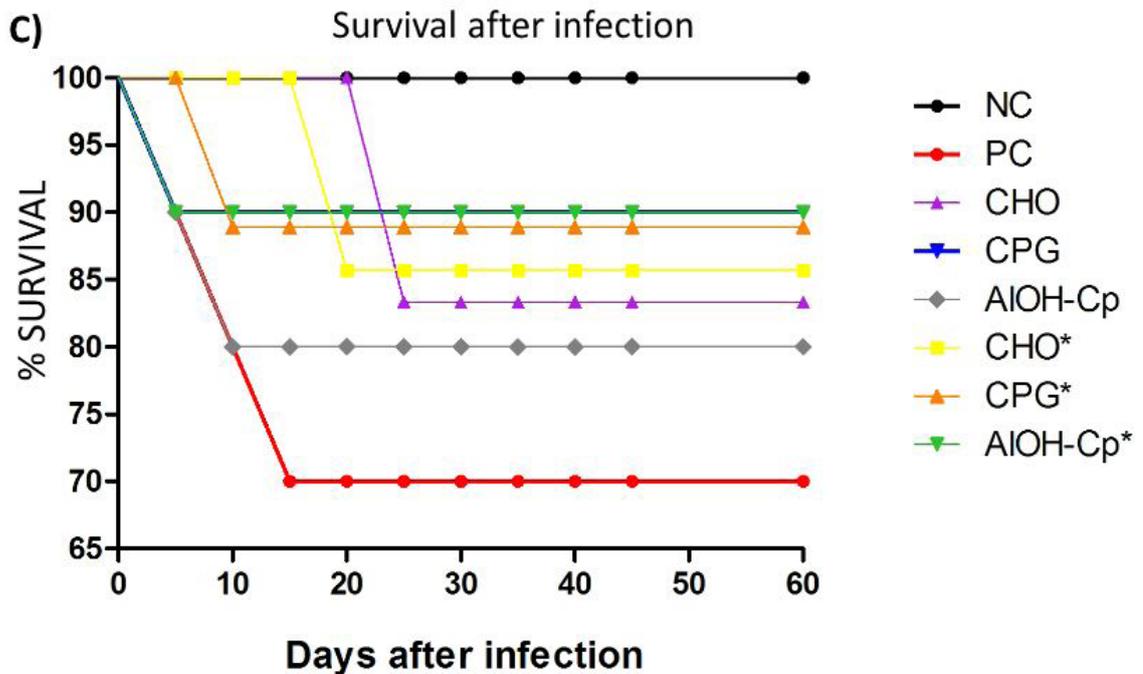

CFU analyzes were performed 30 and 60 DPI by *P. brasiliensis*. It was possible to observe 30 DPI the CHO and CPG groups were able to reduce the fungal load to undetectable levels when associated with rPb27. The group CHO adjuvant associated or not with rPb27 could reduce fungal load (Figure 4D).

D) 30 DPI
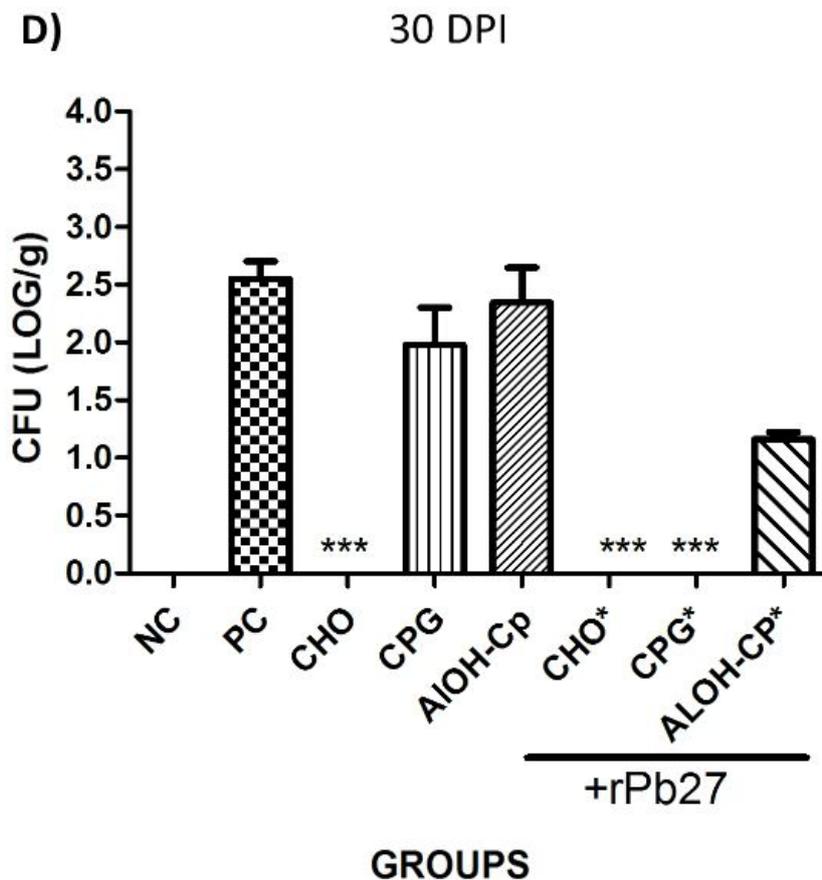
At the euthanasia point of 60 DPI, regardless of the used adjuvant, all groups that received rPb27 during immunizations showed a reduction in fungal load when compared to PC. It is possible to observe that animals immunized with CHO* remained undetectable levels of fungal load, and Cp-ALOH group were able to reduce fungal load even if not associated with rPb27 (Figure 4E).

E) 60 DPI
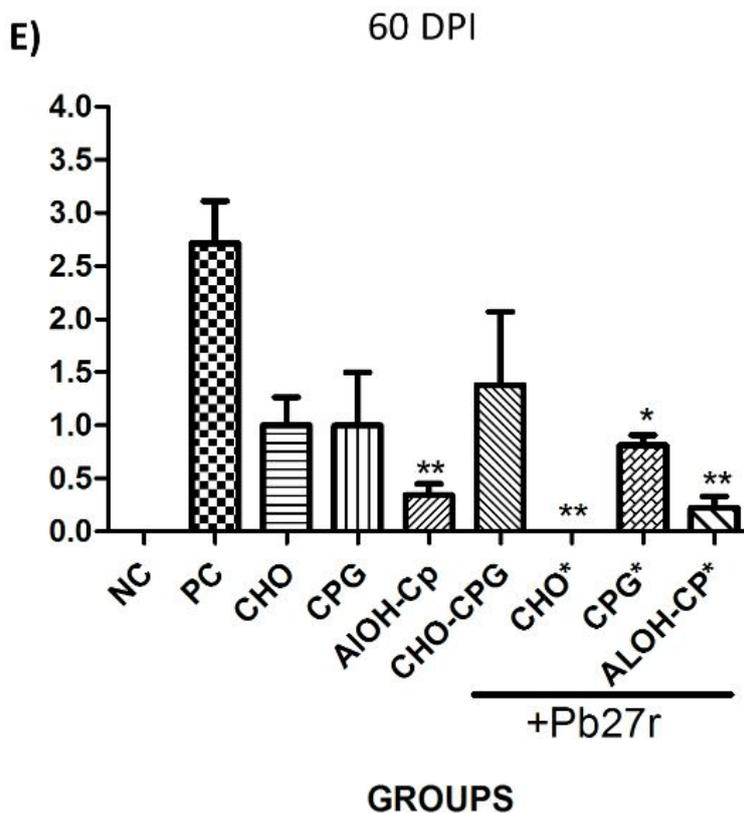
F) Experimental design
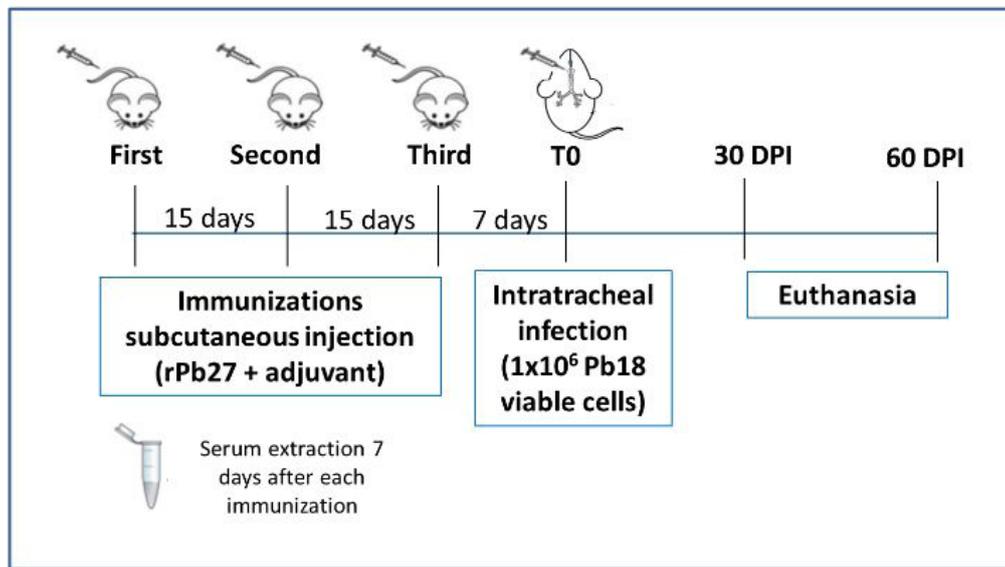
Figure 4: Protection assay

Panel is showing *in vivo* protection assay using chitosan adjuvant associated with rPb27: (A) Antibodies production during immunization. Specific IgG antibodies against rPb27 were quantified in the sera of animals using ELISA assay; (B) Antibodies production after the third immunization. Specific IgG anti rPb27 were quantified in the sera of animals using ELISA assay; (C) Survival percentages of mice during experimental infection; (D) Colony-forming units detected in the lungs of mice at 30 DPI; (E) and at 60 DPI; (F) Experimental design of in vivo protection assay. The groups were: (NC) negative control, (PC) positive control, (CHO) stimulated with chitosan adjuvant, (CPG) stimulated with CPG synthetic oligodeoxynucleotide and (ALOH-Cp) stimulated with aluminum hydroxide associated with *C parvum*. (CHO*) stimulated with chitosan adjuvant associated with rPb27, (CPG*) stimulated with CPG synthetic oligodeoxynucleotide associated with rPb27 and (ALOH-Cp*) stimulated with aluminum hydroxide associated with *C parvum* and rPb27. *Indicates significant differences at (P < 0.05), **at (P < 0.01) and ***at (P < 0.001) compared to the negative control

Histological evaluation was performed after 60 days of infection. The CHO and CPG groups presented compact granulomas with a restricted inflammatory response when compared to the ALOH-Cp group in which the lungs analyzed had a high incidence of inflammatory infiltrates. The groups immunized with adjuvant and rPb27 had fewer lung lesions in general, but among them, it was possible to observe that the CHO* and ALOH-Cp* groups presented lungs with clear airways and low inflammatory response. Also, although the CHO* group did not present CFUs after 60 days of infection, during histological analysis, it was possible to observe fungal cells at some points in the lung by histology, possibly inviable cells (Figure 5).

Discussion and Conclusion

The rPb27 has immunoprotective properties that make it a candidate for the development of a PCM vaccine. In research conducted in our laboratory has shown significant protection in the lungs, spleens, and livers of mice when used in prophylactic protocols [8,11]. In this work, we associated rPb27 with two new adjuvant formulations (CHO and CPG) and compared with the adjuvant used in previous studies by our research group (ALOH-Cp).

It was observed that all immunized animals obtained anti-Pb27 specific IgG production, showing efficiency in immunization independent of the adjuvant used (Figure 4A and B). The survival of the animals during 60 days post infection was evaluated. The groups presented survival rates between 90 and 80%, which is higher than PC.

The CHO, CHO* and CPG* groups demonstrated higher ability to reduce fungal load after 30 DPI, surprisingly approaching the fungal load to zero in the lungs of the animals in this group. This result in the early stages of infection had never been obtained by our group before. At 60 DPI, as expected by the protective effect of the protein, all groups immunized with rPb27 obtained a significant reduction of the fungal load when compared to the PC, highlighting the CHO* group that maintained the fungal lung load at undetectable levels. The ALOH- Cp group obtained a significant reduction, compared to the control. These results corroborate with previous studies, which obtained results similar to this, reaching the fungal load at undetectable levels in 90 days in animals immunized with ALOH-Cp [10,11].

When we compared these results to histology, we were able to observe the modulation of the immune and inflammatory response by rPb27 described in previous studies. All the immunized groups presented reduction of inflammatory infiltrate compared to the

PC showing that rPb27 have protection effect independent of the adjuvant used. In addition, it is possible to observe an additional effect on immunization since the CHO* and ALOH-Cp* groups presented reduced inflammatory infiltrate, showed clear airways and low fungal load after 60 days (Figure 5).
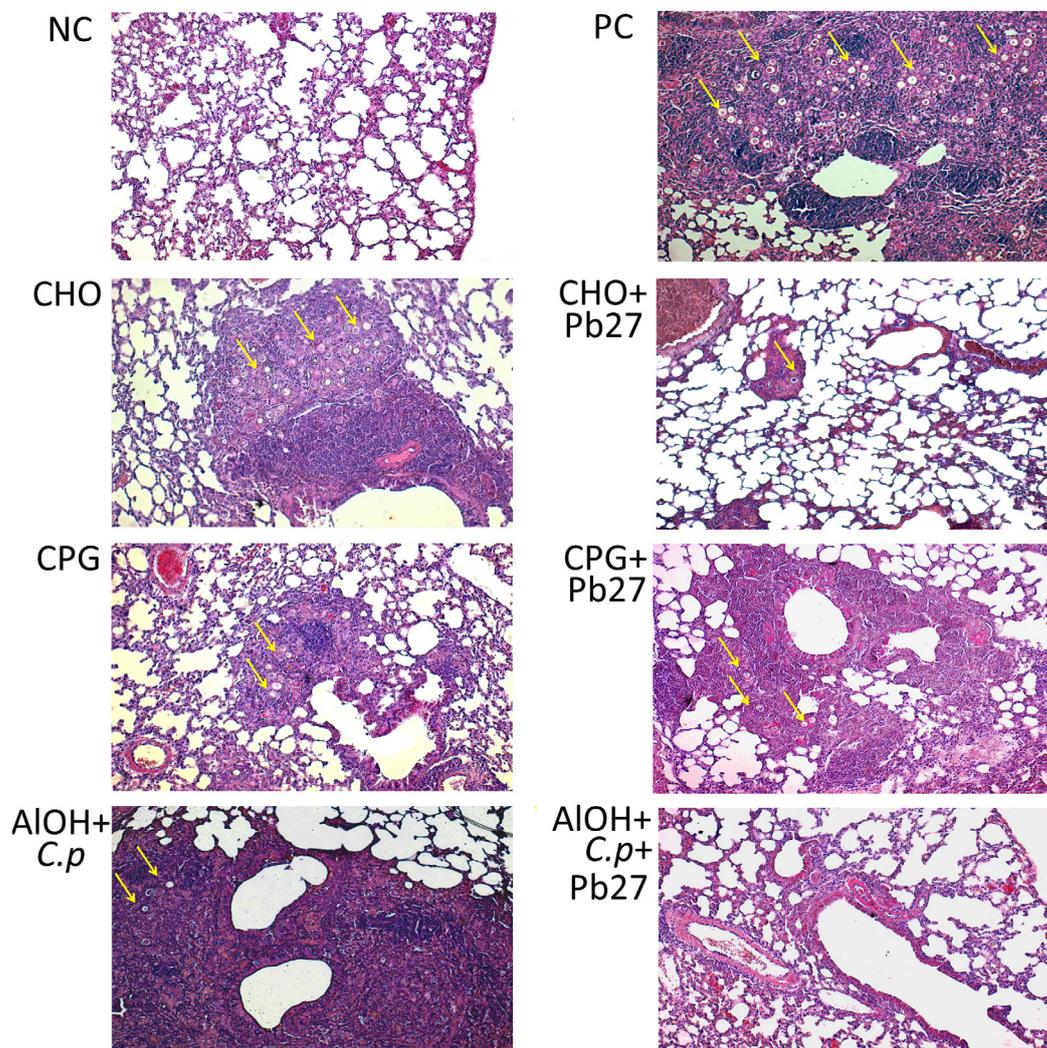
Figure 5: Histological sections of lungs stained with HE 60 days after infection. After 60 days of infection with *P. brasiliensis,* mouse lungs were stained with HE and analyzed by histology. A representative image of each group is shown at high magnification (10X) in the panel. The groups are as follows: The yellow arrows indicate the location of fungal cells in the tissue. (NC) negative control, (PC) positive control, (CHO) stimulated with chitosan adjuvant, (CPG) stimulated with CPG synthetic oligodeoxynucleotide and (ALOH-Cp) stimulated with aluminum hydroxide associated with *C parvum*. (CHO*) stimulated with chitosan adjuvant associated with rPb27, (CPG*) stimulated with CPG synthetic oligodeoxynucleotide associated with rPb27 and (ALOH-Cp*) stimulated with aluminum hydroxide associated with *C parvum* and rPb27

Adjuvants are essential for increasing the immune response to vaccine antigens. They have different modes of action and should be selected for use based on the type of immune response desired for a particular vaccine. To select an adjuvant, it is desired that it be stable, biodegradable, inexpensive to produce, and promotes adequate immune and inflammatory response [31,32].

In this work, in vitro macrophage assays, all adjuvants tested were able to induce increased phagocytic activity relative to controls. The adjuvants that have CPG in their formulation obtained a higher phagocytic index compared to the other groups. However, the macrophages function depends on their activation state, which gives them a microbicidal activity. In non-activated macrophages, *P. brasiliensis* acts as an intracellular pathogen and can survive and replicate within these cells. As a result, the variations observed in the immune response during PCM related to resistance and susceptibility are dependent on fungal suppression mediated by macrophages [33]. Therefore, not only the phagocytic capacity but also the elimination capacity of the fungus inside macrophages is important for resistance in PCM. When we compared macrophage microbicidal activity after stimulation with the adjuvants, it was also possible to observe that all the adjuvants are in some way activators of the fungicidal activity in macrophages. When compared to the controls all the stimulated groups were able to reduce the fungal load in the interior of macrophages. However, cells stimulated with the CPG, and CHO adjuvants have higher fungicidal capacity when compared to the ALOH-cp adjuvant (Figure 2). When associated with rPb27, CPG induced a more significant protective effect, though this protection capacity has

been reduced in 60 days. CPG *in vitro* responses such as high phagocytic index and microbicidal capacity of macrophages may be related to the CPG activation of macrophages by TLR9 way, this mechanism is described as being important cell activator of innate immunity and efficient in eliminating intracellular pathogens [34,35]. Recent studies have shown that TLR9 activation is related to initial protection against PCM, a possible effect seen during immunizations using CPG as adjuvant, since CPG is a TLR9 agonist [36,37]. The results obtained here demonstrate that the activation of TLR9 may be important in protection against PCM and that as adjuvant the CPG is promising, however, adaptations in the protocol and/or association of this adjuvant with other formulations will be necessary for the protective effect to be lasting.

Finally, the *in vitro* CHO adjuvant obtained high fungicidal capacity and was highlighted by IL-1β cytokine production, in addition, it induced lower levels of IL-10 and IL-6. The mechanism of activation of the fungicidal effect of the CHO adjuvant seems to be related to its induction of the cytokine IL-1β (Figure 3). In a previous study, Tavares and coworkers 2013 demonstrated that IL-1β signaling plays an important role in the killing of *P. brasiliensis* by macrophages [38]. The NLRP3 inflammassoma responds to *P. brasiliensis* and plays an important role in defense of the host against the fungus by the production of IL-1β [38,39]. Further studies should be performed to better understand the mechanism of induction of fungicidal activity by these adjuvants [38]. In the prophylactic assay animals immunized with this adjuvant obtained satisfactory levels of survival at all times evaluated. This adjuvant was efficient in the reduction of the fungal load even when not associated with Pb27r, with a histological profile formed by compact granulomas and clear airways. The results obtained with chitosan without the recombinant protein, such as the reduction of the fungal load in the early stages of the infection, are possibly due to the antimicrobial effect inherent to this polymer and already reported in previous studies [40,41]. Another possible explanation would be the similarity of the chemical structure present in the chitosan with the chitin present in fungi, so that there may be the possibility of recognition of the fungus by the immune system. Further study for understanding the induction of chitosan protection should be performed [42].

The results obtained in this work made allow us to conclude that chitosan adjuvant, when associated with rPb27, induces additional protection against PCM. The use of this adjuvant in the prophylactic vaccine formulation was effective in targeting the immune response without exacerbated inflammatory response and no impairment of the pulmonary airways. Due to the properties presented, as well as the characteristics of biocompatibility, low cost and excellent possibility of use in humans, chitosan presents itself as a suitable candidate to compose a vaccine formulation against PCM associated with Pb27r.

Acknowledgments

Supplementary data

The representative video is showing P. brasiliensis inside the macrophage on phagocytosis assay by confocal microscopy.

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