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Antifungal and immunomodulatory activity of a novel cochleate for amphotericin B delivery against *Sporothrix schenckii*

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ABSTRACT

Introduction: Sporotrichosis is an emergent subcutaneous mycoses caused by species of the *Sporothrix schenckii* complex. Amphotericin B (AmB) remains the main antifungal drug for the treatment of systemic infections, but its use is limited by toxicity reasons. AFCo3 is a novel cochleate containing detoxified LPS, which exhibits drug delivery and immunomodulating properties. Here, AFCo3 was used as the vehicle for AmB to evaluate the immunomodulatory and antifungal efficacy against *S. schenckii in vitro* and *in vivo*. Methods and results: The minimum inhibitory concentrations of AFCo3-AmB and AmB were 0.25 and 1 μg/mL respectively. The minimum fungicidal concentration was 0.5 μg/mL for AFCo3-AmB and 2 μg/mL for AmB. AFCo3-AmB was less cytotoxic than AmB for peritoneal macrophages, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and reduced the AmB-induced hemolysis in murine erythrocytes. AFCo3-AmB improved the intracellular killing of phagocytized yeast and it enhanced the *in vitro* production of IL-1β, TNF-α and NO in peritoneal macrophages. Moreover, AFCo3-AmB was more effective than AmB in reducing spleen and liver fungal burden after repeated (five days) intraperitoneal administration of 5 mg/kg of AmB, in a Balb/c model of systemic infection, associated to a significant induction of Th1/Th17 response. Finally, blood chemistry revealed that AFCo3-AmB did not cause changes suggestive of nephrotoxicity, such as increases in total proteins, albumin, creatinine and blood urea nitrogen that were caused by free AmB. Conclusions: AFCo3-AmB exhibited a significant immunomodulator action, reduced toxicity and improved antifungal action against *S. schenckii*, suggesting a potential use as AmB delivery for systemic sporotrichosis treatment.

1. Introduction

Sporotrichosis is an emergent subcutaneous mycosis with worldwide distribution and alarming increasing incidence, especially in tropical and subtropical regions [1,2]. Human and animal sporotrichosis are caused by several species of the *Sporothrix schenckii* complex including: *Sporothrix schenckii sensu stricto*, *Sporothrix brasiliensis* and *Sporothrix globosa*, the species of greatest clinical and global epidemiological importance [3]. The disease is generally acquired by the traumatic inoculation with soil, plants, and organic matter contaminated with the fungus or, more rarely, by inhalation of conidias. Recently the zoonotic transmission, especially from cat scratches, is gaining relevance, mostly in Brazil [4]. Clinical manifestations of sporotrichosis depend mainly on host immune competence. Localized subcutaneous forms are observed in immunocompetent individuals, while immunocompromised patients are predominantly affected by disseminated and systemic forms, especially in patients with HIV infection [5,6]. In this way, several studies have demonstrated the importance of the innate and specific immune system for the control of the infection [7–13].

Itraconazol and terbinafine are the antifungal drugs used for the treatment of uncomplicated cutaneous sporotrichosis in humans, but several fungal isolates have shown resistance [14]. On the other hand, amphotericin B (AmB) which was introduced in the 1950s, still remains the most valuable drug reserved for systemic sporotrichosis [15]. AmB binds preferentially to ergosterol in fungal plasma membranes, although it also interacts with sterols, such as cholesterol, in host cells, which largely explains its known toxicity. Thus, application of AmB is hampered by the long-lasting therapy associated with severe adverse effects, including: nephrotoxicity, neurotoxicity and hepatotoxicity [16]. Accordingly, many efforts have been made to develop more effective and less toxic formulations of AmB, including the use of target delivery, such as it encapsulation in liposomes [17,18] and cochleates

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[19–22]. Despite the existence of diverse experimental AmB formulations, to our knowledge, there are no studies evaluating the effect of these new formulations against *S. schenckii* complex species. In addition, the combined antifungal and immunomodulation therapy has been proposed as a promissory alternative against sporotrichosis, but this strategy has not been sufficiently studied [23].

Cochleates are a novel lipid-based system with a unique multi-layered structure consisting of a large, continuous, solid lipid bi-layer sheet rolled into a spiral, with no internal aqueous space [21]. Adjuvant Finlay Cochleates 3 (AFCo3) is a cochleate containing purified and non-toxic LPS derived from *Neisseria meningitidis* B, which has the properties of a vaccine adjuvant and drug carrier. The presence of detoxified LPS as pathogen-associated molecular patterns (PAMPs) in their composition, confers immunomodulating properties to this structure. This novel cochlear microparticle can be formulated with antibiotics/chemotherapeutic agents with the aim of using them to improve the treatment of several veterinary and human infectious diseases [24,25]. In this study was evaluated the *in vitro* and *in vivo* antifungal activity of AFCo3-AmB against *S. schenckii sensu stricto*, including the toxicity and the immunomodulatory effects in comparison with free AmB.

2. Materials and methods

2.1. Preparation of AFCo3-AmB formulations

AFCo3-AmB was prepared according to the methodology described for AFCo3 production [24], adapted for the formulation with AmB. Briefly, the LPS from *N. meningitidis serogroup B*, previously purified by an industrial process in the Finlay Institute (Havana, Cuba) under strict Good Manufacturing Practices (GMP), was detoxified (LPSd) in sodium hydroxide (NaOH) 0.7 N and placed into an oven at 100 °C for 4 h. The pH was neutralized with hydrochloric acid 1:2 (*v*/*v*), and the concentration of LPSd was adjusted to 40 mg/mL in a re-suspension solution (Tris 30 mM, 1% sodium deoxycholate (DOC)). The solution that was thereby obtained, was filtered through a filter with a pore size of 0.2 mm. Through a process of continuous agitation (330 rpm) and slow dripping (2.2 mL/min), the formation solution ($CaCl_2$ 10 mM, NaCl 100 mM) was added. In this moment AmB (Amphotericin B dissolved in sodium deoxycholate, Sigma) was added and adjusted at a concentration of 0.5 mg/mL. The formation of cochlear structures was evidenced by the presence of a white precipitate and subsequent observation with an optic microscope. Once the cochlear structures were formed, the process continued to the wash step. Wash solution (Tris 10 mM, $CaCl_2$ 5 mM) was added under a process of continuous agitation (300 rpm) and a third volume of medium was dropped at 5 mL/min. After 30 min of rest without agitation, the process continued with the step of centrifugation at 1500 rpm for 15 min at 4 °C. During the processing of the cochlear structure sediment, the supernatant was completely discarded and the pellet slowly re-suspended in Tris 10 mM, DOC 0.25%, and $CaCl_2$ 2.5 mM at the same volume of the formation. All of these processes were performed in a closed-loop cycle under completely sterile conditions and the AFCo3-AmB suspension was conserved in sterile hermetically closed vials (20 mL). The stability of cochleates was evaluated after incubation for at least 3 months at 4 °C and for 7 days at 37 °C by microscopic observation, quantification of LPS and AmB in the supernatant of the wash and in the precipitate after centrifugation.

2.1.1. Quantification of LPS

The content of LPS was evaluated in the final formulation through the determination of 2-keto-3-deoxyoctonoic acid (KDO) using the thiobarbituric acid (TBA) method described by Osborn. The percentage of incorporation of LPS in AFCo3 (B) was determined using the calculation (I × 100) / T wherein "I" is the concentration of LPS in the precipitate and "T" is the concentration of LPS in total [24].

2.1.2. Quantification of AmB and incorporation percentage

The determination of AmB was carried out by high-performance liquid chromatography [26]. The content of AmB was quantified in the supernatant of the wash and in the precipitate upon centrifuging containing the AFCo3-AmB cochleates. The percentage of incorporation of AmB in AFCo3 was determined using the calculation (I × 100) / T wherein "I" is the concentration of AmB in the precipitate and "T" is the concentration of AmB in total.

2.2. Microorganism and culture conditions

Sporothrix schenckii ATCC 16345, originally obtained from a human case of diffuse lung infection (Baltimore, MD) and kindly provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), was used for all experiments. For mice infection, a piece of the fungal mycelium grown on Mycosel (BD Biosciences) agar tubes was transferred to an Erlenmeyer flask containing 100 mL of brain-heart infusion broth (Difco) and then cultured for 5 days at 37 °C with constant shaking at 150 rpm. After that, an aliquot containing 2×10^7 yeast cells was transferred to a new medium and cultured for 7 days more at the same conditions in order to achieve a virtually 100% mycelium-to-yeast conversion in a logarithmically growing culture.

2.3. Antifungal susceptibility testing

2.3.1. Minimum inhibitory concentration (MIC)

Susceptibility tests were performed using microdilution techniques based on the Clinical and Laboratory Standards Institute (CLSI) protocols M27-A3 (for the yeast form) [27]. AmB and AFCo3-AmB were tested over a final concentration range of 16 to 0.078 μg/mL. One hundred microliters of the prepared cell suspension (1×10^3 to 5×10^3 CFU/mL) was added to each well of 96-well microtiter plates containing 100 μL of previously prepared antifungal drugs in RPMI 1640 to bring the drug dilutions and inoculums to the final desired test concentrations. After this step, the final concentration of DMSO in test wells became 1%. Growth and sterility controls were included for each isolate tested (growth control, RPMI medium with DMSO and organisms but with no drug added; sterility control, RPMI medium only, with no organisms or drug added). The inoculated plates were incubated at 35 °C for 5 days. The MIC was defined as the lowest concentration at which there was complete inhibition of growth. Susceptibility test was performed in triplicate on three different days (independent experiments).

2.3.2. Determination of fungal viability by microplate Alamar Blue assay (MABA)

The MABA was employed according to the manufacturer's instructions (Invitrogen), adding 20 μL to the well of Alamar Blue at 72 h and the plates were incubated for an additional 24 h, totaling 4 days for the MIC final reading. The lowest antifungal agent concentration that substantially inhibited the growth of the organism was visually determined at the point at which there was no change in the original blue colour of the reagent [28].

2.3.3. Determination of the minimum fungicidal concentration (MFC)

MFC was determined after 5 days of incubation by removing 10 μL of the contents from all wells showing no visible growth and spreading them onto Sabouraud dextrose agar plates. The plates were incubated at 35 °C for 72 h. The lowest antibiotic concentration in which no cell colonies were observed after 4 days of incubation at 30 °C was defined as the MFC.

2.4. Preparation of peritoneal exudate cells (PECs)

Thioglycollate-elicited PECs were harvested from male Balb/c mice of six weeks 3 days after i.p. inoculation with 3% sodium-thioglycollate,

which was performed by washing the peritoneal cavity with 5.0 mL of sterile PBS, pH 7.4. The cells were cleaned twice by centrifugation at 200*g* for 5 min at 4 °C and washing with sterile PBS, pH 7.4. The cells were then resuspended in RPMI-1640 medium (Sigma) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 5×10^{-2} M mercaptoethanol, and 5% inactivated fetal calf serum (Sigma), referred to as RPMI-1640 complete (RPMI-1640C) medium. PECs were manually counted in a Neubauer chamber (Boeco, Germany), and the concentration was adjusted to 5×10^6 PEC/mL in RPMI-1640C for use in the following experiments. 0.1 mL PEC suspensions containing 5×10^6 cells/mL in RPMI-1640C were added to each well of a 96-well tissue culture plate and incubated for 1 h at 37 °C with a supply of 5% CO_2. Next, non-adherent cells were removed by discarding the supernatants and refilling each well with 0.1 mL of RPMI-1640C medium.

2.5. Determination of cytotoxic activity in peritoneal macrophages

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). Briefly, AFCo3-AmB or AmB were dispensed in triplicate at different concentrations (0.078 to 16 μg/mL) in 96-well microplates with adherent peritoneal macrophages and incubated for 48 h with a supply of 5% CO_2 at 37 °C with three wells without drug as the control. The supernatant was firstly collected for cytokine studies and 100 μL MTT (0.5 mg/mL) was added to macrophages in the well and incubated for 4 h. The plate was spun down at 1000*g* for 10 min at 4 °C and the supernatant was aspirated. The product of the MTT reduction and the insoluble purple formazan crystals were solubilized in 100 μL acid isopropanol and the optical density at 570 nm was spectrophotometrically determined using a microplate reader (Multiskan Ascent, Labsystems). The results were expressed as percentage of viable cells compared with untreated control wells.

2.6. In vitro hemolysis assay

The toxicity in erythrocytes of AFCo3-AmB was evaluated by investigating its hemolytic potential in comparison with free AmB. Hemolysis assay was performed using fresh mouse blood in 96-well plates, as previously described. In brief, the serum was removed from the blood by centrifugation and the erythrocytes were then washed three times with saline solution containing 0.85% of NaCl and 10 mM of $CaCl_2$. Following the last wash, the erythrocytes were collected by centrifugation at 1500 rpm for 5 min. The stock solution of erythrocytes was prepared by diluting 200 μL of erythrocytes into 9.8 mL of saline solution. The first well of each line was used as negative control (100 μL of 10% blank AFCo3 in saline solution or 100 μL of 1% DMSO, the vehicle used for AmB solubilisation). The second well of each line was used as positive control, in order to obtain 100% of hemolysis (100 μL of 0.02% Triton X-100 in saline solution). The subsequent wells contained 100 μL of solution of either AmB or AFCo3-AmB dissolved in DMSO. The remaining wells were filled with 100 μL of saline solution for the serial dilutions. Then, each well received 100 μL of the stock solution of erythrocytes. The plate was incubated for 1 h and then centrifuged at 1500 rpm for 10 min. The supernatant was transferred to another plate and the hemolysis (%) was measured with a microplate reader (Multiskan Ascent, Labsystems) at 540 nm. The % hemolysis was calculated according to the following equation [29].

$$\text{Hemolysis } (\%) = \left(\frac{\text{Abs}_s - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0}\right) \times 100$$

where Abs_s is absorbance of the sample treated with AmB or AFCo3-AmB, Abs100 is absorbance of 100% lysed and Abs0 is absorbance of unlysed sample treated with PBS, pH 7.4.

2.7. Measurement of the ex vivo release of IL-1β, TNFα and nitric oxide (NO) by peritoneal macrophages

Peritoneal macrophages were treated with AmB, AFCo3-AmB, empty AFCo3 or lipopolysaccharide (LPS) as positive control for 24 h and cell-free supernatants were assayed for IL-1β and TNFα using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) Kit (BD Bioscience-Pharmingen) according to the manufacturer's instructions. Colorimetric reactions were measured at 450 nm with wavelength correction at 570 nm (Microplate Reader, Multiskan Ascent, Labsystems). For the evaluation of NO content, the Griess reaction was used [30]. Briefly, 100 μL of supernatant was incubated with 100 μL of Griess reagent (1% sulfanilamide (Sigma Chemicals, St. Louis, MO, USA), 0.1% N-(1-naphthyl)-ethylenediaminedihydrochloride (NED, Sigma Chemicals, St. Louis, MO, USA) and 2.5% phosphoric acid in water). The NO concentration was determined using the same microplate reader at 540 nm, with reference to a standard curve ($NaNO_2$).

2.8. Determination of intracellular antifungal activity in peritoneal macrophages

This study was adapted according to previously described methodologies [31,32]. Briefly, monolayers of macrophages were cultured as described above. After 24 h of incubation, opsonized *S. schenckii* was added to the monolayers, resulting in a *S. schenckii*-to-macrophage ratio of 1:8. The yeasts were opsonized by incubation of a washed stationary-growth-phase suspension with 10% normal mouse serum (Balb/c) under continuous rotation at 8 rpm for 15 min at 37 °C, and then the yeasts were washed twice in PBS. After a 30-min uptake period, the un-ingested yeasts were removed by washing the monolayer. The macrophages were re-incubated for 48 h in the presence of AFCo3-AmB or AmB in culture medium supplemented with 5% fetal bovine sera; concentrations ranged from 2 to 0.03 μg/mL for AmB, according to the previous results of the viability assay. Control monolayers were re-incubated with either PBS or empty AFCo3 in appropriate dilutions. After incubation, the culture medium was carefully removed and the monolayers were gently washed with 200 μL of distilled water to lyse macrophages. One hundred microliters of cell homogenates was assayed for the presence of viable yeasts in Petri dishes containing Mycosel agar, incubated at 30 °C for 5 days. Colony forming units (CFU) per sample were determined by counting the number of fungi growing in the culture plate. All assays were done with five wells per condition in three independent experiments. The occurrence of effective phagocytosis of *S. schenckii* yeast by macrophages was controlled in parallel by incubation under the same conditions in glass slides and microscopic observation.

2.9. Fungal burden study in spleen and liver after in vivo treatment

Balb/c mice, 5–7 weeks old purchased from "Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório" (CEMIB), Campinas University (UNICAMP, Brazil) were intraperitoneally inoculated with 10^6 of *S. schenckii* yeast cells in 200 μL of PBS. Two days after infection five mice in each group were treated with AmB or AFCo3-AmB (i.p. route) at either 1 or 5 mg/kg. Control groups were included: PBS or empty AFCo3. Mice were treated daily from days 2 to 6 post-challenge. On the 7th day, mice were euthanized and the spleens and the livers were removed for assessment of the systemic fungal load. Adequate dilutions of the spleen or liver macerate were inoculated in duplicate on Mycosel agar plates and the resulting CFU were counted. Non-infected mice were treated in a similar way as above to study the comparative profiles of cytokines released from splenocytes and blood chemistry endpoints for nephrotoxicity associated to the AmB treatment. All animal procedures were performed according to the guidelines of SBCAL/COBEA (Sociedade Brasileira de Ciência em Animais de Laboratório).

2.10. Preparation of total splenocytes and measurement of the ex vivo release of cytokines

Spleens were aseptically removed and passed through a 100 μm cell strainer into a Petri dish containing 2 mL of PBS with the aid of a syringe plunger. For red cell lysis, the resulting suspension was mixed with 6 mL of a 0.17 M ammonium chloride solution and then incubated on ice for 5 min. The splenocytes were then separated from the supernatant by centrifugation at 300 ×*g* for 5 min at 4 °C, washed once with 3 mL of RPMI complete medium and then resuspended in 1 mL of the same medium. The cell concentration was determined by microscopy using the Trypan blue exclusion test and then the splenocytes were adjusted to 5×10^6 cells/mL in RPMI complete medium. For the measurement of the *ex vivo* release of IFNɣ, IL-4 and IL-17A, splenocytes were cultured for 24 h at 37 °C and 5% CO_2 on flat bottom 48-well tissue culture plates in the presence of HKss, at a splenocyte-to-yeast ratio of 1:5. Final concentrations were 2.5×10^6 splenocytes/mL and 1.25×10^7 yeast cells/mL. Concanavalin A (0.25 μg/mL in RPMI complete medium) and RPMI alone were used as positive and negative controls, respectively. Cytokines were measured by ELISA kits (eBioscience), according to the manufacturer's instructions.

2.11. Blood chemistry measurements

With the aim of monitoring the effect of the different treatments on blood chemistry parameters associated to AmB nephrotoxicity, samples of whole blood from treated and non-infected mice were collected by cardiac puncture. The blood samples were allowed to clot and the serum was separated by centrifugation and stored in Eppendorf tubes at −80 °C until analysis. The measure of total proteins (TP), albumin (Alb), creatinine (Cr) and blood urea nitrogen (BUN) were performed using an automatic analyzer 7180 (Hitachi High-Technologies Co., Tokyo, Japan).

2.12. Statistical analysis

Statistical analysis was performed by GraphPad Prism ver. 6.01 software. Data are expressed as mean ± standard error of mean (SEM) of three independent experiments. Analysis of variance (ANOVA) was performed by using one- or two-way analysis with multiple comparisons test, as indicated. The *t*-test with correction for multiple comparisons by the Holm–Sidak method was also used. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Generation and characterization of AFCo3-AmB cochleates

A cochleate-based formulation of AFCo3 containing AmB was successfully prepared. It was observed that after several attempts the formulation presented the same characteristics, confirming the reproducibility of the preparation method according to the authors [24]. Fig. 1 shows a microscopic view of the AFCo3-AmB cochleates with the typical cigar-like microstructures, between 5 and 10 μm on average. After obtaining AFCo3-AmB, the concentrations of LPS and AmB were analyzed as active components of the formulation. There was no apparent release of AmB from AFCo3, observed after incubation for at least 3 months at 4 °C and for 7 days at 37 °C. Between 98 and 99.6% of added AmB was detected in the cochleate with the procedure used, as evidence of the efficacy of the method and stability of the formulation throughout the assessment period (Table 1).

3.2. Antifungal susceptibility test

The MICs and MFCs of free AmB and AFCo3-AmB against *S. schenckii* are presented in Table 2 and Fig. S1. The data indicated that the fungistatic (MIC) and fungicidal (MFC) activity of AmB was improved after incorporation in AFCo3. The MIC was similar using both M27-A3 and MABA methods.

Fig. 1. Typical cigar-like microstructures of AFCo3-AmB, between 5 and 10 μm (400×). Scale bars represent 10 μm.

3.3. Cytotoxic activity in peritoneal macrophages and erythrocytes

The cytotoxic effect of AFCo3-AmB and free AmB on peritoneal macrophages is shown in Fig. 2. AFCo3-AmB treatment was found to be less cytotoxic than free AmB to macrophages even at concentrations as high as 16 μg/mL and at lower concentrations ($P < 0.05$). Interestingly in our study, a marked stimulation of the metabolic activity was observed in macrophages in doses between less than 2 μg/mL and 0.50 μg/mL for free AmB and between 2 and 0.125 for AFCo3-AmB. In concentrations lower than 0.50 μg/mL the toxicity of free AmB was higher than AFCo3-AmB. NaOH (0.1 N) was used as a positive control of cytotoxicity, while AFCo3 without AmB did not exhibit a deleterious effect in macrophages. On the other hand, comparative hemolytic properties of AFCo3-AmB were investigated by treating erythrocytes obtained from Balb/c mice with either AFCo3-AmB or free AmB, as shown in Fig. 3. In this test the protective effect of AFCo3 against the AmB-induced hemolysis was observed above 0.5 μg/mL and protection was directly proportional to the AmB concentration.

3.4. Determination of IL-1β, TNF-α and NO production by peritoneal macrophages

Peritoneal macrophages from Balb/c mice were challenged *ex vivo* with either AFCo3-AmB, free AmB or empty AFCo3 to evaluate their effect on the innate immune response. Detectable levels of IL-1β, TNF-α and NO were induced in macrophages by AmB, empty AFCo3 and AFCo3-AmB in almost all concentrations. Interestingly the highest concentration (16 μg/mL) of AmB and AFCo3-AmB, did not stimulate the highest production of pro-inflammatory cytokines and NO, as occurs

Table 1
Quantification of LPS, AmB in AFCo3-AmB and determination of the percentage of AmB incorporation.

LPS	AmB	Percentage of AmB incorporation
40 μg/mL	0.5 mg/mL	98–99.6%

Abbreviations: AmB, amphotericin B; AFCo3, Adjuvant Finlay Cochleate 3; LPS, lipopolysaccharide.

Table 2
In vitro direct antifungal activity of AFCo3-AmB and free AmB against *S. schenckii* ATCC 16345.

Evaluated substances	MICs (μg/mL)		MFC (μg/mL)
	M27-A3	MABA	
AFCo3-AmB	0.25	0.25	0.5
AmB	1	1	2

Abbreviations: MIC: minimum inhibitory concentration (expressed as μg/mL of AmB). MFC: minimum fungicidal concentration (expressed as μg/mL of AmB). M27-A3: Clinical and Laboratory Standards Institute CLSI (document M27-A3); MABA: microplate Alamar Blue assay. The empty AFCo3 did not evidence direct antifungal activity.

with empty AFCo3. The highest production of inflammatory mediators induced by AFCo3-AmB was detected in this study at 8 μg/mL, with proportional activity reduction in lower concentrations (Fig. 4A, B, C). LPS-activated PEC cells as positive control produced the highest level of pro-inflammatory cytokines and NO.

3.5. Determination of intracellular antifungal activity in peritoneal macrophages

The intracellular antifungal activity of AFCo3-AmB in comparison with free AmB was evaluated *ex vivo* on *S. schenckii* yeast previously phagocytized by peritoneal macrophages and the results are shown in Table 3. The presence of intracellular phagocytized yeast was verified microscopically (Fig. S2). The antifungal activity of AFCo3-AmB against intracellular *S. schenckii* was found to be superior to that of the reference free AmB. The minimal concentration causing intracellular fungal killing was 0.5 μg/mL for free AmB and 0.12 μg/mL for AFCo3-AmB. Empty cochleates did not show any antifungal activity in this model.

3.6. Fungal burden in spleen and liver after in vivo treatment

The comparative antifungal effect of AFCo3-AmB and AmB against infective *S. schenckii* was also evaluated *in vivo.* During the seven days of the study the animals were observed clinically and there were no detected clinical symptoms of toxicity. No significant difference was observed in body weight gain between the experimental groups, although the group infected and treated with AmB5 showed a mild tendency to lose weight over the length of the study, but it was not statistically significant (Fig. S3). At the 7th day the fungal load in the spleen and liver was evaluated as an expression of systemic infection and the results are shown in Fig. 5. The average CFU count obtained from spleen and liver macerates yielded significant differences between untreated and treated animals with AFCo3-AmB and AmB ($p < 0.05$). Mice treated

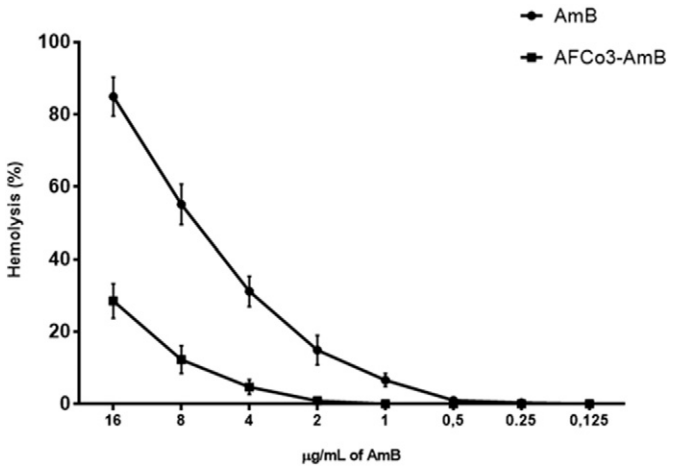

Fig. 3. Effect of amphotericin B (AmB) on the percent of hemolysis of murine erythrocytes treated with different concentrations of AmB, or AmB-AFCo3. Empty AFCo3 did not exhibit hemolytic activity (not shown).

with the lowest dose of AmB and AFCo3-AmB (1 μg/mL) had a reduction of CFU count in these organs in comparison with controls, but there was no difference between them. However, statistical differences between both groups were detected when using a higher daily dose of AmB (5 μg/mL), since mice treated with AFCo3-AmB showed less CFUs in the spleen and liver, in comparison with mice treated with free AmB. The results also showed no statistical difference between those mice treated with empty AFCo3 and the control group. This result evidences that AFCo3 used as a vehicle of AmB also improves its antifungal effect against *S. schenckii in vivo*.

3.7. Ex-vivo release of cytokines from splenocytes

In order to address to what extent the treatment with AmB, empty AFCo3 and AFCo3-AmB could impact cytokine production of the profiles of Th1, Th2 and Th17A, groups of infected or not infected mice were treated during five days with these formulations. On the seventh day of the study, the *ex vivo* release of IFN-γ, IL-4 and IL-17A in infected and non-infected mice were measured in culture supernatants of total splenocytes challenged with Hkss. The mice treated with either AFCo3-AmB or empty AFCo3, stimulated the production of IFN-γ and IL-17A in both infected and non-infected mice, but more intensely in infected groups. AmB did not stimulate the production of these cytokines, but a tendency to the reduction of these cytokines directly proportional

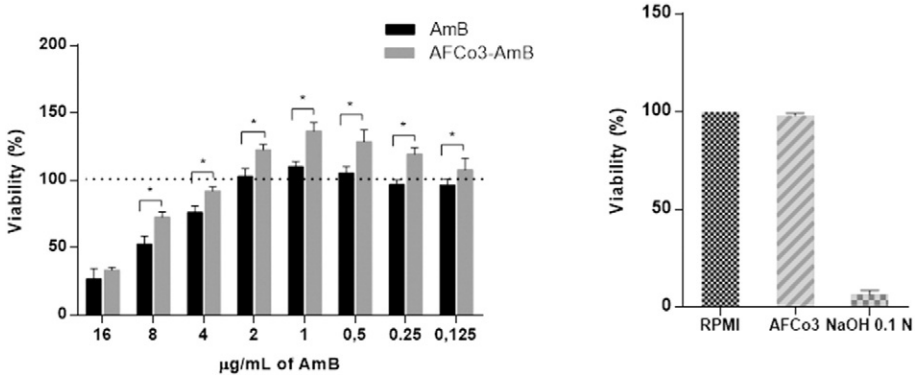

Fig. 2. Viability of peritoneal macrophages treated with AFCo3-AmB, free AmB and empty AFCo3. Adherent cells from peritoneal exudate cells were exposed to AFCo3-AmB and free AmB at different concentrations of AmB for 24 h followed by MTT assays. Data represent the mean ± SD from three independent experiments (*$p < 0.05$ AFCo3-AmB *versus* AmB group). Empty AFCo3 did not affect the macrophage viability. NaOH 1 N was used as a positive control of hemolysis.

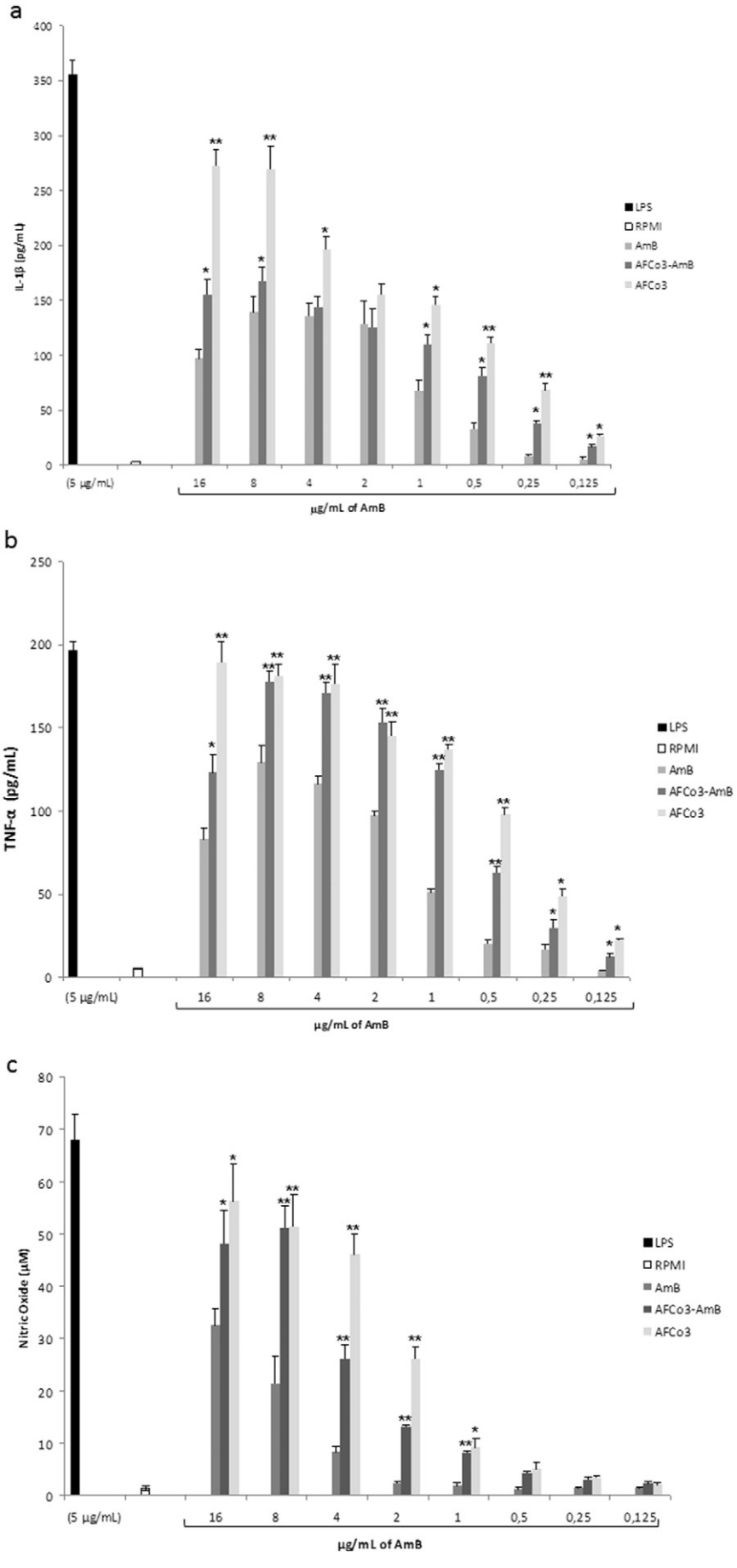
a
IL-1β (pg/mL)
400
350
300
250
200
150
100
50
0
LPS
RPMI
AmB
AFCo3-AmB
AFCo3
(5 µg/mL)
16
8
4
2
1
0,5
0,25
0,125
µg/mL of AmB
b
TNF-α (pg/mL)
250
200
150
100
50
0
LPS
RPMI
AmB
AFCo3-AmB
AFCo3
(5 µg/mL)
16
8
4
2
1
0,5
0,25
0,125
µg/mL of AmB
c
Nitric Oxide (µM)
80
70
60
50
40
30
20
10
0
LPS
RPMI
AmB
AFCo3-AmB
AFCo3
(5 µg/mL)
16
8
4
2
1
0,5
0,25
0,125
µg/mL of AmB

Table 3
Intracellular antifungal activity at various concentrations of AmB against phagocytized *S. schenckii* yeast after 48 h of incubation: effective killing expressed as CFU/10 μL (>99%).

Evaluated compounds	Concentrations of AmB (μg/mL)						
	2	1	0.5	0.25	0.12	0.06	0.03
AmB	–	–	–	5 ± 3	17 ± 7	58 ± 12	177 ± 33
AmB-AFCo3	–	–	–	–	–	23 ± 9	138 ± 42
AFCo3	134 ± 22						
Non-treated	151 ± 14						

– Absence of fungal growth (effective killing).
The *S. schenckii* yeast/macrophage ratio was 1:8. "Non-treated" are monolayers of macrophages/phagocytized yeast, incubated with the solvent of the compounds.
Abbreviations: AmB, amphotericin B; AFCo3, Adjuvant Finlay Cochleate 3; CFU, colony forming unit.

to the doses was observed in infected groups. The production of IL-4 by splenocytes was significantly reduced in infected mice treated with AmB. On non-infected mice there were no detected significant productions of IL-4 in the treated groups. The results showed a preferential Th1/Th17 profile induced by the infection that was increased by the treatment with AFCo3 and AFCo3-AmB (Fig. 6).

3.8. Blood chemistry measurements

Blood chemistry analysis of treated mice indicated that 5 mg/kg of free AmB induced a significant increase in TP, Alb, Cr and BUN, compared with the control group (Fig. 7). No significant changes in these endpoints were observed in the remaining groups.

4. Discussion

Antifungal prescription remains a challenge for the treatment of opportunistic disseminated mycosis because of uncertainties regarding toxicity and fungal resistance, leading to sub-therapeutic antifungal drug concentrations and poorer clinical outcomes [32]. In this way, many efforts are made to develop preventive and therapeutic methods against fungal invasive infections based in the stimulation of antifungal immune response and improving the delivery of classic antifungals [16,32–35].

New approaches for reducing the AmB toxicity and maintaining or improving its antifungal activity would represent a significant advance in the management of systemic fungal infections. In this regard, diverse lipid-based formulations such as liposomal formulations, lipid complexes, lipid emulsions and colloidal dispersions, have been developed and introduced into clinical practice. However, their use remains limited by cost, stability, and toxicity [32,35].

Multi-lamellar cylindrical micelles known as cochleates are one of the drug delivery platforms that attracted attention recently as a means to enhance and optimize the delivery of pharmaceuticals. Cochleates have shown to be effective in the therapeutic oral delivery of hydrophobic drugs, including antifungals such as AmB. Encapsulation of AmB into cochleates results in stable, nontoxic and highly efficacious AmB lipid particles, facilitating systemic delivery of AmB [19–22,36–38]. Cochleates are formed spontaneously when calcium ions are added to phosphatidylserine in physiological saline. These structures can be envisioned as membrane fusion intermediates, which facilitate the interaction with biological membranes [38]. Because of the hydrophobicity of AmB molecules, they integrate particularly well with the cochleate structure, providing the additional benefit of protecting AmB from exposure to harsh environmental [22,38].

AFCo3 is a formulation of deoxycholate-based cochleate microparticles containing AmB that has proven efficacy as an adjuvant vaccine and as a drug carrier [24,25]. As occurs for lipid-based carriers, the entrapment efficiency into AFCo3 depends on the nature of the drug. Hydrophobic drugs would have a high affinity for the hydrophobic interior of the lipid bilayers to minimize the interaction with water, as demonstrated by the high entrapment efficiency obtained with AmB [22,24,38]. During the process of AFCo3-AmB elaboration, the efficacy of AmB absorption was >98%, accordingly, almost all the AmB used was absorbed in the cochleate due to the high hydrophobic nature of this drug. In addition to AmB, detoxified LPS was incorporated with the aim of reducing the endotoxicity and to retain its immunostimulatory activity. The detoxified LPS was evaluated using the *Limulus* test before use, as part of the quality studies, confirming the reduction of LPS endotoxicity (data not shown). Moreover, the process was microscopically monitored to verify the typical cigar-like microstructures of the cochleates.

The *in vitro* antifungal activity of AFCo3-AmB against *S. schenckii* ATCC 16345 yeast was evaluated using CLSI M27-A3, a reference method for broth dilution antifungal susceptibility recommended for yeast fungi [27]. Another method recommended for the mycelial phase is: CLSI M38-A2 [39], but all our studies were performed with the yeast parasitic phase of *S. schenckii*. In this case, the reference protocols M27-A3 was adapted for use with dimorphic fungi by increasing the incubation period to five days in order to compensate for the slow growth of this fungus *in vitro* [40]. In addition, owing to *S. schenckii* being a dimorphic fungi and with no methods sufficiently standardized in these cases, it was evaluated in parallel with the Alamar Blue test, as was previously proposed for the genus Paracoccidiodes [28]. Similar results in the susceptibility to AmB was observed using both methods. AFCo3-AmB exhibited superior antifungal effect in at least two dilutions in comparison with free AmB. This enhanced activity may be attributed to the membrane fusion capability and an increased cell cochleate contact as it was described for other similar structures [38]. In this way, it is hypothesized that cochleate could facilitate the interaction between AmB and fungal ergosterol, thereby increasing the antifungal action. More mechanistic studies are necessary to elucidate how this phenomenon occurs.

The cytotoxicity of AmB observed in mammalian cells, including its hemolytic effect, remains a concern and new AmB formulations must overcome this problem. The mechanism proposed for the toxic effect of AmB on the cell membrane is derived from its interaction with sterols in bilayer membranes, causing pore formation, in turn leading either to its destruction or to the inhibition of membrane repair [41]. In this study, AFCo3-AmB exhibited a reduced cytotoxicity in comparison with free AmB, evaluated in murine peritoneal macrophages and erythrocytes. This effect associated to the improvement of the antifungal activity, is suggesting a favourable efficacy-toxicity balance for AFCo3-AmB. Interestingly, the increase in the viability was also observed in macrophages treated with AFCo3-AmB at concentrations less than or equal to 2 μg/mL of AmB. According to the mechanisms of the MTT assay, the yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH, resulting in intracellular purple formazan [42]. It implies that cells exhibiting elevated viability are developing an active metabolic activity. In this way, an increased production of pro-inflammatory mediators, IL-1, TNF-α and NO, was also observed. Surprisingly, a slight increased viability was also detected in macrophages treated with AmB at concentrations between 2 and 0.5 μg/mL. In this respect, the activity of AmB, inducing the production of pro-inflammatory cytokines and Reactive Oxygen Intermediates (ROIs) have previously been reported, and it was suggested that this immunomodulating effect can work synergistically with its direct antifungal effect [43]. On the other hand, the production of pro-inflammatory mediators correlates well with the cytotoxicity results, since it is

Fig. 4. Levels of pro-inflammatory cytokine IL-1β (A), TNF-α (B) and nitric oxide (C) produced by peritoneal macrophages after exposure with AmB, AFCo3 or AmB-AFCo3 measured by ELISA. AFCo3 represents the correspondent dilutions of empty AFCo3 in relation to AFCo3-AmB (*$p < 0.05$ or **$p < 0.01$ compared with the AmB group).

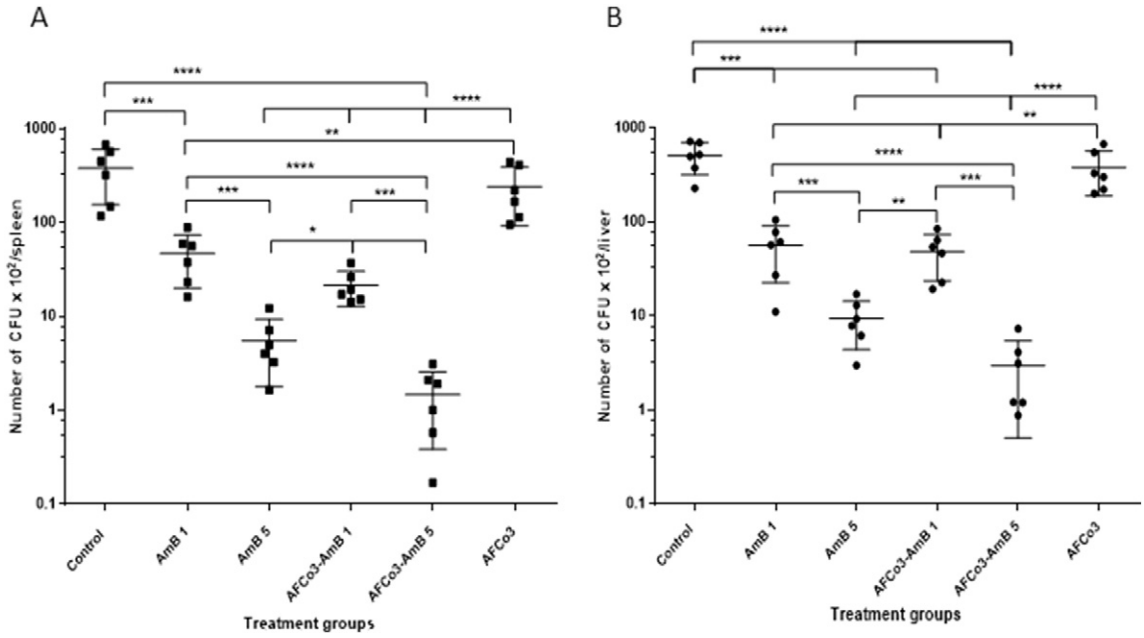

Fig. 5. Spleen and liver tissue burden of mice infected with 10^6 CFU of *S. schenckii via* intraperitoneal treatment and mice treated intraperitoneally two days later with either AmB at 1 or 5 mg/kg; AFCo3-AmB at 1 or 5 mg/kg; empty AFCo3 or sterile PBS only (control). The treatment lasted from the 2nd to the 6th day post infection and mice were euthanized at the 7th day. Statistical significance was determined by the *t*-test with correction for multiple comparisons by the Holm–Sidak method. The confidence interval was set at 95% ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$). Results are presented as the mean ± SD of 3 animals from two independent experiments.

possible that a reduction of cell viability in peritoneal macrophages observed at higher concentration of AmB and AFCo3-AmB, may have caused the lower production of cytokines in comparison with empty AFCo3. The observed protective effect of AFCo3 against the AmB-induced hemolysis confirmed its cytoprotective action. In a controlled drug-delivery system like AFCo3, AmB is incorporated into a polymeric network structure in such a way that the drug is slowly released and in addition, the external exposition is reduced. Both mechanisms may explain the reduced cytotoxicity in this context as occurs with other drug delivery systems [41,44].

During the infectious process, *S. schenckii* cells are phagocytized by tissue macrophages. But in spite of the fungicidal mechanisms to kill invading pathogens, *S. schenckii* can survive inside the macrophage, especially in an immunosuppressed host [45,46]. Intracellular fungal survival of *S. schenckii*, supporting a hostile environmental condition, is mediated by different virulence factors, and it appears to be the basis for latency, disseminated disease and resistance to eradication by antifungal agents [47]. Thus, another important aspect of the pharmacological interest for new antifungal formulations is the ability to destroy the invading intracellular yeast. In this study, when the effect of AFCo3-AmB over the phagocytized *S. schenckii* was evaluated, a dose dependent reduction of the intracellular CFU was observed. In the same way, AmB-AFCo3 exhibited a superior intracellular antifungal effect in comparison with free AmB. This effect can be explained by an improvement of the AmB delivery within macrophages mediated by the cochleate and possibly also by a stimulation of these cells through the interaction between Toll-like receptor 4 (TLR4) and LPS contained in the AFCo3 structure [24,25]. In this way, the TLR4 signalling pathway is involved in the antifungal mechanism against *S. schenckii* [13].

Several studies using different cochleates containing AmB in the murine model of candidiasis [19,20] and aspergillosis [48], evidenced their potential use as carriers for antifungal treatment. Here, the ability of AFCo3 modulating the immune response and improving the antifungal action of AmB against *S. schenckii* was also evidenced *in vivo* using a model of systemic fungal infections, previously developed in our lab [9]. The reduced fungal burden in the spleen and liver, with a similar effect to those observed in the group treated with AmB, suggests that AFCo3 cochleates are promising vehicles for AmB delivery. Besides the interaction with biological membranes favouring the drug delivery, it is possible that AFCo3 improves the biodistribution of AmB. Future pharmacokinetic studies should be performed to confirm this hypothesis.

The immunomodulatory effect observed *in vitro* by AFCo3-AmB was confirmed in the study *in vivo*, where a preferential Th1/Th17 cytokine profile of T helper cell responses was observed without an apparent significant impact in the production of IL-4, cytokine characteristic of the Th2 profile. Another important finding was that the early stimulation of IFNγ/IL-17 production by infection was reduced by the treatment with AmB. However, in infected mice treated with AFCo3-AmB an elevated production of these cytokines was detected, suggesting a possible synergistic effect. Previous studies evidenced that the immunomodulatory effect of AFCo3 largely lies in the presence of detoxified LPS in its composition, being able to activate the innate immune response and induce a Th1 profile in this way [24,25]. Here we demonstrated that in addition to a Th1 profile, AFCo3 is also able to stimulate a Th17 response. However, in spite of the importance of the Th1/Th17 response in the immunity against *S. schenckii* [9,34], in this study the strong stimulation of these responses alone was not sufficient to reduce the fungal load in the spleen and liver. Possibly, the short duration of the present investigation did not allow observing the impact of these responses induced by the cochleate. More studies are necessary to evaluate this issue.

AmB is known to induce channel formation across kidney cell membranes, disruption of postendocytic trafficking and direct vasoconstriction. Thus, its therapeutic use is mainly hampered by its nephrotoxicity [52–54]. In this study, increased levels of TP, Alb, Cr and BUN were observed in serum from mice treated with 5 mg/kg of AmB, in comparison with the control (non-treated) as evidence of a nephrotoxic effect [53], while there were no detected evidences of renal dysfunction caused by AFCo3 and AFCo3-AmB. We hypothesized that AmB, loaded in the cochleates is delivered more efficiently into the fungal cells, by improving both the transfer through the cell wall and the interaction with the fungal cell membrane [38]. Moreover, the presence of LPS in the AFCo3 structure can allow targeted delivery of AmB to macrophages, which minimizes undesirable reactions in other tissues and reduces the AmB toxicity. Several toxicological studies performed in AFCo1, another related cochleate, even more complex than AFCo3, revealed that it is potentially safe when it was used by an intranasal

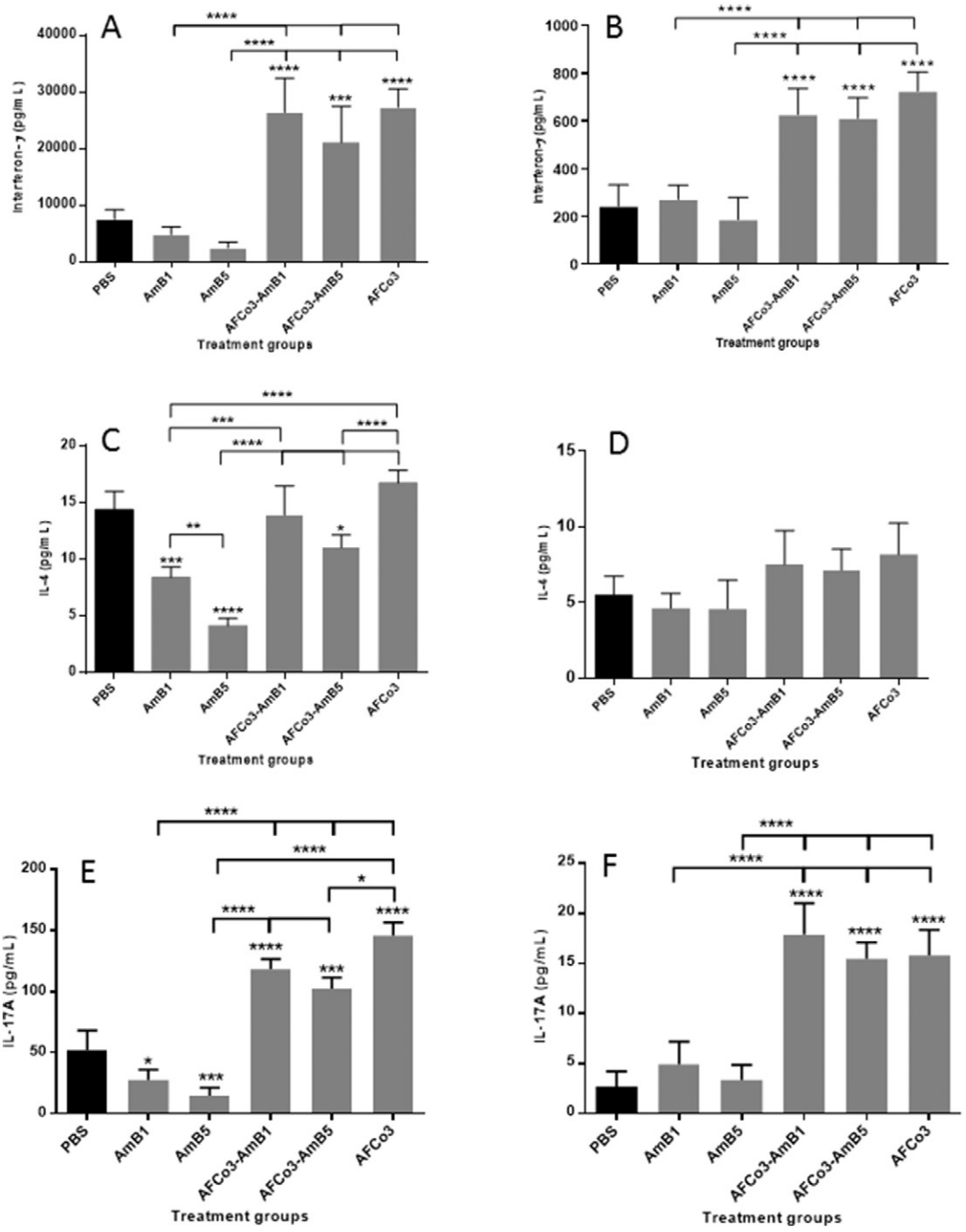

Fig. 6. *Ex vivo* release of Th1, Th2 and Th17 effector cytokines: mice were intraperitoneally inoculated with 10^6 *S. schenckii* yeast cells in PBS (infected: A, C, E) or sterile PBS only (non-non-infected: B, D, F) and treated intraperitoneally two days later with either AmB at 1 or 5 mg/kg; AFCo3-AmB at 1 or 5 mg/kg; empty AFCo3 or sterile PBS only (control). At the 7th day, mice were sacrificed for the removal of the spleen and the resulting splenocytes were cultured for 24 h in the presence of HKss. Cytokines IFN-γ, IL-17A and IL-4 were measured in the supernatant by ELISA, in duplicate, according to the manufacturer's instructions. Statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$). The asterisks over the error bar indicate that the groups differ significantly with respect to the control group. Results are presented as the mean ± SD of 3 animals from two independent experiments.

route as evidenced by the absence of local and systemic signs of toxicity [49–51]. Currently, other toxicological studies in rats are being made to evaluate the safety profile of AFCo3-AmB as a vehicle for drug delivery in laboratory animals.

In summary, this is the first report showing the effect of a cochleate-AmB formulation against *S. schenckii*. The experiments presented in this report revealed that AFCo3-AmB is able to reduce the cytotoxic effect and improve the antifungal *in vitro* activity of free AmB against *S. schenckii*. Moreover, this new formulation exhibited an immunostimulatory effect that can be also involved in the growth reduction of phagocytized yeast by macrophages and in the reduction of fungal load in systemic infection. Thus, the combined effect of AFCo3-AmB, favouring AmB delivery, the immunomodulatory effect and reduced toxicity, can act synergistically to eradicate the fungal infection more effectively than free AmB. Although no definitive conclusions can be reached at this time, all these results suggest a potential for therapeutic application AFCo3-AmB against sporotrichosis. Further studies are necessary to evaluate

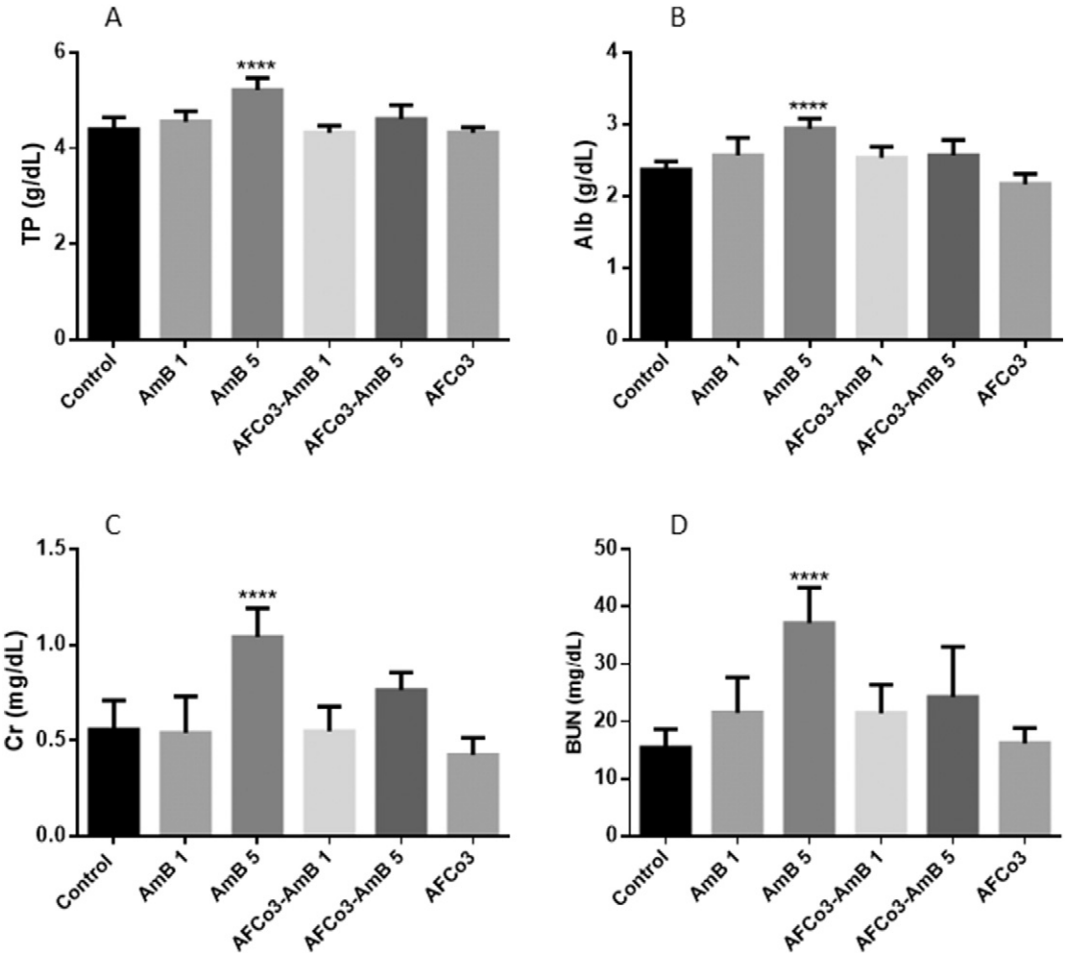

Fig. 7. Blood chemistry measurements. Mice were intraperitoneally inoculated with either AmB at 1 or 5 mg/kg; AFCo3-AmB at 1 or 5 mg/kg; empty AFCo3 or sterile PBS only (control). At the 7th day, blood samples were collected intracardially from the mice and serum were analyzed for total proteins (TP), albumin (Alb), creatinine (Cr) and blood urea nitrogen (BUN). Data are expressed as means ± S.D. The statistical significance of differences between treated and control groups was determined with Dunnett's multiple comparison test. (****$p < 0.0001$).

the post-treatment survival of mice infected with the highest inoculum in long lasting studies using other species of the *S. schenckii* complex to support the results of this first report.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2016.09.008.

Competing interests

The authors declare that they have no competing interests.

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