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Comparative efficacy and toxicity of two vaccine candidates against *Sporothrix schenckii* using either Montanide™ Pet Gel A or aluminum hydroxide adjuvants in mice

Deivys Leandro Portuondo [a], Alexander Batista-Duharte [a], Lucas Souza Ferreira [a], Cleverton Roberto de Andrade [b], Camila Quinello [a], Damiana Téllez-Martínez [a], Maria Luiza de Aguiar Loesch [a], Iracilda Zeppone Carlos [a,*]

[a] São Paulo State University (UNESP), School of Pharmaceutical Sciences, Department of Clinical Analysis, Araraquara, SP, Brazil
[b] São Paulo State University (UNESP), School of Dentistry, Department of Physiology & Pathology, Araraquara, SP, Brazil

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ABSTRACT

Sporotrichosis is an important zoonosis in Brazil and the most frequent subcutaneous mycosis in Latin America, caused by different *Sporothrix* species. Currently, there is no effective vaccine available to prevent this disease. In this study, the efficacy and toxicity of the adjuvant Montanide™ Pet Gel A (PGA) formulated with *S. schenckii* cell wall proteins (ssCWP) was evaluated and compared with that of aluminum hydroxide (AH). Balb/c mice received two subcutaneous doses (1st and 14th days) of either the unadjuvanted or adjuvanted vaccine candidates. On the 21st day, anti-ssCWP antibody levels (ELISA), the phagocytic index, as well as the *ex vivo* release of IFN-γ, IL-4, and IL-17 by splenocytes and IL-12 by peritoneal macrophages were assessed. Cytotoxicity of the vaccine formulations was evaluated *in vitro* and by histopathological analysis of the inoculation site. Both adjuvanted vaccine formulations increased anti-ssCWP IgG, IgG1, IgG2a, and IgG3 levels, although IgG2a levels were higher in response to PGA +CWP100, probably contributing to the increase in *S. schenckii* yeast phagocytosis by macrophages in the opsonophagocytosis assay when using serum from PGA+CWP100-immunized mice. Immunization with AH+CWP100 led to a mixed Th1/Th2/Th17 *ex vivo* cytokine release profile, while PGA+CWP100 stimulated a preferential Th1/Th2 profile. Moreover, PGA+CWP100 was less cytotoxic *in vitro*, caused less local toxicity and led to a similar reduction in fungal load in the liver and spleen of *S. schenckii*- or *S. brasiliensis*-challenged mice as compared with AH+CWP100. These results suggest that PGA may be an effective and safe adjuvant for a future sporotrichosis vaccine.

1. Introduction

Sporotrichosis is an emergent subcutaneous mycosis in tropical and subtropical regions, with isolated cases and outbreaks reported worldwide [1,2]. The disease is caused by different *Sporothrix* species, which are ubiquitous environmental saprophytes that can be isolated from soil and plant debris. In the environment, they can increase their virulence and cause infections to humans and other animals upon traumatic lesions with contaminated materials [3]. Over the last years, the zoonotic transmission of sporotrichosis through the bite or scratch of sick cats and other animals became an important cause of concern, mainly in Brazil [4,5].

Several pathogenic *Sporothrix* species have been described, including *S. brasiliensis*, *S. globosa*, *S. mexicana*, *S. lurie*, and *S. schenckii sensu stricto* [6]. In Brazil, the most frequently involved species in the cat-human zoonotic transmission is *S. brasiliensis* [5]. Sporotrichosis can assume many clinical forms, including fixed cutaneous or lymphocutaneous and disseminated forms, the latter

Abbreviations: ssCWP, *S. schenckii* cell wall protein; AH, aluminum hydroxide; PGA, Montanide™ Pet GeL A; gp70, glycoprotein of 70 kD; NIS, serum from non-immunized mice.

* Corresponding author at: São Paulo State University – UNESP, School of Pharmaceutical Sciences, Department of Clinical Analysis, Rodovia Araraquara-Jaú – km 1, 14800-903 Araraquara, SP, Brazil.

E-mail addresses: deivysleandro@gmail.com (D.L. Portuondo), batistaduhartea@gmail.com (A. Batista-Duharte), gigabreath@hotmail.com (L.S. Ferreira), clevertonroberto72@gmail.com (C.R. de Andrade), caquinello@hotmail.com (C. Quinello), damianatellezm@gmail.com (D. Téllez-Martínez), ma_luizaloesch@hotmail.com (M.L. de Aguiar Loesch), carlosiz@fcfar.unesp.br (I.Z. Carlos).

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of which has been mainly reported in immunocompromised individuals [6,7]. Given the renewed epidemiological importance of sporotrichosis and difficulties associated with the conventional antifungal drugs, different strategies are being investigated for prevention and treatment of this disease [8,9].

Several immune mechanisms have been shown to play a role in resistance against *S. schenckii* [9-17], impelling the use of immunomodulation tools for the management of sporotrichosis. Previously, we studied two aluminum hydroxide (AH)-adsorbed *S. schenckii* cell wall proteins (ssCWP)-based vaccine formulations and demonstrated induction of a strong specific immune response in vaccinated mice [18]. Furthermore, sera from those mice conferred protection against *S. schenckii* infection after passive transference in non-vaccinated and non-infected mice. In that study, a local inflammatory reaction at the inoculation site of immunized mice was observed.

Local reactions and tumors at the inoculation site have been associated with alum-adjuvanted vaccines in genetically predisposed cats, ferrets, and dogs [19–22]. Montanide™ Pet Gel A (PGA) is a ready-to-disperse polymeric adjuvant designed to improve the safety and efficacy of vaccines for companion animals, especially in cats [23].

Considering the high prevalence of animal-to-human transmission and the predominance of the highly virulent species *S. brasiliensis* as the etiological agent in cats [5,24], the objective of this work was to compare PGA and AH regarding their safety and effectiveness in inducing a protective immune response against this species when formulated with ssCWP. This study will help us choose a better adjuvant for a future anti-*Sporothrix* veterinary vaccine.

2. Materials and methods

2.1. Animals

Male 5–7 week-old specific-pathogen-free (SPF) Balb/c mice were purchased from the Multidisciplinary Center for Biological Research (CEMIB), University of Campinas, São Paulo, Brazil. Five mice per group were housed in microisolator cages and maintained under SPF conditions. This work was approved by the Institutional Ethics Committee for Animal Use in Research (Protocol CEUA/FCF/CAR 30/2012) and was in accordance with the National Institutes of Health Animal Care guidelines.

2.2. Microorganism and growth conditions

Sporothrix schenckii ATCC 16345 sensu stricto, isolated from a patient with diffuse lung infection (Baltimore, USA), and *S. brasiliensis* 250, isolated from a feline sporotrichosis case in Brazil, were kindly provided by the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Mycelial-to-yeast conversion of both isolates was performed in 100 ml of brain–heart infusion broth (Difco) for 7 days at 37°C with continuous agitation at 150 rpm [15]. After that, an aliquot containing 2×10^7 or 2×10^5 yeasts from *S. schenckii* or *S. brasiliensis*, respectively, was transferred to a fresh medium and cultured for 5 more days at the same conditions.

2.3. Extraction of ssCWP

ssCWP extraction was performed as previously described [18].

2.4. Adjuvants and vaccine formulation

Aluminum hydroxide (AH) gel adjuvant was bought from Invivogen (EUA); Montanide™ Pet Gel A, an adjuvant composed of a highly stable dispersion of microspherical particles of sodium polyacrylate in water, was kindly provided by SEPPIC (France). The AH-ssCWPs formulation was prepared by mixing 0.1 mg of ssCWPs with an amount of AH equivalent to 0.1 mg of Al^{3+} (AH+CWP100) in a total volume of 100 μl and an adsorption time of 40 min [18]. The same antigen amount was formulated with 5% PGA (PGA+CWP100), according to the manufacturer's instructions. In mice, the 0.1 mg dose of Al^{3+} corresponds approximately to the maximum approved dose for human vaccines [25].

2.5. In vitro cytotoxicity

Mice were intraperitoneally (i.p) injected with 3 ml of a 3% sodium thioglycollate (Difco) solution 3 days before euthanasia. Peritoneal cells were harvested, plated in 96-well plates (5×10^5 cells/ml) in complete RPMI-1640 medium (cRPMI) and incubated overnight. Non-adherent cells were removed and macrophages were incubated with 100 μL of cRPMI containing either CWP100, AH+CWP100, PGA+CWP100, AH (100 μg of Al^{3+}), or 5% PGA at 37°C in a 5% CO_2 atmosphere. cRPMI or NaOH 0.1 N were used as negative or positive controls, respectively. After 20 h, cytotoxicity was determined using the MTT assay [26].

2.6. Immunization schedule

Balb/c mice (n = 5) received two subcutaneous (s.c) injections (0.1 ml) on the back of the neck on days 0 and 14 with CWP100, AH+CWP100, PGA+CWP100, or PBS alone as negative control. Serum obtained 1 week after the second immunization was heat-inactivated at 56°C for 30 min, aliquoted and stored at −20°C for further use.

2.7. Serum antibody titration and opsonophagocytosis assay

Both assays were conducted as described by Portuondo et al. [18]. Shortly, serum levels of anti-ssCWP IgG, IgG1, IgG2a and IgG3 were measured by ELISA. For the opsonophagocytosis assay, thioglycollate-elicited peritoneal macrophages were co-cultured in a 1:4 ratio with opsonized or non-opsonized *S. schenckii* yeasts in LabTek® slides (Nunc) for 2 h at 37°C. After that, the slides were stained with Giemsa and phagocytic activity was expressed using the phagocytic index (mean number of phagocytosed yeasts per macrophage).

2.8. Flow cytometry analysis

Sporothrix brasiliensis yeasts obtained as described on item 2.2 and then washed thrice with PBS at 4°C, were incubated at 37°C for 1 h with anti-ssCWP sera (1/20) from AH+CWP100- or PGA+CWP100-immunized or non-immunized mice. After that, the yeasts were washed with PBS and incubated with FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) (1/50) for 1 h at room temperature (RT). After washing, the binding of serum antibodies to the surface of the yeasts was determined using a flow cytometer (BD Accuri C6, BD Biosciences).

2.9. Cytokine induction assay

Thioglycollate-elicited peritoneal macrophages and total splenocytes were harvested from immunized mice and cultured in cRPMI for 24 h at 37°C and 5% CO_2 in the presence of ssCWPs. Final concentrations were 2.5×10^6 cells/ml and 40 μg of ssCWPs/ml in cRPMI; concanavalin A (0.25 μg/ml) or *Escherichia coli* O111B lipopolysaccharide (10 μg/ml) were used as positive controls for macrophages or splenocytes, respectively; cRPMI alone was used as negative control. The following supernatant-accumulated

cytokines were measured by ELISA (eBioscience) according to the manufacturer's instructions: IL-12, IFN-γ, IL-4, and IL-17.

2.10. Protection assay

Mice were immunized as described previously. Seven days after the second immunization, mice were i.p. challenged with 10^6 *S. schenckii* ATCC 16345 or *S. brasiliensis* 250 yeasts in 200 μl of PBS. Protection was assessed by determining the number of colony forming units (CFUs) recovered from the spleen and liver of mice on day 5 post-infection when the peak of systemic fungal burden is expected [15,18].

2.11. Gross necropsy and histopathology

The subcutaneous tissue and muscles where the vaccine formulation was inoculated were trimmed and preserved in 10% formalin according to the standardized methodology. Later, the paraffin embedded tissues were sectioned and stained with hematoxylin and eosin and examined using a light microscope. Relevant gross lesions were microscopically examined in all animals.

2.12. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-test using Graph Pad Prism 5. In this study, a p value of <0.05 was considered significant. The results are expressed as the mean ± SD.

3. Results

3.1. In vitro cytotoxicity

Cytotoxicity varied greatly between formulations, as follows, from most to least cytotoxic: NaOH (88.6% ± 1.7%), AH (82.0% ± 2.1%), PGA (64.0% ± 6.1%), AH+CWP100 (56.6% ± 1.8%), PGA+CWP100 (40.2% ± 2.1%), and CWP100 (20.4% ± 3.6%) (Fig. 1). There was no significant difference between AH and NaOH, or AH +CWP100 and PGA alone. This result shows that PGA and AH-adsorbed antigens seem to inhibit cytotoxicity, as both AH+CWP100 and PGA+CWP100 exerted significantly less cytotoxicity than the respective adjuvants alone. Furthermore, PGA was significantly less cytotoxic than AH, alone or in formulation with the antigen, suggesting PGA is safer than AH, at least in the context of this particular vaccine formulation.

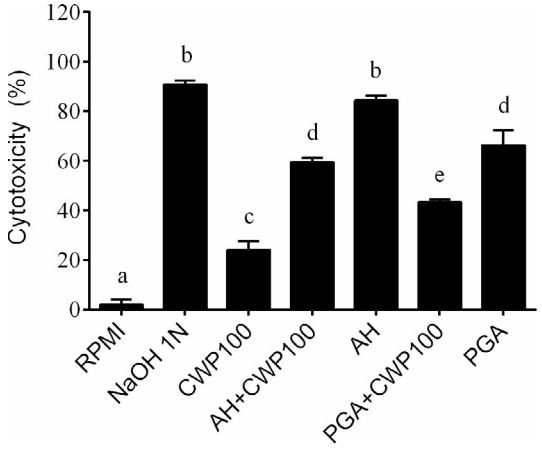

Fig. 1. *In vitro* cytotoxicity of the different vaccine formulations. Mice were intraperitoneally injected with 3 ml of a 3% sodium thioglycollate aqueous solution. Three days post-inoculation, mice were euthanized and peritoneal exudate cells were harvested. The cells were incubated with cRPMI (negative control), 1 N NaOH (positive control), PBS, CWP100, AH (100 μg of Al^{3+}), AH+CWP100, PGA+CWP100, or 5% PGA and 20 h later cell viability was determined by the MTT assay. The results are presented as the mean ± SD of three independent experiments and statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences ($p < 0.05$) between treatments. CWP (cell wall proteins), AH (aluminum hydroxide), PGA (Montanide™ Pet Gel A).

3.2. Antibody response and phagocytosis

The PGA- or AH-adjuvanted formulations induced higher anti-ssCWP IgG, IgG1, IgG2a, and IgG3 antibody levels as compared to CWP100 alone, whereas PGA+CWP100 induced significantly higher IgG2a levels than AH+CWP100 (Fig. 2A–D). In another set of experiments, we found that phagocytic activity was almost absent

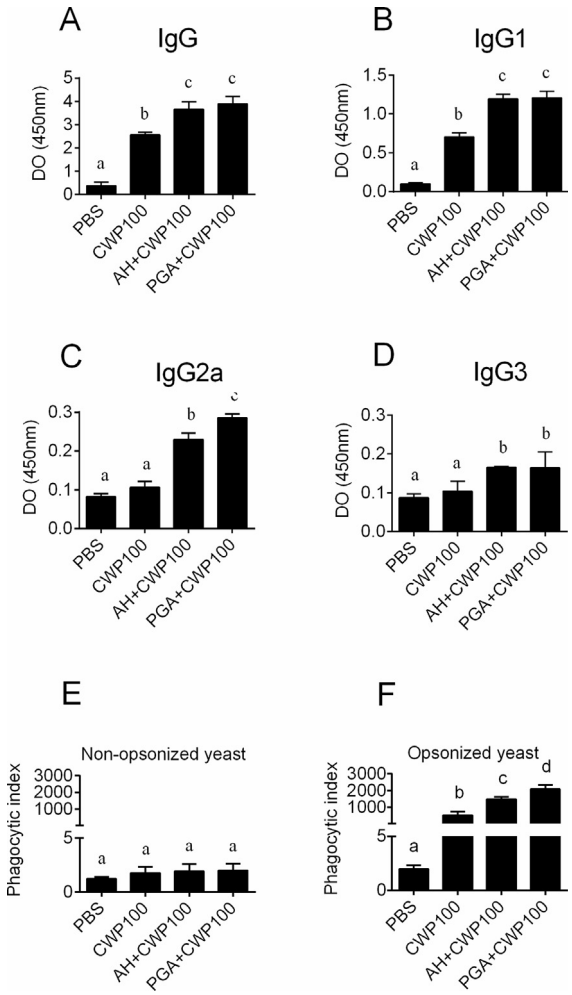

Fig. 2. Immunization with both the PGA- and AH-adjuvanted formulations markedly enhanced antibody response to ssCWPs which enhanced the phagocytic killing of *S. schenckii* yeasts. Balb/c mice (n = 5) were immunized (s.c.) twice with CWP100, AH+CWP100, PGA+CWP100, or PBS as negative control. Serum collected 7 days after the second immunization was used to determine ssCWP-specific IgG (A), IgG1 (B), IgG2a (C), and IgG3 (D) levels by ELISA or as opsonizing serum in the opsonophagocytosis assay. Thioglycollate-elicited peritoneal macrophages harvested from immunized mice were incubated with opsonized or PBS-treated *S. schenckii* yeasts (at a 1:4 macrophage to yeast ratio). (E and F) Phagocytic index (mean number of phagocytosed yeasts per macrophage) for non-opsonized or opsonized yeasts, as indicated. The results are presented as the mean ± SD of 5 mice from one of three independent experiments and statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences ($p < 0.05$) between treatments.

in the presence of non-opsonized yeasts across all groups (Fig. 2E), but it was notably enhanced when yeasts were opsonized with serum from CWP100-, AH+CWP100-, or PGA+CWP100-vaccinated mice, but especially the latter (Fig. 2F).

3.3. Flow cytometry

Flow cytometry analysis revealed the presence of *S. brasiliensis* cross-reactive antibodies in the anti-ssCWP serum from AH+CWP100- or PGA+CWP100- vaccinated mice (Fig. 3).

3.4. Ex vivo cytokine induction

Immunization with AH+CWP100 or PGA+CWP100 induced greater *ex vivo* release of IL-12 by macrophages and of INF-γ by splenocytes (Fig. 4A and B) compared to CWP100 alone, although IL-12 levels were higher in response to PGA+CWP100 than AH+CWP100 (Fig. 4A). In contrast, immunization with AH+CWP100 led to greater *ex vivo* release of IL-4 and IL-17 by splenocytes compared with the other formulations (Fig. 4C and D). These results suggest PGA+CWP100 induces a Th1-biased cytokine profile and confirm the Th1/Th2/Th17 balanced profile in AH+CWP100-vaccinated mice previously described by Portuondo et al. [18].

3.5. In vivo protection assay

Five days after challenge with the fungi, the number of CFUs in spleen and liver was lower in AH+CWP100- and PGA+CWP100-immunized mice compared to control mice (Fig. 5). No statistical difference was found between AH+CWP100- and PGA+CWP100-immunized mice. Both ssCWP-based vaccine candidates provided protection against *S. schenckii*- or *S. brasiliensis*-challenge in mice.

3.6. Histopathological assessment

As expected, CWP100-immnunized mice showed no macroscopic lesions at the injection site (Fig. 6A). Nevertheless, microscopic evaluation showed a slight inflammatory response with neutrophils, some striated muscle fiber degeneration, and subcutaneous edema (Fig. 6B–C). AH+CWP100 injection, on the other hand, produced palpable nodules at the injection site in all animals, varying in size between 1 and 5 mm (Fig. 6D) and microscopically characterized by an intense granulomatous reaction, abundant macrophages, neutrophils, and fibroblasts, as well as a loss of continuity in striated muscle fibers (Fig. 6E–F). In contrast, PGA+CWP100-injected animals showed no macroscopically visible nodules (Fig. 6G) but had a moderate inflammatory infiltrate with abundant macrophages and neutrophils upon microscopic evaluation (Fig. 6H and I).

4. Discussion

Robust adjuvant action is often associated with toxicity, which is influenced by direct interactions of the adjuvant/antigen formulation with the tissue at the inoculation site, causing an inflammatory reaction associated with systemic effects [21,27-29]. AH is a universally accepted adjuvant for human and veterinary vaccines and several experimental antifungal vaccines use AH in their composition [30]. However, growing concerns regarding efficacy and toxicity has stimulated the search for alternative adjuvants [27]. Here, AH was chosen as a "reference adjuvant" for comparison with PGA, a new polymeric adjuvant reported to have high safety and efficacy profiles, especially for companion animals [25,31]. We previously demonstrated that an AH-based vaccine formulation using purified ssCWPs was immunogenic and able to induce protective antibodies against this *S. schenckii* in mice [18]. However, most epidemic outbreaks in Brazil are associated with the zoonotic transmission of *S. brasiliensis* [5]. In light of this, we aimed to evaluate the comparative efficacy of two vaccine candidates in a

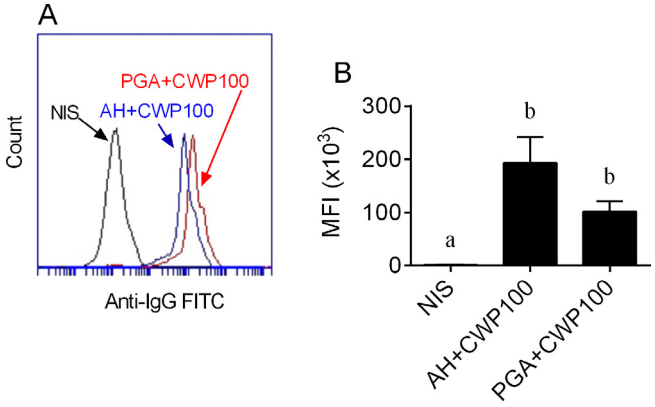

Fig. 3. Flow cytometry analysis showing the reactivity of the anti-ssCWP serum with *S. brasiliensis* yeasts. A *S. brasiliensis* Ss250 yeast suspension was previously incubated with anti-ssCWP serum obtained from Balb/c mice (n = 5) immunized (s.c.) twice with AH+CWP100 or PGA+CWP100, or with serum from non-immunized mice (NIS). After washing, the cells were exposed to a FITC-conjugated rabbit anti-mouse IgG and analyzed using a flow cytometer. (A) Representative histogram from one independent experiment. (B) Median fluorescence intensity (MFI). The results are presented as the mean ± SD of three independent experiments and statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences ($p < 0.05$) between treatments. CWP (cell wall proteins), AH (aluminum hydroxide), PGA (Montanide™ Pet Gel A).

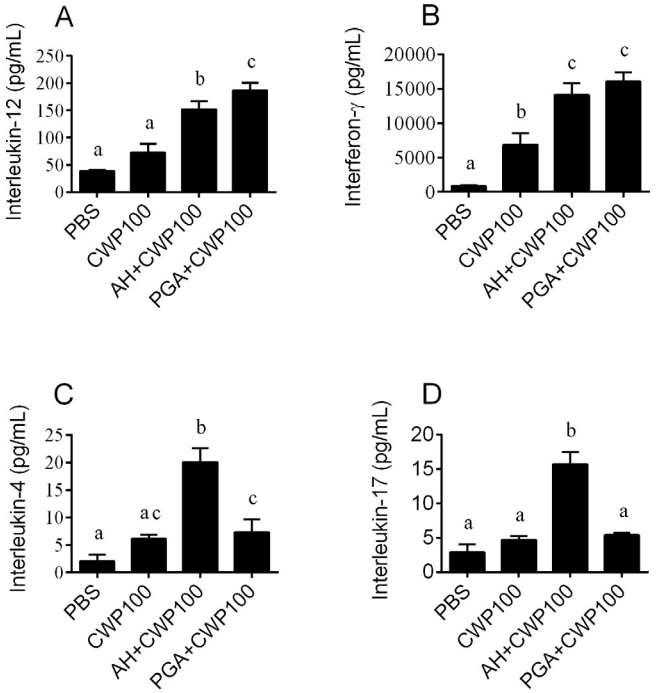

Fig. 4. Cytokine profile induced by vaccination with AH+CWP100 or PGA+CWP100. *Ex vivo* release of INF-γ (A), IL-12 (B), IL-4 (C), or IL-17 (D) by ssCWP-stimulated splenocytes from vaccinated Balb/c mice (n = 5). The results are presented as the mean ± SD of 5 mice from one of three independent experiments and statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences ($p < 0.05$) between each treatments. CWP (cell wall proteins), AH (aluminum hydroxide), PGA (Montanide™ Pet Gel A).

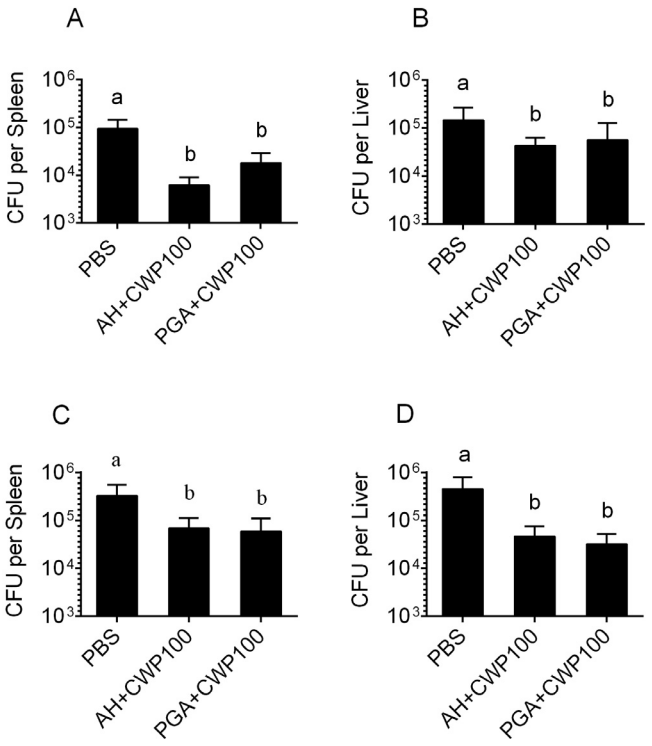

Fig. 5. Vaccination with AH+CWP100 or PGA+CWP100 was able to reduce the fungal burden. Balb/c mice (n = 5) were immunized (s.c.) twice with the indicated formulations. One week after the boost, mice were i.p. challenged with *S. schenckii* ATCC 16345 or *S. brasiliensis* Ss250 and five days after infection the protection was assessed by the number of CFUs recovered from the spleen and liver of mice. Fungal burden in the spleen (A) and liver (B) of *S. schenckii*-challenged mice. Fungal burden in the spleen (C) and liver (D) of *S. brasiliensis*-challenged mice. The results are presented as the mean ± SD of 5 mice from one of three independent experiments and statistical significance was determined by one-way ANOVA using Tukey's multiple comparisons test and a 95% confidence interval. Different letters represent significant differences ($p < 0.05$) between treatments. CWP (cell wall proteins), AH (aluminum hydroxide), PGA (Montanide™ Pet Gel A).

model of experimental infection using both *S. schenckii* and *S. brasiliensis*.

A comparative evaluation of the *in vitro* cytotoxicity of AH and PGA, alone or formulated with the antigen was performed. Despite the high cytotoxicity exhibited by the adjuvants alone, when they were formulated with the antigen, occurred a significant reduction of toxicity, suggesting a protective effect against membrane damage owing to the presence of the antigen in the final formulation. A similar effect was observed on guinea pig erythrocytes when AH and calcium phosphate were pre-adsorbed with ovalbumin, drastically reducing the hemolytic effect exerted by both adjuvants alone [32], suggesting the antigen dose could be optimized to generate maximum efficacy and minimum toxicity in a given vaccine formulation. Our *in vitro* results matched the *in vivo* toxicity detected at the inoculation site. As expected, the AH formulation caused a granulomatous inflammatory response in the subcutaneous tissue while the PGA formulation caused only a mild local inflammatory response. Deville et al. [22] reported similar results in a comparative study between both adjuvants using different animal models. Together with the presence of abundant fibroblasts in the subcutaneous tissue of PGA+CWP100-immunized mice, these results suggest PGA formulations could be safer due to a faster recovery of the subcutaneous tissue. In another recent study using different adjuvants, the existence of a high correlation in the magnitude of the direct tisular irritation detected in the HET-CAM (hen's egg test on chorioallantoic membrane) model and *in vivo* local toxicity was demonstrated [33]. Our *in vitro* cytotoxicity assay using murine peritoneal macrophages showed a similar correlation with the *in vivo* toxicity at the inoculation site for both AH and PGA.

Several *S. schenckii* cell wall antigens are immunogenic. Nascimento et al. [34] reported that *S. schenckii*-infected mice develop a humoral response, particularly of the IgG1 and IgG3 subclasses, against gp70, a key immunodominant antigen of the *S. schenckii* cell wall. The relevance of this antigen was confirmed in a study where passive transference of IgG1 monoclonal antibodies (mAb) against gp70 (P6E7), before or during *S. schenckii* infection, caused a significant reduction in the number of CFUs in the spleen and liver of mice [35]. Another study also demonstrated that P6E7 was able to reduce the fungal burden in these organs in mice infected with virulent *Sporothrix* isolates, especially *S. brasiliensis* [36]. Moreover, opsonization of *S. schenckii* yeasts with PE67 led to increased phagocytosis and TNF-α production by macrophages [37]. As a whole, these studies evidence the protective effect of antigen-specific antibodies against both species. In a recent study, Alba-Fierro et al. [38] demonstrated the immunogenicity of an immunodominant 60 kDa glycoprotein from the *S. schenckii* cell wall and suggested its potential in a vaccine candidate.

We previously showed that a serum containing mostly IgG1 and IgG2a antibodies, obtained from mice that had been immunized with a ssCWP-based AH-adjuvanted vaccine candidate, conferred protection upon passive transference in mice [18]. Here, the PGA-adjuvanted formulation induced a higher IgG2a level than the AH formulation. This could explain, at least in part, the higher phagocytic index obtained when *S. schenckii* yeasts were opsonized with serum from PGA+CWP100- as compared with AH+CWP100-immunized mice. IgG2a antibodies are very effective in upregulating antibody responses, primarily via Fc-receptors and T cells [39]. Furthermore, the higher IgG2a levels induced by the PGA formulation suggest a Th1-prone response as IgG2a is regarded as a Th1-pattern subclass [40-42]. This was confirmed by the cytokine profile induced by the PGA formulation (i.e., high IL-12 and IFNγ and low IL-4 levels). Maia et al. [43] showed a predominantly Th1 *ex vivo* cytokine release profile in the initial stages of infection and a Th2 predominance in later stages. Aligned with this, our group has previously shown the occurrence of M1 and M2 macrophages in the initial and late phase of the *S. schenckii* infection, respectively, matching this cytokine pattern [44]. M1 and M2 macrophages are stimulated by Th1 and Th2 cytokines, respectively [45].

Surprisingly, in this study the PGA formulation induced lower IL-17 levels than the AH formulation. Despite the recent findings indicating the importance of IL-17 to host resistance against *S. schenckii* [15], it seems the comparatively low induction of IL-17 by vaccination with the PGA formulation did not affect its effectiveness in our model. Some studies have shown that adjuvants from the Montanide™ Gel line exert their immunoadjuvant effect through a similar mechanism to that of AH (i.e., by forming a depot at the injection site that favors antigen presentation and immune response induction). These adjuvants adsorb proteins on their surface, favoring the slow release of antigens, recruitment of inflammatory and antigen-presenting cells and therefore the induction of immune response [22,31,46].

Conclusive evidence of protective immunity induction by the two vaccine candidates was found in the reduction of fungal load in the spleen and liver of vaccinated mice, which was similar for both candidates. Our results suggest that several mechanisms may be involved in the post-vaccinal protection conferred by the PGA formulation, including induction of opsonizing antibodies and an adequate balance of Th1 and Th2 cytokines. Further studies are necessary to ascertain the role played by these mechanisms, as well as others, in the post-vaccinal defense.

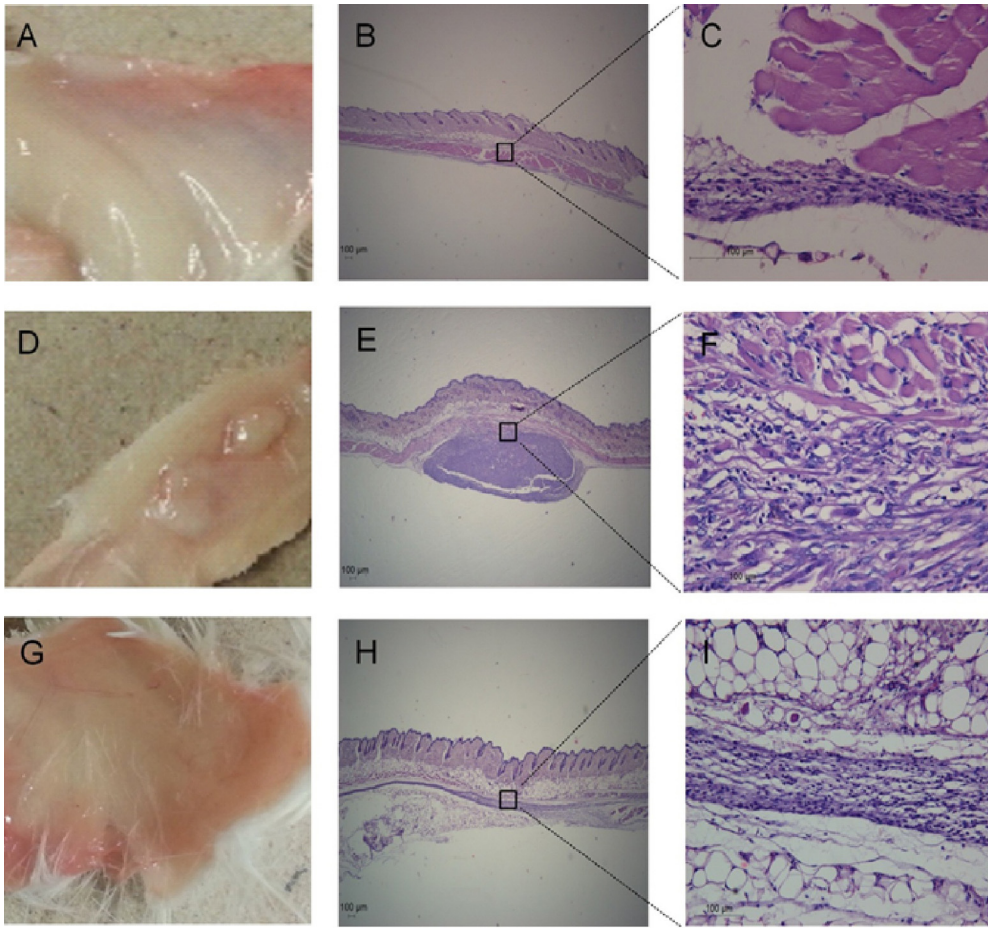

Fig. 6. Histopathological evaluation of the injection site. (A and G) Macroscopic findings: absence of lesions in CWP100- or PGA+CWP100-vaccinated mice, respectively. (B and C) Microscopic findings: slight inflammation with neutrophils, striated muscle fibers and subcutaneous edema in the skin of CWP100-vaccinated mice. (D) Macroscopically visible subcutaneous nodules in AH+CWP100-vaccinated mice, measuring about 1 mm in diameter. (E and F) Foreign body granulomas with abundant macrophages and neutrophils in AH+CWP100-vaccinated mice. (H and I) Moderate inflammatory infiltrate with abundant macrophages, neutrophils and fibroblasts. H&E, 2.5× and 20× magnification. CWP (cell wall proteins), AH (aluminum hydroxide), PGA (Montanide™ Pet Gel A).

Interestingly, an antigen from the low virulent *S. schenckii* ATCC 16345 strain [47] was able to confer protection against *S. schenckii* and *S. brasiliensis*, evidencing the existence of conserved immunodominant antigens between these species. Such cross-reactivity could prove beneficial for the simultaneous protection against many *Sporothrix* spp., besides offering useful information for the identification of conserved immunodominant antigens that could aid the development of a single multi-antigenic vaccine against *Sporothrix* infection. Moreover, using an antigen from a low virulent species contributes to reduce the risk of a hazardous contamination during the manufacturing process.

In conclusion, this study showed that PGA is able to confer the same level of protection as AH with the benefit of only minimal local reactions, making it a valuable alternative for a future anti-*Sporothrix* veterinary vaccine. Furthermore, the *S. brasiliensis* cross-reactivity could be useful for the multispecies immunoprophylaxis within the *Sporothrix* genus. Additional studies are needed to evaluate the effectiveness of this formulation in preventing infections caused by other *Sporothrix* species.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.05.04.

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