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Protective effect of fungal extracellular vesicles against murine candidiasis.

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Abstract

Extracellular vesicles (EVs) are lipid bilayered compartments released by virtually all living cells, including fungi. Among the diverse molecules carried by fungal EVs, a number of immunogens, virulence factors and regulators have been characterized. Within EVs, these components could potentially impact disease outcomes by interacting with the host. From this perspective, we previously demonstrated that EVs from *C. albicans* could be taken up by and activate macrophages and dendritic cells to produce cytokines and express costimulatory molecules. Moreover, pre-treatment of *Galleria mellonella* larvae with fungal EVs protected the insects against a subsequent lethal infection with *C. albicans* yeasts. These data indicate that *C. albicans* EVs are multi-antigenic compartments that activate the innate immune system and could be exploited as vaccine formulations. Here we investigated whether immunization with *C. albicans* EVs induces a

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AUTHOR CONTRIBUTION STATEMENT

L.N. conceived the presented idea. G.V. and L.H. carried out the experiments. F.C.G.R. characterized the vesicles. L.N., A.J.G., M.L.R, A.V., A.R. and J.D.N contributed to the interpretation of results. G.V. and L.N. wrote the manuscript with input from all authors.

CONFLICTS OF INTEREST

The authors state that there are no conflicts of interests.

protective effect against murine candidiasis in immunosuppressed mice. Total and fungal antigen-specific serum IgG antibodies increased by 21 days after immunization, confirming the efficacy of the protocol. Vaccination decreased fungal burden in the liver, spleen and kidney of mice challenged with *C. albicans.* Splenic levels of cytokines indicated a lower inflammatory response in mice immunized with EVs when compared with EVs+Freund's adjuvant (ADJ). Higher levels of IL-12p70, TNFα and IFNγ were detected in mice vaccinated with EVs+ADJ, while IL-12p70, TGFβ, IL-4 and IL-10 were increased when no adjuvants were added. Full protection of lethally challenged mice was observed when EVs were administered, regardless the presence of adjuvant. Physical properties of the EVs were also investigated and EVs produced by *C. albicans* were relatively stable after storage at 4, −20 or −80 °C, keeping their ability to activate dendritic cells and to protect *G. mellonella* against a lethal candidiasis. Our data suggest that fungal EVs could be a safe source of antigens to be exploited in vaccine formulations.

Keywords

Extracellular vesicles; *Candida albicans*; vaccines; fungal pathogenesis

INTRODUCTION

Candida albicans is regularly found as part of the human microbiota, colonizing the oral cavity, skin, and the gastrointestinal and genitourinary tracts (Iliev and Leonardi, 2017). Nonetheless, it is also one of the major opportunistic fungal pathogens, causing superficial cutaneous-mucosa (oral and vaginal) and life threatening disseminated infections (Kim and Sudbery, 2011; Gow et al., 2012). This species is the most frequent pathogen causing recurrent vulvovaginal candidiasis (RVVC), affecting over 370 million woman during their lifetime (Denning et al., 2018). In addition, *C. albicans* causes a systemic disease in immunocompromised patients, the so-called invasive candidiasis (Pappas et al., 2018a). Oral and vulvovaginal candidiasis (VVC) in patients that are not critically ill are usually treated with azole derivatives, such as fluconazole (Sobel, 2016). However, echinocandins recently became the initial choice for treatment of RVVC and systemic candidiasis (Cornely et al., 2012b; Pappas et al., 2016b; Bassetti et al., 2018b). Amphotericin B is also recommended to combat systemic candidiasis, but only when azoles and echinocandins are limited by intolerance, resistance or unavailability (Cornely et al., 2012a; Pappas et al., 2016a). Despite the recent changes and advances in treatment, systemic candidiasis remains associated with high mortality rates (Pappas et al., 2018b; Bassetti et al., 2018a). Moreover, there are reports of increasing numbers of *C. albicans* strains resistant to current antifungal drugs (Fisher et al., 2018; Pappas et al., 2018b). Thus, the development of alternative therapeutic strategies and prophylactic tools is imperative.

Considering that systemic candidiasis is usually associated with altered immune status, such as neutrophil or $CD4^+$ T cells immunodeficiencies, adjunctive therapies that stimulate effector functions in the immune response are potential alternatives (Cassone and Rappuoli, 2010; Cassone, 2013; Kullberg BJ, van de Veerdonk F, 2014; Scriven et al., 2017). This stimulation should be specially effective when the pre-existing immunity is modified or strongly reduced by immunosuppressing conditions (Cassone, 2013). In this context, live

attenuated strains, cytoplasm and cell wall extracts as well as purified proteins and conjugated polysaccharides have been explored in vaccine formulations to control disseminated candidiasis in murine models (Vilanova et al., 2004a; Thomas et al., 2006b; Wu et al., 2007; Raska et al., 2008b; Saville et al., 2009; Li et al., 2011a; Ahmad et al., 2012; De Bernardis et al., 2012; Sui et al., 2017a). Although attenuated strains have provided full protection (Saville et al., 2009) they are particularly risky under conditions of limited immune response. Immunization with cell wall (Thomas et al., 2006a) and cytoplasmic (Ahmad et al., 2012) fungal extracts are protective against murine candidiasis, but the methods of extraction and the batch-to-batch differences are problems that have yet to be circumvented. In addition, it is likely that the methods used for antigen extraction from the cell wall promote changes the native structure of the immunogens. For instance, native cryptococcal glucuronoxylomannan is biologically different from the detergent-extracted polysaccharide (Frases et al., 2008). A number of univalent vaccines, formulations carrying a single antigen, have been investigated in mice models and two of them tested in human clinical trials (Vilanova et al., 2004b; Raska et al., 2008a; Li et al., 2011b; De Bernardis et al., 2012; Sui et al., 2017b). However, according to Cassone (Cassone and Rappuoli, 2010; Cassone, 2013), the use of univalent formulations would be able to induce protection limited to specific body niches sites. Indeed, the use of univalent vaccine formulations seems to protect mice and humans in VVC and RVVC, but not against disseminated candidiasis (Cassone, 2013). Thus, the development of a safe and well-designed multivalent vaccine formulation could promote protection to different targets and against disseminated candidiasis. The major limitations include the elevated cost of development and the ability to determine the perfect combination of antigens.

Recent studies suggest that fungal extracellular vesicles (EVs) may represent a new alternative for the development of multivalent vaccine formulations (Rodrigues et al., 2014; Joffe et al., 2016; Nimrichter et al., 2016; Rizzo et al., 2017; Colombo et al., 2019). Fungal EVs were isolated in 2007 from the culture supernatant of *Cryptococcus neoformans* (Rodrigues et al., 2007a). Since then, fungal EVs have been characterized in several fungal species including *C. albicans, Candida glabrata, Paracoccidioides brasiliensis, Sporothrix brasiliensis, Cryptococcus gattii, Histoplasma capsulatum, Saccharomyces cerevisiae, Pichia fermentans, Malassezia sympodialis, Alternaria infectoria* and *Aspergillus fumigatus* (Albuquerque, 2004; Oliveira et al., 2010b; Vallejo et al., 2011; Silva et al., 2014; Vargas et al., 2015; Rayner et al., 2017; Bielska et al., 2018; Ikeda et al., 2018; Leone et al., 2018; Souza et al., 2019b). As multi-antigenic compartments, fungal EVs carry a number of native structures such as proteins, pigments, polysaccharides, lipids and nucleic acids (Rodrigues et al., 2007a; Rodrigues et al., 2008; Eisenman et al., 2009; Vallejo et al., 2011; Vargas et al., 2015; Da Silva et al., 2015; Nimrichter et al., 2016). Some of these components are conserved among the different species, but others are species-specific (Nimrichter et al., 2016). EVs isolated from pathogens contain a diverse array of virulence factors and regulators as well as highly immunogenic components that could directly contribute to disease development. *In vitro,* these EVs are able to stimulate macrophages and dendritic cells (Vargas et al., 2015; Da Silva et al., 2016; Zamith-Miranda et al., 2018). They regulate cytokine production in phagocytes, macrophage polarization and the expression of co-stimulatory molecules in dendritic cells (DCs) (Vargas et al., 2015). In addition, pre-

treatment of *Galleria mellonella* larvae with fungal EVs stimulated a protective response against a lethal challenge with *C. albicans* or *C. neoformans* (Vargas et al., 2015; Colombo et al., 2019). In other models, the exposure of host cells to fungal EVs have been also associated with disease development, as demonstrated for *C. neoformans* and *S. brasiliensis* (Huang et al., 2012; Ikeda et al., 2018). In addition, fungal EVs seem to participate in the development of antifungal resistance and as a messenger compartment for virulence transference (Bielska et al., 2018; Mitchell et al., 2018).

Based on the composition and biological activities of *C. albicans* EVs we investigated whether these compartments could be used as a multivalent antigenic vaccine formulation in a lethal murine candidiasis model. We initially developed an intraperitoneal prime-boost immunization murine model using *C. albicans* EVs and then evaluated total and specific serum immunoglobulin levels, fungal burden in different tissues, cytokine production in the spleen and mice survival during a lethal challenge with yeasts of *C. albicans.* Then we tested the stability and morphological properties of fungal EVs stored at low temperatures using dynamic light scattering and transmission electron microscopy. Stored EVs were tested according to their ability to activate murine bone-marrow derived DCs and protect *G. mellonella* larvae challenged with a lethal inoculum of *C. albicans* yeasts. Our results demonstrated that fungal EVs are stable formulations with the potential to combat candidiasis.

MATERIAL AND METHODS

Culture of fungal cells.

C. albicans strain 11 is a clinical bloodstream isolated from a 46-year-old male patient, kindly provided by Dr. Marcos Dornelas (Laboratory of Microbiology and Mycology, State Institute of Hematology Arthur de Siqueira Cavalcanti, HemoRio). The strain was stored in solid Sabouraud/glycerol agar medium and maintained at −80 °C. Yeast cells were cultured in liquid Sabouraud for 48 hours at 30 °C under agitation (150 rpm).

Preparation of fungal EVs (Vargas et al., 2015).

Fungal EVs were isolated from *C. albicans* culture supernatants. Yeasts were inoculated in a 100 mL Erlenmeyer flask containing 20 mL of liquid Sabouraud medium and cultured under agitation (150 rpm) at room temperature for 48 hours. The pre-inoculum was then transferred to a 1000 ml Erlenmeyer flask containing 400 ml of liquid Sabouraud medium and cultivated under the same conditions. The yeasts were separated from the culture supernatant according to the protocol previously described in our laboratory (Vargas et al., 2015). The whole preparation was developed at 4 °C. Briefly, the culture was centrifuged at 4,000 x *g* for 15 minutes. The supernatants were collected and further centrifuged at 15,000 x *g* for 15 minutes to remove cell debris. Residual cells and debris were removed after a step of filtration using a 0.8 μm membrane filter (Merck Millipore). The cell-free supernatant was concentrated about 25-fold using an Amicon ultrafiltration system (100 kDa membrane). The concentrated supernatant was then centrifuged at 100,000 x *g* for 1 hour. The pellet was washed twice with 0.1 M phosphate-buffered saline (PBS) pH 7.4. Fungal EVs were suspended in PBS and aliquots were plated onto Sabouraud agar plates to confirm the

absence of any contaminant, confirmed by no colony observation. Quantification of EVs was developed using the quantitative Amplex Red Sterol Assay Kit (Invitrogen) and the BCA Protein Assay Kit (ThermoFisher). Quality control of EVs preparation was performed by dynamic light scattering (DLS) as described below (Vargas et al., 2015).

Detection of antibody classes.

Serum pool of five animals was used for antibody detection. For IgM and global IgG analysis, goat anti-mouse IgM or IgG (SouthernBiotech, AL), 1 μg/mL diluted in PBS, were pre-immobilized to half-area-high binding 96 well ELISA plates, following incubation at 4 °C overnight. Afterwards, the plates were subjected to four washes with PBS and blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour and 30 minutes at room temperature. BSA was removed and the plates were washed with PBS, serum samples were diluted 1:100 in PBS-1% BSA and serially three-fold and five-fold diluted to determine the concentration of IgM and IgG, respectively. IgM and IgG (1 μg/mL) diluted 1: 3 and 1: 5, respectively, were used as the standard curve. The samples were incubated at 4 °C overnight. Plates were washed four times with PBS and incubated with 50 μL of goat anti-mouse IgM (1:4000) and IgG (1:8000) (SouthernBiotech, AL) conjugated to peroxidase (HRP) diluted in PBS 1% BSA and incubated at room temperature for 1 h 30 min. At the end, the plates were again subjected to four washes with PBS and then developed with TMB (3,3′, 5,5;-tetramethylbenzidine) (Thermo Fisher); the reaction was stopped with 50 μL of 3 N HC1 and the reading performed on a 450 and 650 nm filter microplate reader (iMark, BioRad, US).

Protein extraction.

To extract proteins from *C. albicans* an adaptation of the method used by Dojnov and colleagues was used (Dojnov et al., 2007). Briefly, *C. albicans* yeasts (10^8 cells) were suspended in 1 mL of lysis buffer (0.06 M Tris HC1, pH 6, 10% glycerol, 5% 2-mercaptoethanol) and the suspension was submitted to 6 cycles of 1 min in ultrasonic bath (22 KHZ) alternating with 30 sec pauses. Suspensions were centrifuged at 5,000x*g* for 15 min and the supernatants collected. The cells were suspended in the same lysis buffer and transferred to 2 mL tubes containing glass beads (0.5-0.75 mm) (1:3 v/v). The cells were disrupted using a Thermo Savant Fastprep FP120 device using 10 cycles of 2 min, with 2 min alternating pauses at 4 °C. The samples were then centrifuged at 5,000 x*g*-for 15 min. The supernatants (cytosolic proteins) were saved and the pellets, containing the cell wall antigens, were suspended in lysis buffer supplemented with 2% SDS and boiled for 2 min at 100 °C for protein extraction. After centrifugation under the same conditions described above all the supernatants were combined and the pellets discarded. Total protein was quantified using the BCA protein quantification kit (Pierce, US).

Detection of antibody subclasses.

Serum pooled from five animals was used for antibody detection. For IgG subclass determination, 50 μl/well of antigens (EVs, proteins from *C. albicans* yeasts and BCG) were added to half-area-high binding 96 well ELISA plates in a final concentration of 10 μg/ml and the samples were incubated overnight incubation at 4 °C. The wells were subjected to four washes with PBS and blocked with PBS containing 1% BSA for 90 minutes at room

temperature. Serum samples were diluted 1:40; 1:120; 1:360 and 1:1080 in PBS-1% BSA and then added to the wells. The plates were incubated at 4 °C overnight, followed by four more washing steps with PBS. A final volume of 50 μL of the subclasses reporter antibodies (anti-IgG1, IgG2a, IgG2b and IgG3, diluted 1:1000 in PBS 1% BSA) conjugated to HRP were added to the wells and incubated for 90 min at room temperature. The wells were again subjected to four washes with PBS and then developed with TMB; the reaction was stopped with 50 μL of 3 N HC1 and read on a 450 and 650 nm filter microplate reader (iMark, BioRad, US).

Mouse model.

Female BALB/c mice (6-8 weeks, 11 animals per group) were immunized intraperitoneally four times, one week apart, with 200 μL of an EV suspension at a final sterol concentration of 10 μM in PBS, formulated or not with Freund's adjuvant (ADJ) (Sigma-Aldrich) diluted 1:1 (v/v) in the EV suspension (fresh preparations kept at 4 °C up to 36 hours). PBS and PBS-Adjuvant (ADJ) (Sigma-Aldrich) were included as controls. Four days after the third boost the animals were immunosuppressed with cyclophosphamide (200 mg/kg) intraperitoneally. Under these conditions, cyclophosphamide induces mice neutropenia with maximum effect between days 3 and 4 (Segal E, 1981). Neutropenia is usually restored 10 post-administration. Three days later, the animals were inoculated intraperitoneally with a lethal inoculum of *C. albicans* ($3x10^7$ yeasts per mouse). Blood was collected at days 0, 7, 14, 21, 25 and 28 days. The blood was centrifuged at 10,000 x *g* for 10 min, and the serum (upper phase) was collected and stored at −80 °C for further analysis. All mice were treated according to the ethical guidelines for animal experimentation (Protocol 01200.001568/2013-17, approved in 2019 by CEUA, UFRJ). The number of deaths was evaluated daily. Survival curves were plotted, and statistical analyses were performed using the log—rank (Mantel—Cox) survival test.

Fungal burden and cytokine measurements.

Following the immunizations and immunosupression, three and five days after fungal infection mice were euthanized and the organs (kidney, liver and spleen) excised, weighted and homogenized in 5 ml of PBS for analysis of the fungal load by the enumeration of colony forming units (CFU) and determination of the cytokines profile. The CFU were determined after plating successive dilutions of organs homogenate onto Sabouraud agar and incubation at 30 °C for 48h. Homogenates were also used for the measurement of cytokines. The cytokines TNF-α, IL-12, IL-10, TGF-β, IL-4, IL-6 and INF-γ were quantified using commercial kits from eBioscience (US) according to the manufacturer's protocols. Statistical analysis was performed using one-way analysis of variance (ANOVA), and the difference between groups were analyzed by Tukey post test.

EVs stability at low temperatures.

To investigate the stability of the fungal EVs, 200 μL aliquots (final concentration of 10 μM based on sterol content) were stored at distinct temperatures; 4 °C, −20 °C and −80 °C. After 7 days, the aliquots were thawed at room temperature and then their dimensions were analyzed by two independent approaches. First, EVs were analyzed using dynamic light scattering (DLS) in a 90Plus / BIMAS Multi-Angle Particle Size Analyzer (Brookhaven

Instruments) as described (Vargas et al., 2015). In addition, the EVs were submitted to NTA on an LM10 nanoparticle analysis system, coupled with a 488-nm laser and equipped with an $_{S}$CMOS camera and a syringe pump (Malvern Panalytical, Malvern, United Kingdom) as detailed by Reis and colleagues (Reis et al., 2019). All samples were 700-fold diluted in filtered PBS. Samples were injected using a syringe pump speed of 100, and three videos of 60 s duration captured per sample, with the camera level set to 15, gain set to 3, and viscosity set to that of water (0.954 to 0.955 cP). For data analysis, the gain was set to 10 to 15 and the detection threshold was set to 2 to 3 for all samples. Levels of blur and maximum jump distance were automatically set. The data were acquired and analyzed using the NTA 3.0 software (Malvern Panalytical). Finally, EVs were visualized by transmission electron microscopy (TEM) using a negative staining technique. Briefly, the EVs suspension was adhered to 200-mesh copper grids previously treated with Formvar 0.3% and discharged at PELCO easiGlow™ Glow Discharge Cleaning System. The EVs were then negatively contrasted with 1% (m/v) uranyl acetate. The micrographs were performed in an JEOL 1200EX 80 kV electronic microscope. A fresh preparation of EVs was used as control for DLS, NTA and EM analyses.

Cytokine production by dendritic cells treated with fungal EVs.

To investigate whether storage would impact fungal EVs ability to activate host cells, bone marrow-derived DCs were treated with pre-stored EVs and the levels of IL-6 and IL-10 evaluated. Bone marrow-derived DCs were obtained from femur and tibia of BALB/c female mice (4 to 12 weeks old) following the protocol established by Lutz et al (Lutz et al., 1999). Briefly, femur and tibia of BALB/c mice (female), aged 4 to 12 weeks, were removed. After removal of the musculature and tendons, the intact bones were left in 70% ethanol for 2 to 5 minutes for disinfection followed by washing with sterile PBS. Both ends were sheared with scissors and the bone-marrow washed with Hanks' medium containing 2% FBS using a syringe with a 0.45 mm diameter needle. The supernatant was then centrifuged for 5 minutes at 1500 rpm and suspended in 2 mL of RPMI and 10% FBS. Cells were counted in Neubauer's chamber and plated at the final suspensions of 10^6 cells in 10 ml of RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM MEM ("Minimal Essential Medium") nonessential amino acids, 50 μM 2-β-mercaptoethanol and 20 ng/mL rGM-CSF (recombinant granulocyte macrophage - colony stimulating factor) at 37 °C and 5% CO_2. Cells were then incubated for 3 days at 37 °C and 5% CO_2. On day 2, 10 mL of RPMI supplemented with 10% FBS and 20 ng/mL rGM-CSF was added. On day 10 the cells were washed in RPMI medium supplemented with 10% FBS, counted and plated at 10^5 cells per well in 96-well plate. Bone-marrow derived DCs were then incubated with *C. albicans* EVs (at final concentration of 10 μM sterol per well) and incubated overnight under the same conditions. The supernatants were collected and the cytokines IL-6 and IL-10 were determined by enzyme-linked immunosorbent assay as recommended by the manufacturer eBioscience (US). Lipopolysaccharide (LPS) (Sigma) (1 μg/mL) and PBS were used as a positive and negative control respectively. Statistical analysis was performed using one-way analysis of variance (ANOVA), and the difference between groups were analyzed by Tukey post test.

Galleria mellonella infection.

G. mellonella larvae in the final instar larval stage were selected according to similarity in weight (0.10—0.15 g). Larvae (20 per group) were inoculated with 10 μL of EV suspensions (10 μM per insect, based on sterol quantification) using an 30G insulin syringe into the haemocoel through the last proleg as described by Brennan *et al.* (Brennan et al., 2002). The same volume of PBS was used as negative control. The larvae were then placed in sterile Petri dishes and kept in the dark at 37 °C for two days. Subsequently, all larvae were inoculated with 10 μL of a suspension containing 2×10^5 yeasts of *C. albicans* (strain 11). A control group received only the EVs to determine whether these compartments are toxic to the larvae. Larvae were kept under the same conditions above and their mortality was monitored by checking twice daily. Death was assessed by the lack of movement in response to stimulation. Survival curves were plotted, and statistical analyses were performed using the log-rank (Mantel–Cox) survival test and displayed results represent the mean percentage survival of larvae from all assays.

Statistics.

Statistical analyses were performed with the GraphPad 5 software (La Jolla, CA). Group comparisons were submitted to one-way analysis of variance (ANOVA) with Bonferroni or Tukey post test, according to each experiment. Survival analysis were performed by Log-rank (Mantel-Cox) test.

RESULTS

Fungal EVs from *C. albicans* activate the humoral immune response.

To investigate the ability of *C. albicans* EVs to stimulate the humoral response we carried out a prime-and-boost immunization protocol using EVs stored at 4°C, the same conditions used for all the studies previously performed in our laboratory (Oliveira et al., 2010a; Vargas et al., 2015). Figure 1 shows our vaccination strategy. We first aimed to determine the number of immunizations required to induce antibody production in mice. Prior to the first immunization step, serum from all mice was collected. Then EVs, EVs + ADJ, ADJ or PBS were inoculated intraperitoneally, and the serum collected before each of the three additional boosts to follow up the production of IgM and IgG. We detected an increase of total IgM one week after the first immunization only with EVs + ADJ (day 7) (Figure 2A). However, total IgG increased after the third immunization (day 21), for all groups except for the PBS treated mice (control) (Figure 2A). Based on these results we decided to immunize the mice four times before infection.

We then investigated the specificity of the IgM and IgG produced after immunization against *C. albicans* protein extract (Ca-Ptn, Figure 2B) and EVs (and Figure 2C). In general, we observed a slight increase in IgM production against both Ca-Ptn and EVs at day 7, with low variation during later periods. Similar to serum IgG concentration, a significant increase of IgG anti-Ca-Ptn and EVs at day 21 was observed in mice immunized with EVs + ADJ and EVs. The predominant IgG classes reactive to Ca-Ptn were IgG1 from mice immunized with EVs + ADJ. IgG1 was also the major class recognizing EVs antigens when mice were immunized with EVs + ADJ or EVs (Figure 1). In addition, the increased reactivity of IgG

antibodies from mice immunized with EVs + ADJ was highly associated with BCG antigens present in the ADJ (Figure S1).

Vaccination with EVs decreased fungal burden in mice tissues.

To determine whether treatment with *C. albicans* EVs can control *C. albicans* dissemination in immunosuppressed mice, the immunization protocol was followed by a single dose of cyclophosphamide (CP) (Figure 1) and two days later the mice were infected intraperitoneally with a lethal inoculum of *C. albicans* yeast cells. Three- and five-days post-infection, fungal burden in the kidney, spleen and liver were determined (Figure 3). Immunization with fungal EVs and EVs + ADJ significantly decreased the CFU in all organs, suggesting a protective effect. When the CFU was compared between the formulations containing EVs the presence of adjuvants decreased the CFU at day 3 in spleen and liver, and at day 5 in all organs.

Immunization with *C. albicans* EVs induces different profiles of cytokines.

In order to better understand the impact of immunization with fungal EVs during the immune response we also measured the cytokines produced in the spleens of mice at different time-points after infection (3 and 5 days). Mice vaccinated with fungal EVs produced higher of IL-12p70 at days 3 and 5, while treatment with EVs + ADJ displayed substantially high levels for this cytokine at day 3, returning to levels similar to EVs stimulation at day 5 (Figure 4A). Also, day 3 demonstrated an increase of TNF-α production when mice were immunized with EVs and EVs + ADJ (Figure 4B). After 5 days all conditions showed an increased production of TNFα, but only EVs + ADJ displayed a statistically significant raise (Figure 4B, $p<0,05$). Remarkably, only spleens from mice immunized with EVs showed a higher level of IL-10 (day 3), TGF-β (days 3 and 5) and IL-4 (day 3) (Figure 4C, D and E). The cytokines IFN-γ and IL-6 were not detected after 3 days of infection; however, both increased for all mice after 5 days. A higher and significant level was observed for both cytokines only when fungal EVs + ADJ were used as a vaccine formulation (Figure 4F and G).

Immunization with EVs protected mice against a lethal infection with *C. albicans*

After a lethal infection with *C. albicans* yeast cells, all non-immunized or ADJ-immunized mice died by 15 days of infection (Figure 5). However, immunization with EVs alone or EVs-ADJ formulations induced full protection, with all mice surviving until the end of the experiments ($p<0.05$).

Fungal EVs from *C. albicans* were preserved after storage at low temperatures.

Considering the protective effects of fungal EVs, we evaluated their stability as a potential parameter for determining their future applicability as vaccine candidates. Maintenance of EV dimensions have been proposed as a parameter of vesicular stability (Wolf et al., 2012; Almeida et al., 2017). We therefore adopted EV dimensions in association with microscopic observation to evaluate the effects of storage on EV stability. Initial experiments were performed to investigate the stability of fungal EVs after storage at 4, −20 and −80 °C. The average diameter size was evaluated using DLS and distribution of EVs after storage showed

a very modest increase in diameter by DLS analysis (Figure 6). For all samples, a bimodal distribution was visualized. Two populations of fungal EVs were detected, ranging between 30-75 and 140-230 nm. TEM of fresh and stored EVs demonstrated the presence of round shaped bilayered membranous structures within the size rates observed by DLS analysis. In order to confirm the size of the EVs we also evaluated the samples using nanoparticle tracking analysis (NTA). Minimal changes in size were observed when the EVs were stored at 4, −20 or −80 °C, in comparison to fresh samples (Figure 6). In all tested conditions the population of EVs ranged between 60 and 350 nm. However, EVs kept at 4 °C showed a less homogeneous distribution in size. Finally, no agglutination or membrane fragments were observed in TEM micrographies or DLS analysis, since DLS is also able to detect micelles or smaller particles (Moniruzzaman et al., 2010; Stetefeld et al., 2016).

Fungal EVs induced production of IL-6 by dendritic cells.

We recently demonstrated that *C. albicans* EVs modulate the activity of bone marrow-derived DCs, suggesting that these compartments could activate immune cells involved directly with the innate response (Vargas et al., 2015). To investigate whether storage would modify the ability of *C. albicans* EVs to activate DCs we examined IL-6 and IL-10 production after overnight stimulation with these compartments. Independent of the storage conditions all fungal EVs induced IL-6. However, stimulation with EVs kept at −80 °C was significatively reduced when compared with fresh and that storage at −20 °C. On the other hand, IL-10 production was minimal for all EV samples (Figure 7).

Pretreatment with fungal EVs decreased the lethality of *C. albicans* in a *G. mellonella* model of infection.

We used *G. mellonella* larvae to study whether fungal EVs storage could impact their ability to protect the insect against a lethal challenge with *C. albicans*. Fungal EVs were administered in concentrations previously described by our group as capable of significantly reduce larvae mortality and fungal burden (Vargas et al., 2015). The *G. mellonella* survival data indicated that all fungal EVs tested were able to protect the larvae from *C. albicans* infection, independent of their storage conditions (Figure 8). However, fresh fungal EVs resulted in the highest survival rates (62%).

DISCUSSION

Fungal EVs can directly modulate the innate immune system with a promising potential to trigger the development of adaptive responses (Vargas et al., 2015). Since fungal EVs carry a combination of native immunogens in a cell-free system, they may be a safe source of immunogens for the development of vaccine formulations. In fact, the immunomodulatory activity of fungal EVs has been clearly demonstrated in several *in vitro* models (Oliveira et al., 2010a; Vargas et al., 2015; Joffe et al., 2016; Da Silva et al., 2016; Baltazar et al., 2018; Bielska et al., 2018; Souza et al., 2019a), as well as their ability to prolong the survival of invertebrate models of infection with *C. albicans* or *C. neoformans* (Vargas et al., 2015; Colombo et al., 2019). However, the protective effects of fungal EVs in mammalian models of fungal infections have not yet been demonstrated, which hampers the design of fungal

EV-based vaccine formulations. In this study, we demonstrated that fungal EVs efficiently protect immunosuppressed mice against a lethal *C. albicans* infection.

As demonstrated previously by our group, DCs treated with *C. albicans* EVs exhibited an increase in MHCII and CD86 expression, suggesting that these compartments could mediate an efficient communication between the innate and the adaptive immune response (Vargas et al., 2015). To investigate this possibility, we developed a protocol for mice immunization using fungal EVs in the presence or absence of classic vaccine adjuvants. Our results demonstrated that one initial vaccination and three additional boosts are required to stimulate a significant increase in antibody production. We found that most of the serum antibodies that recognize fungal antigens, both *C. albicans* crude extract and EVs, belong to the IgGl subclass. Although our data indicate that the addition of adjuvants promotes a polyclonal B cell response, further studies are required to determine whether the antibodies produced after immunization with *C. albicans* EVs are specific and/or effector components of the immune response.

The reduced fungal burden in the spleen, liver and kidney of mice immunized with EV formulations clearly shows that vaccination decreases *C. albicans* dissemination. It is noteworthy that during these time intervals, mice are still neutropenic and susceptible to fungal dissemination, supporting the efficiency of the vaccination protocol in immunosuppressed animals. These data corroborate with the full protection observed in the survival experiment using both EVs or EVs+ADJ as vaccine formulations. However, the cytokine profile in the spleen of mice was distinct when immunization with EVs and EVs +ADJ were compared suggesting a different protective immunological regulation for both formulations. Although the pro-inflammatory cytokines IL12p70 and TNFα increased at day 3 for both conditions, IL-4 and TGFP were significantly higher in the absence of adjuvants. In general, IL-4 is associated with TH1 inhibition and has a pivotal role inducing a TH2 response and susceptibility to candidiasis (Puccetti et al., 1994; Tonnetti et al., 1995). However, IL-4 can also play a protective role in candidiasis. Presence of IL-4 was required for protection through a mechanism involving the induction of $CD4^+$ TH1 response (Romani et al., 1994; Spaccapelo et al., 1995). An increased production of IL-4 was detected in memory $CD4^+$ T cells after a secondary challenge with *C. albicans* yeasts (Romani et al., 1996). Corroborating with that, TGFP seems to be involved with acquired resistance and its presence is required for optimal TH1 development and long-term anticandidal activities (Spaccapelo et al., 1995). Of note, the participation of TGFP as an inducer of memory CD4 cells in mice has been reported (Swain et al., 1991). Interestingly, TGF-β was able to enhance both memory phenotype of antigen-specific murine TH1 cells and their effector function in experimental autoimmune encephalomyelitis (Weinberg et al., 1992).

A higher inflammatory activity mediated by EVs+ADJ was confirmed at day 5, when increased levels of TNFα, IFNγ and IL-6 were detected. In mice immunized with EVs the IL-6 increase in combination with the presence of TGFP could trigger the development of a TH17 response (Mangan et al., 2006), also supported by the potential TH1 inhibitory activity of IL-4. Excepting for the increase in IL-10 and TGFP at day 5, all the cytokines detected after EV immunization were similar to PBS and ADJ. Under these conditions, the cytokines associated with anti-inflammatory responses would be important for restoring

normal conditions. Based on our data we could not rule out that the infection was resolved faster when the EVs were used alone. However, the fungal burden was similar during days 3 and 5 after immunization with EVs or EVs+ADJ, which argues against this hypothesis. The addition of adjuvant apparently favored an inflammatory response.

Our results confirm that fungal EVs are promising multi-antigenic compartments to be exploited as vaccine formulations. However, the mechanisms by which EVs mediate this protection remains to be determined. A number of components carried by *C. albicans* EVs could be individually and / or collectively involved in the protective effects. Indeed, proteomic analysis of *C. albicans* EVs was previously performed and a list of proteins successfully used in murine vaccination models were characterized, including (i) enolase-1 (Enol), (ii) MP65, (iii) Bgl-2, (iv) the agglutinin-like sequence protein 3 (Alsp3) and (v) the secreted aspartyl protease 2 (Sap2) (Vargas et al., 2015; Gil-Bona et al., 2015b). Thus, the mechanism by which the fungal EVs induce protection is most probably dependent on a combination of antigens that stimulates: (i) phagocyte activation with the upregulation of cytokines (Oliveira et al., 2010a; Vargas et al., 2015), (ii) activation of antigen presenting cells (Vargas et al., 2015), with potential induction of TH17 response and (iii) antibody production. The presence of mannosylated proteins and the mechanism of internalization of *C albicans* EVs suggests the participation of cell surface receptors (Vargas etal., 2015; Gil-Bona et al., 2015a). This activation could be at least in part be mediated by MP65, a mannoprotein enriched in EVs that modulates the expression of CD86 and MHCII by dendritic cells (Gomez etal., 1996; Pietrella et al., 2006) and induces the production of cytokines, similarly to what has been observed when DCs were incubated with *C. albicans* EVs (Vargas etal., 2015). Two specific proteins carried by *C. albicans* EVs, Als-3 and Sap2, have been extensively investigated in vaccine studies (De Bernardis et al., 2012; Sui et al., 2017b). The N-terminal domain of Als-3 was formulated with aluminum hydroxide as adjuvant (NDV-3 vaccine). In humans, this formulation was safe, tolerable and stimulated an immunogenic response (Schmidt et al., 2012) and is currently in a phase 2 trial. In addition, recombinant Sap2 was incorporated into influenza virosomes (PEV7) and has completed phase 1 clinical trial (De Bernardis et al., 2018). Protection in mice by these antigens is mediated through mechanisms that include shifts in TH1 and TH17 cytokines as well as antibody production (De Bernardis et al., 2012; De Bernardis et al., 2015).

The combination of all these immunogens in their native structure could potentiate the efficacy of *C. albicans* EVs as a vaccine. In fact, the use of combined antigens has been previously investigated before. Torosantucci and colleagues (Torosantucci et al., 2005) conjugated /Z-glucan to CRM197, a non-toxic mutant of diphtheria toxin that functions as a carrier protein, and this formulation protected mice against vulvovaginal and systemic candidiasis. Additional optimized formulations were recently tested by Bundle and colleagues (Bundle et al., 2018) who conjugated β-mannans to β-glucans to obtain more efficient immunogens. Other highly conserved EVs components, such as metabolic enzymes (e.g. from the glycolytic pathway), heat shock proteins and the glycosphingolipid glucosylceramide (GlcCer), potentially stimulate the immune system against a variety of fungal species (Nimrichter et al., 2004; Rodrigues et al., 2007b; Raska et al., 2008a). In combination these antigens could induce crossed protection against other fungal pathogens, which still has to be experimentally addressed for fungal EVs. In other models,

immunization with *C. albicans* aldolase, a metabolic enzyme that is abundant in EVs, conferred protection against *C. glabrata* (Medrano-Diaz et al., 2018). In addition, immunization of mice with GlcCer protected mice against a lethal inoculum of *C. neoformans* (Mor et al., 2016). We previously demonstrated that passive immunization with anti-GlcCer antibodies prolongs the survival of mice challenged with *C. neoformans* (Rodrigues et al., 2007b).

The crossed protection activity of *C. albicans* EVs is currently under investigation in our laboratory, as well as of the potential EV-associated cell surface pattern recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs). EVs produced by *C. albicans* and other fungal species are the vehicles of RNA export (Da Silva et al., 2015). Therefore, it is reasonable to expected that, if released within the host cells, vesicular RNA could potentially activate TLR9. Alternatively, since TLR9 can be recruited to the phagosomes when dectin-1 is activated (Khan et al., 2016), trafficking of EVs to phagosomes could also trigger TLR9 activation. Finally, we cannot rule out the participation of polysaccharides in the activation of innate responses. Recently, Silva and colleagues demonstrated that EVs from *Paraccoidioides brasiliensis* are recognized by DC-SIGN *in vitro* (Peres da Silva et al., 2015). However, such components have not been reported in *C. albicans* EVs.

The use of fungal EVs in vaccine preparations depends on the reproducibility of their protective effects, which is directly related to their stability. Indeed, intact fungal EVs could function as a delivery system similarly to liposomes, preventing antigen degradation, which would increase their relative concentration and facilitate the delivery of fungal components to antigen presenting cells (Giddam et al., 2012; Peres da Silva et al., 2015). Independent of their mechanism for promoting protection, EVs must be carefully isolated and stored to keep their functional epitopes structurally stable. Although fungal EV isolation protocols have been significantly improved to speed up and optimize the preparation of vesicular fractions (Reis et al., 2019), storage is a key issue to be considered and thoroughly investigated, since it directly impacts the applicability of fungal EVs as vaccine candidates. In EVs released by mammalian cells, the impact of storage at different temperatures has been investigated, but the results seem to diverge according to the cell line tested, suggesting that each model must be individually investigated (Sokolova et al., 2011; Gamonet et al., 2017; Maroto et al., 2017; Park et al., 2018; Frank et al., 2018). The effects of storage on fungal EVs are poorly known. In *C. neoformans,* vesicle stability appears to be distinct according to the EV population (Wolf et al., 2012). Larger EVs, varying from 100 to 200 nm, were stable for at least 72 hours at room temperature in conditioned medium. In contrast, smaller vesicles, ranging from 40–60 nm, decreased in size after 24 hours of storage. In our experiments *C. albicans* EVs were kept at lower temperatures for 7 days and their size compared with fresh preparations. Using DLS as a tool to measure size we showed that EVs from *C. albicans* consist of two populations, comparable to what was previously described by our group (Vargas et al., 2015). The particle range measured by DLS after storage was relatively homogeneous and similar to the fresh prepared EVs. A small increase in size was observed in the smaller population of EVs after storage at 4 and −20 °C. The larger vesicles were also slightly increased when storage was carried out at 4 °C. These modest changes could be explained by EV fusion or aggregation. Increasing in EV size has been reported after

freezing, caused by multilamellar formation due to expansion of ice nano- or micro-crystals in the lipid bilayer(Lee et al., 2016). This effect was not evident in our TEM images after storage at −20 and −80 °C. Despite our multiple approaches suggesting that the EVs were stable and intact, the data does not absolutely rule out that there was no disruption and reassembly of the vesicles. However, the absence of membrane fragments or debris, which would be detected both by TEM and DLS, suggests that most of the EVs were intact. The preservation of their dimensions was confirmed by NTA, which revealed EV populations very similar in dimensions independently of storage conditions. However, it is noteworthy that, so far, there are no authoritative methods to determine the size of EVs (Thery et al., 2018). A combination of fluorescent labeling EVs and particle counting technologies, such as NTA, is recommended. However, antibodies and lipid dyes can form particles that could interfere with data analysis. Thus, although there remains a theoretical methodological limitation in our current investigation, our data indicate that although fungal EVs were relatively stable and that the −80 °C temperature was apparently the best storage condition, at least for *C. albicans* fungal EVs.

Since EVs integrity was maintained under different storage conditions, we subsequently investigated whether the immunogens also conserved their antigenic activity. Most importantly, storage conditions did not abrogate the ability of EVs to activate DCs. Treatment with EVs increased the production of the pro-inflammatory cytokine IL-6, consistent with previous data from our lab confirming the activation of DCs through EVs recognition (Vargas et al., 2015). In addition, lower levels of IL-10 were detected. Fungal EV storage at lower temperatures did not affect protection against *C. albicans* in *G. mellonella,* a reliable model to investigate the activation of innate immune responses (Fuchs and Mylonakis, 2006). Our results not only confirmed previous experiments published by our lab, showing that fungal EVs stimulated the innate immune response and protected the insect (Vargas et al., 2015), but also demonstrated that *C. albicans* EVs could be stored at lower temperatures without losing their ability to activate DCs and the innate immune system.

Our present study shows that immunization with *C. albicans* EVs stimulates a protective immune response in a murine model of candidiasis. These results, in association to the stability of fungal EVs preparations, open a new venue for the development of novel and efficient vaccines to prevent candidiasis and potentially other fungal infections. The use of mutant strains lacking virulence factors can also be explored as well as the development of platforms to generate EVs tailored with heterologous antigens originally from pathogenic fungi and expressed in non-pathogenic fungal species. Furthermore, antigens from bacteria, viruses and parasites could also be combined with fungal components for immunological assays of prophylaxis or treatment by boosting the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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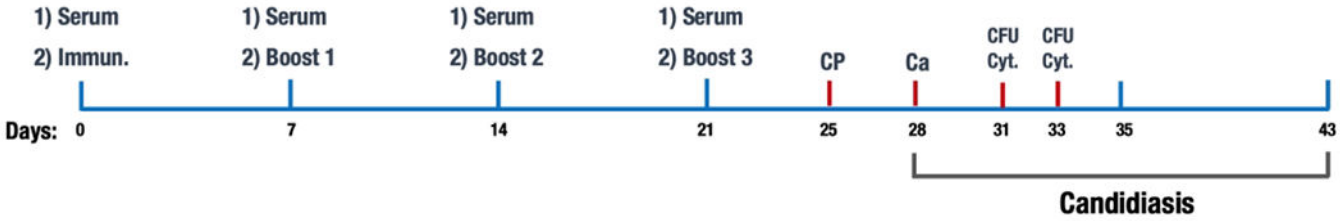

Figure 1 –. Experimental design.

The strategy used to immunize and infect the mice was represented as a function of time. All treatments were intraperitoneal. First immunization was given on day 1 right after serum collection, followed by three boosts and cyclophosphamide treatment (CP). Three days after CP treatment the mice were infected with *C. albicans*. Colony forming units (CFU) and cytokines (Cyt) were measured three and five days after infection.

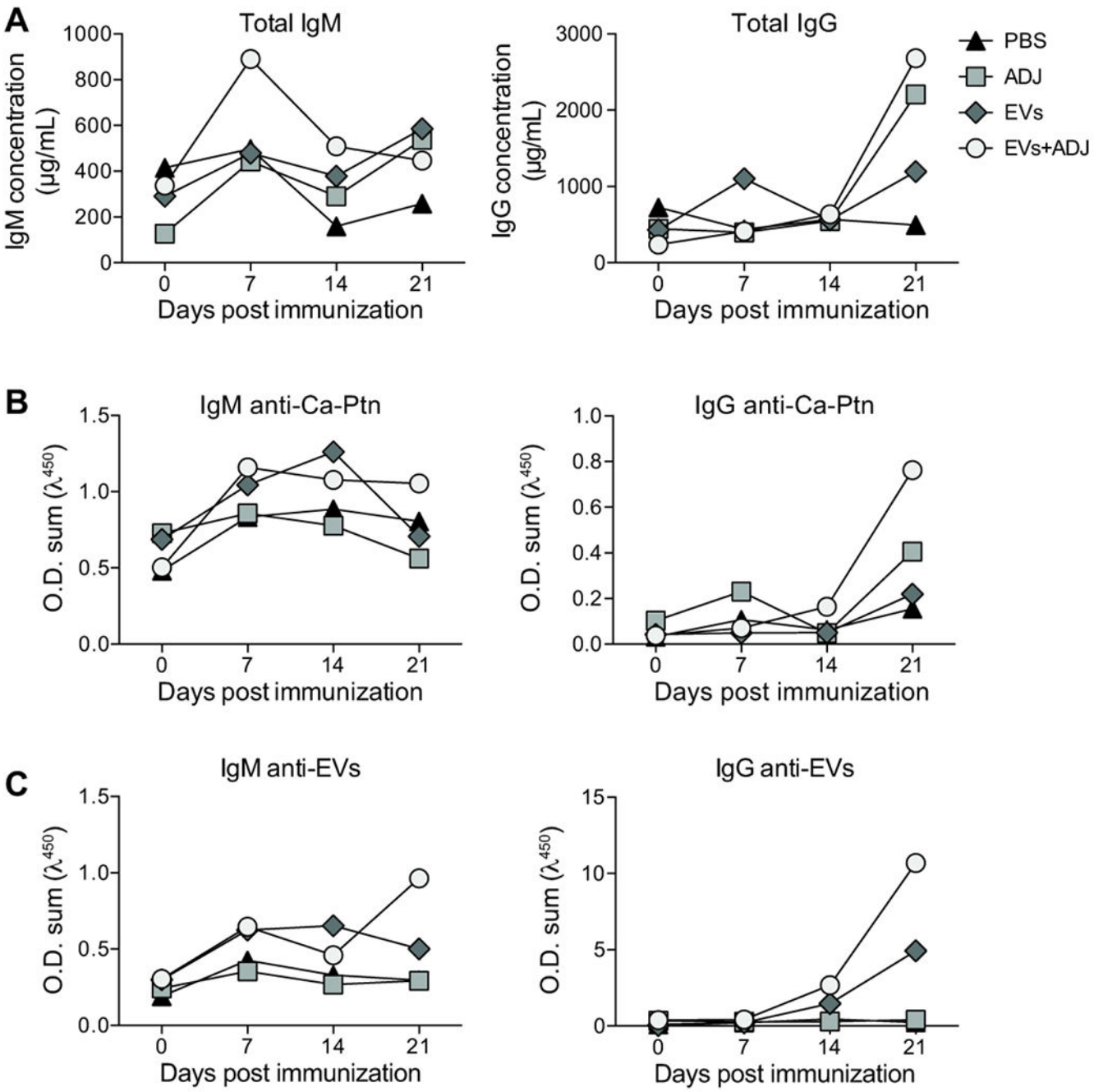

Figure 2- Total and fungal antigen-specific IgM and IgG production in mice immunized with EVs from *C. albicans*.

Mice were vaccinated intraperitoneally with EVs from *C. albicans* with (EVs+ADJ) or without (EVs) complete Freund's adjuvant (ADJ) four times as described in Figure 1. ADJ and PBS were used as controls. Total IgM and IgG (A) were determined. *C. albicans* protein extract (Ca-Ptn) and EVs were used to detect specific IgM (B) and IgG (C). These data represent one of two consistent experiments.

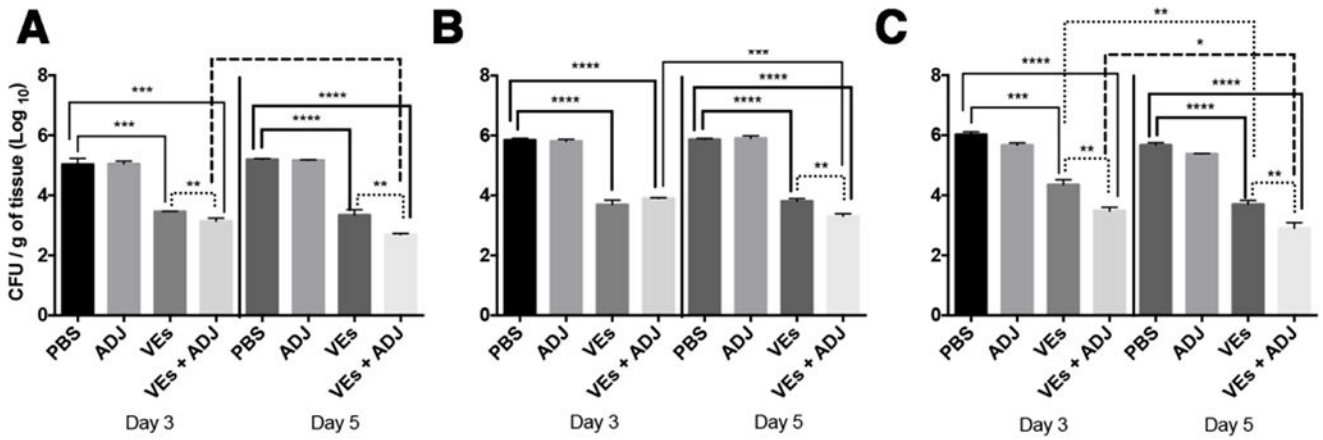

Figure 3- Vaccination with *C. albicans* EVs reduces fungal burden.

Mice were immunized with *C. albicans* EVs, immunosuppressed with cyclophosphamide (CP) and then challenged with a lethal inoculum of *C. albicans* yeasts. Figure shows colony forming units (CFU) in spleen (B), kidney (C) and liver (D) are showed. Results are representative of two independent experiments. Statistical analysis was performed using one-way analysis of variance, and the difference between groups were analyzed by Tukey post test. * $p<0.05$; ** $p<0.001$; *** $p<0.002$ and **** $p<0.0001$.

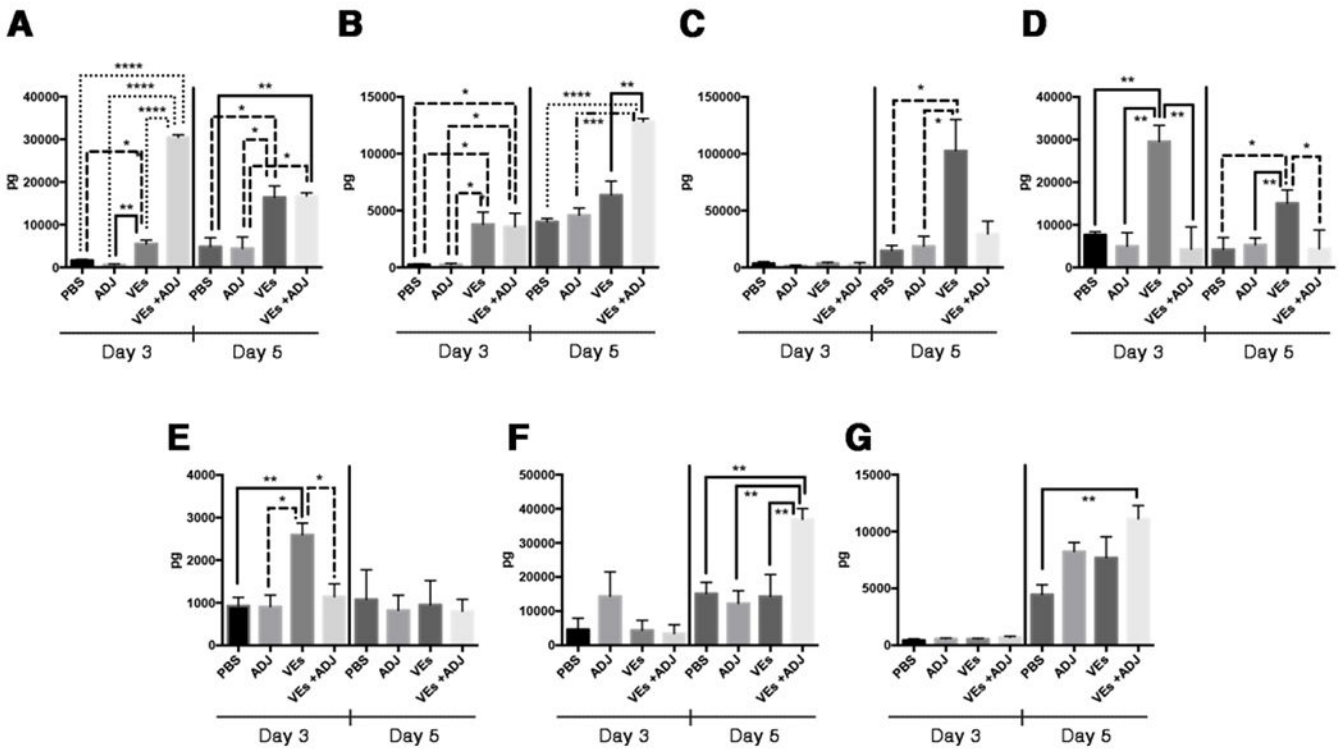

Figure 4 - Vaccination with *C. albicans* EVs modified the cytokine production in response to *C. albicans.*

Mice were immunized, immunosuppressed and infected with *C. albicans* yeasts as described in Figure 5. After three and five days of infection mice were euthanized, and the spleen was excised for cytokine determination. (A) IL12p70, (B) TNFα, (C) IL-10, (D) TGFβ, (E) IL-4, (F) IFNγ and (G) IL-6 were measured. Results are representative of two independent experiments. Statistical analysis was performed using one-way analysis of variance, and the difference between groups were analyzed by Tukey post test. * $p<0.05$; ** $p<0.001$; *** $p<0.002$ and **** $p<0.0001$.

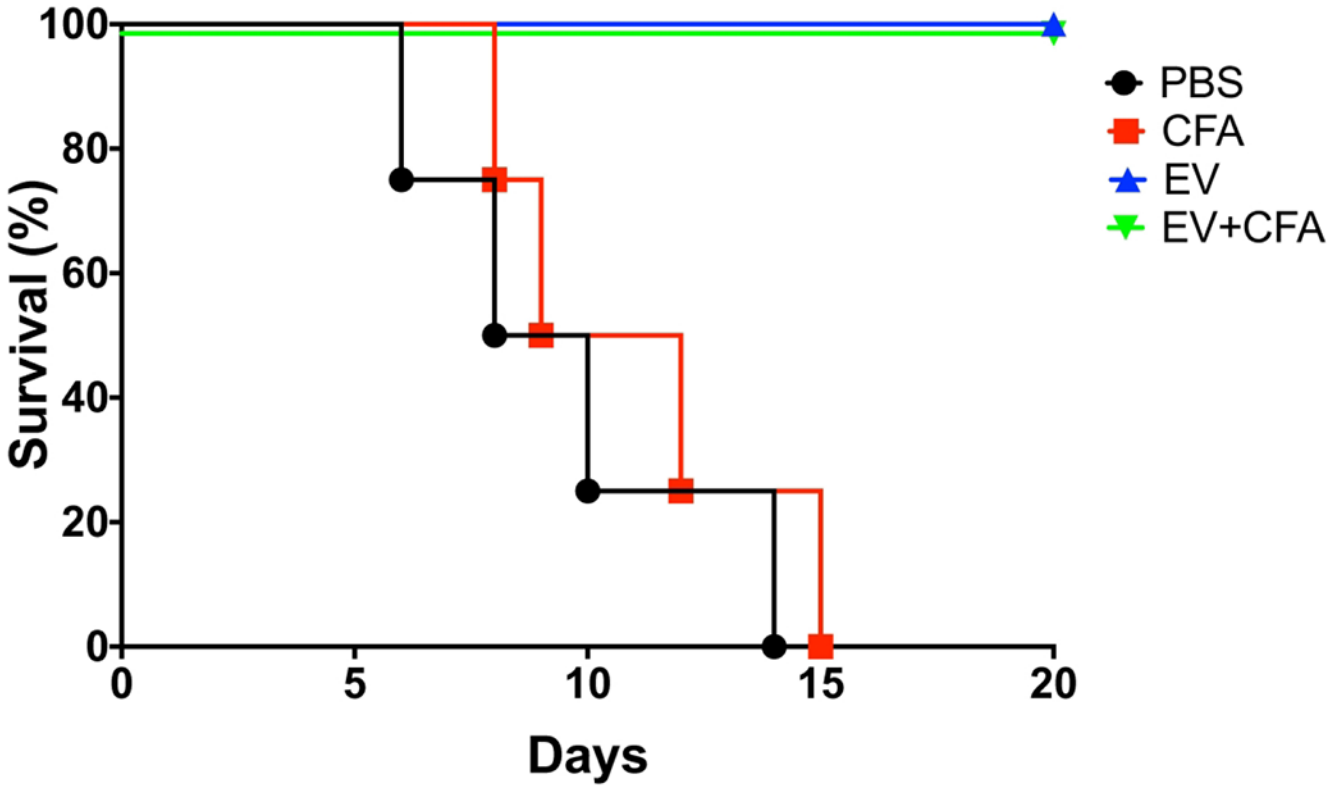

Figure 5- Vaccination with *C. albicans* EVs induced protection against disseminated candidiasis in mice.

Mice were immunized with *C. albicans* EVs, immunosuppressed with cyclophosphamide (CP) and then challenged with a lethal inoculum of *C. albicans* yeasts. The survival curves evidenced the protective effect of EVs and EVs-ADJ immunization. Statistical analysis was performed using one-way analysis of variance, and the difference between groups were analyzed by Log-rank (Mantel-Cox) test, $p<0.0001$. Results are representative of two independent experiments.

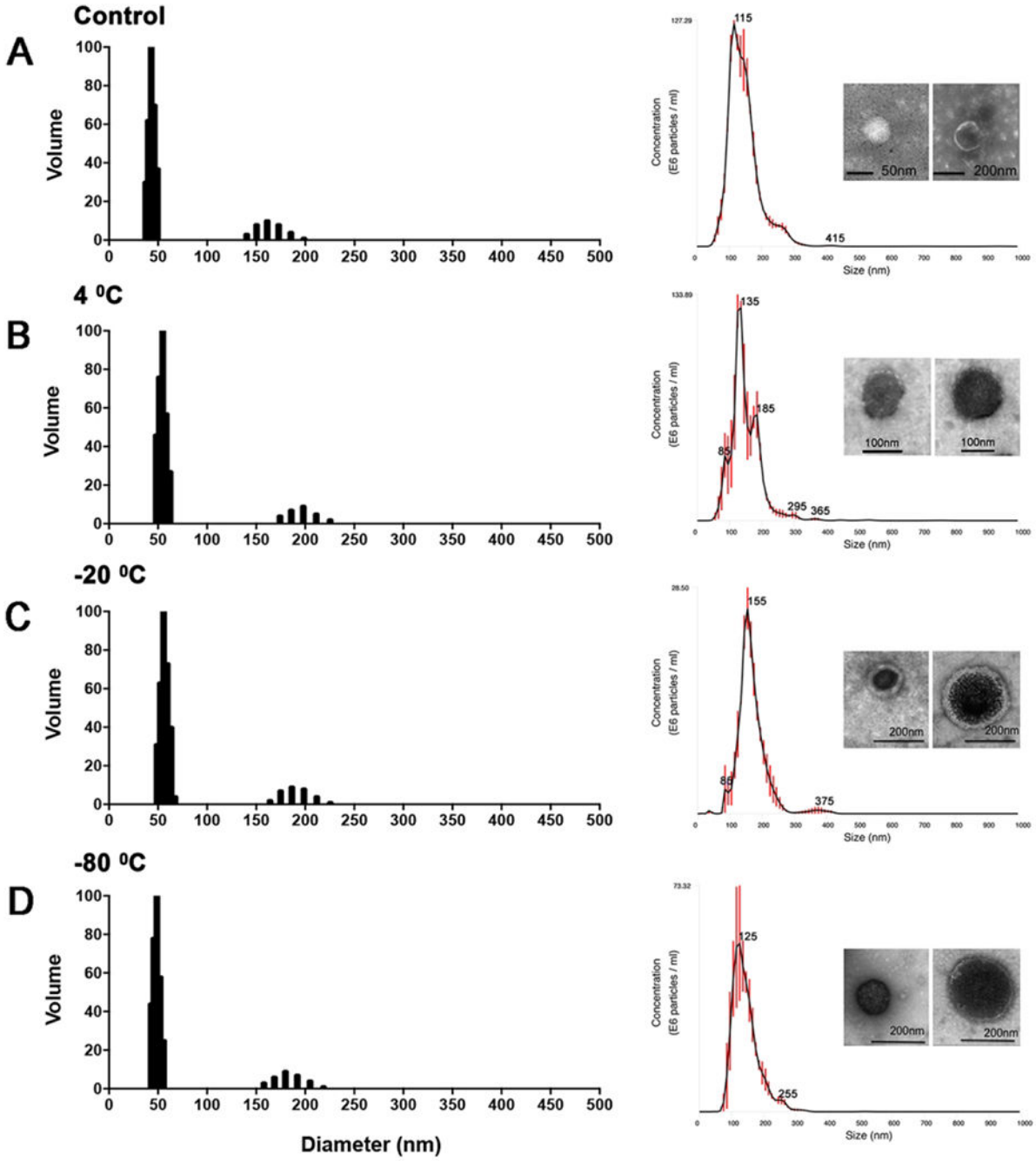

Figure 6- Effect of low temperature storage on fungal EVs size and stability.

DLS, NTA and TEM analysis were performed in fresh (A) and stored fungal EVs at 4 °C (B), −20 °C (C) and −80 °C (D). The data depicted here is representative of two consistent, independent experiments.

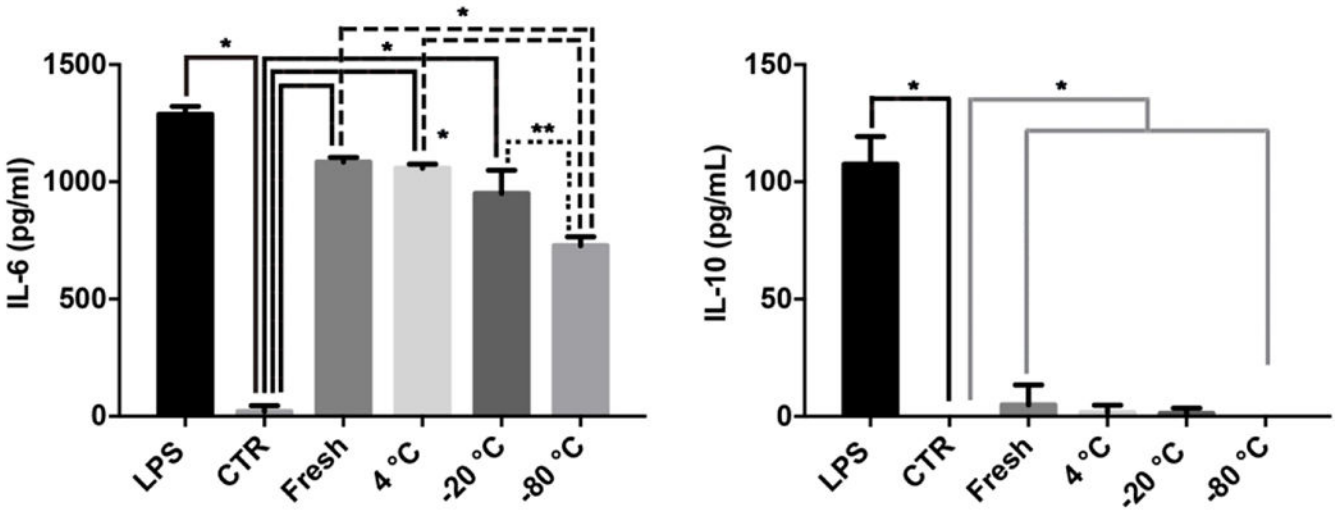

Figure 7–. Effects of storage conditions on the induction of IL-6 and IL-10 in dendritic cells (DCs).

DCs were pretreated with fungal EVs and the levels of IL-6 and IL-10 were determined. Results are representative of two independent experiments. Statistical analysis was performed using one-way ANOVA analysis of variance, and the difference between groups were analyzed by Bonferroni post test, * $p<0.0001$; ** $p=0.0015$.

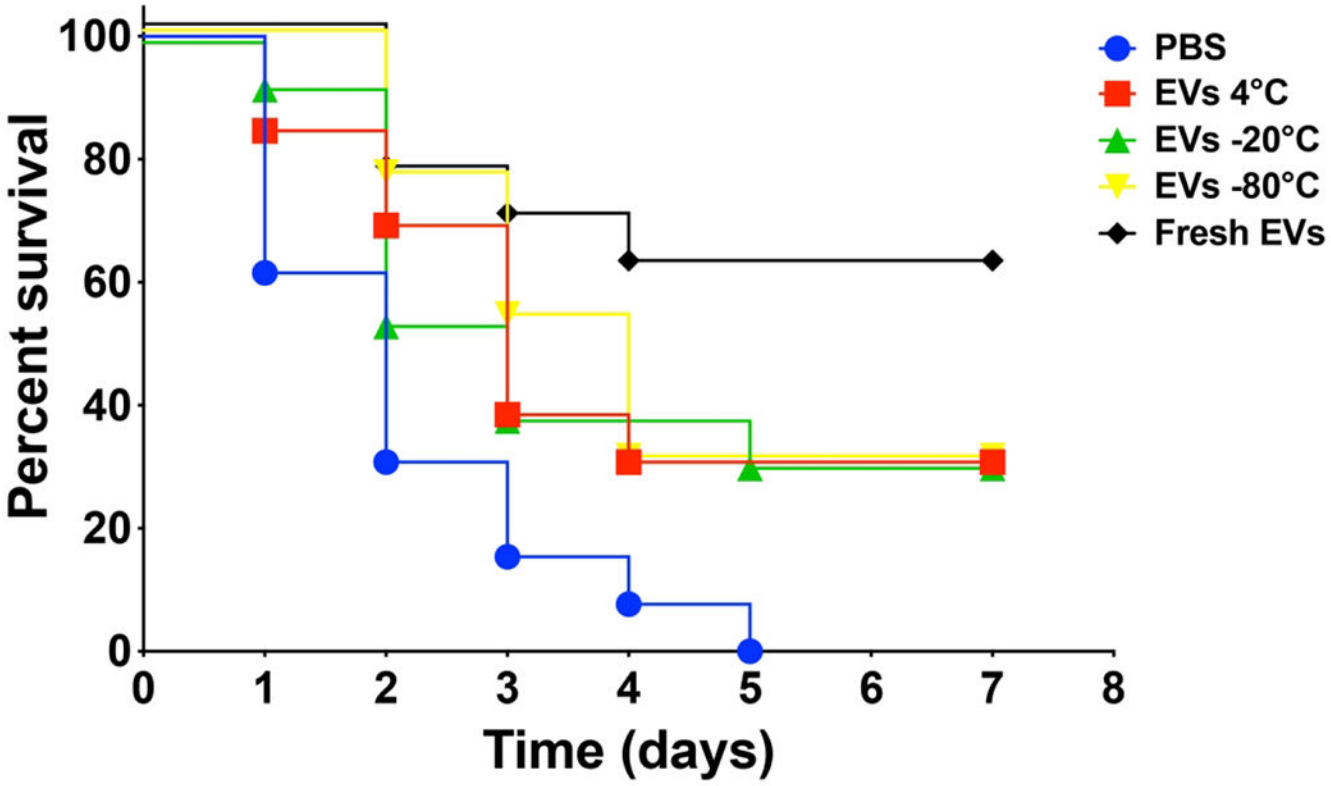

Figure 8 –. Pretreatment with fungal EVs reduced the mortality of *G. mellonella* larvae infected with *C. albicans*.

Survival curves of larvae pretreated with fungal EVs stored under different conditions (10 μl of a 10 μM suspension based on the sterol content) and then infected two days later with a lethal inoculum of *C. albicans* yeasts are shown. Statistical analysis was performed using oneway analysis of variance, and the survival differences between each EV groups and PBS were analyzed by individual paired Log-rank (Mantel-Cox) (Supplemental Table 1). Results are representative of two independent experiments.