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Protective effect of antigen delivery using monoolein-based liposomes in experimental hematogenously disseminated candidiasis

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ABSTRACT

We evaluated the potential of a liposomal antigen delivery system (ADS) containing *Candida albicans* cell wall surface proteins (CWSP) in mediating protection against systemic candidiasis. Treatment of bone-marrow-derived dendritic cells with CWSP-loaded dioctadecyldimethylammonium bromide:monoolein (DODAB:MO) liposomes enhanced and prolonged their activation comparatively to free antigen, indicating that liposome-entrapped CWSP were released more sustainable. Therefore, we immunized mice with CWSP either in a free form or loaded into two different DODAB:MO liposome formulations, respectively designated as ADS1 and ADS2, prior to intravenous *C. albicans* infection. Immunization with ADS1, but not with ADS2, conferred significant protection to infected mice, comparatively to immunization with CWSP or empty liposomes as control. ADS1-immunized mice presented significantly higher serum levels of *C. albicans*-specific antibodies that enhanced phagocytosis of this fungus. In these mice, a mixed cytokine production profile was observed encompassing IFN-γ, IL-4, IL-17A and IL-10. Nevertheless, only production of IL-4, IL-17 and IL-10 was higher than in controls. In this study we demonstrated that DODAB:MO liposomes enhance the immunogenicity of *C. albicans* antigens and host protection in a murine model of systemic candidiasis. Therefore, this liposomal adjuvant could be a promising candidate to assess in vaccination against this pathogenic fungus.

Statement of Significance

This work describes the immunomodulation capacity of the previously validated antigen delivery system (ADS) composed by dioctadecyldimethylammonium bromide (DODAB) and monoolein (MO) lipids incorporating the cell wall surface proteins (CWSP) from *C. albicans*. Here, we not only present the ability of this system in facilitating antigen uptake by DCs in vitro, but also that this system induces higher levels of pro-inflammatory cytokines and opsonizing specific IgG antibodies in serum of mice immunized subcutaneously. We show that the ADS are efficient nanocarrier and modulate the immune response against intravenous *C. albicans* infection favoring mouse protection. In sum, we show that the incorporation of *C. albicans* antigens in DODAB:MO nanocarries are a promising vaccine strategy against *C. albicans* fungal infection.

1. Introduction

Vaccines are routinely used to protect against microbial pathogens. They usually contain antigens as surrogates of the disease-causing microorganism or a product of it. Antigens may include weakened or inactivated forms of the target pathogen, or subunits

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that could be constitutive or extracellular molecules [1]. Nevertheless, the use of attenuated pathogens raises several safety issues due to possible reversion of the phenotype or residual virulence. These safety problems may be circumvented by using subunit vaccines. In such case, univalent subunit vaccines may have their effectiveness limited due to antigen variations in the target pathogens. The use of complex antigen extracts, instead of single molecules, in the immunogenic preparations may be a way to overcome this issue [2]. Yet, the effective implementation of subunit vaccines is frequently impaired by insufficient immunogenicity when administered without adjuvant [3,4]. Therefore, selecting an appropriate adjuvant or delivery system is as important as selecting antigen candidates.

Formulating protein antigens into nanoparticles has emerged as one of the most promising strategies to enhance the immune response to vaccine antigens [5–9]. Cationic liposomes are interesting adjuvants that also serve as carriers for the targeted delivery of antigens to immune cells. These liposomes tightly bind negatively charged antigens, which may render soluble antigens into a particulate form thereby increasing their *in vivo* half-life [7,9]. In fact, cationic liposomes were used as adjuvants in several studies, enhancing cell mediated or humoral immunity, as well as delivery systems for drugs, DNA or peptides [5,6,10]. Major limitations behind the fact that as yet no adjuvant based on liposomes has been registered for human use seem to be their stability, manufacturing and quality assurance problems [11]. However, these limitations may be overcome by tuning physicochemical properties like size, charge and hydrophobicity [12–14]. Recently, a direct comparative study showed that cationic liposomes were most efficient for the induction of effector antigen-specific T cells *in vivo*, than poly-(lactic-coglycolic-acid) (PLGA) nanoparticles or the clinically used adjuvants Montanide ISA-51 and SWE, a squalene oil-in-water emulsion [13].

Cationic liposomes composed by surfactant dioctadecyldimethylammonium bromide (DODAB) have been used as carriers in drug delivery studies [8,15] as well as adjuvants in vaccination strategies, displaying higher colloidal stability than aluminum hydroxide and better efficacy in inducing cellular immune responses [16–18]. The main advantage of DODAB as an adjuvant is that it requires a lipid concentration lower than the concentrations traditionally used in liposomal formulations [19,20]. However, these preparations can be physically unstable and therefore the incorporation of different neutral molecules, such as cholesterol, 1-monopalmitoyl glycerol and trehalose 6,60-dibehenate have shown to improve the stability without undermining their adjuvanticity [21]. In previous studies we have demonstrated that monoolein (MO), when incorporated as helper lipid with DODAB, could act as a stabilizer, conferring fluidity to the DODAB nanoparticle liposomes by favoring lipid chain mobility [22]. We have successfully used DODAB:MO as a mammalian cell transfection system and as a nanocarrier for *in vitro* gene silencing [15,23]. In particular, we demonstrated that liposomes formed by DODAB and MO at DODAB:MO (1:2) molar ratio, assembled mainly as positively spherical bilamellar vesicles with some internal structures [24]. In this way, in a recent report, we explored this formulation, DODAB:MO (1:2), and described the development of two liposomal nanoparticle antigen delivery systems (ADS), ADS1 and ADS2, loaded with *Candida albicans* cell wall surface proteins (CWSP) as antigens. These ADS assembled as stable negatively charged spherical nanoparticles with an average particle size of approximately 280 nm, indicating that the CWSP readily associated with the liposomes [25]. This efficient adsorption onto the liposomes and their size, mimicking that of natural pathogens, induced a strong, humoral and cell-mediated immunity when compared with free CWSP [26]. *C. albicans* is an opportunistic human pathogen and is by far the most common cause of fungal invasive infections [25]. Despite the availability of new antifungal agents, candidemia is the fourth most common bloodstream infection in hospitalized patients both in the United States and in many European countries [27–29].

Consequently, antifungal vaccines are currently considered one of the most appealing and cost effective strategies against *Candida* infections [27,30–32]. As far as we know, only two vaccines against *C. albicans* infections have completed Phase I clinical trials, the adhesin-like substance 3 (Als3) with aluminum hydroxide as the adjuvant (NDV-3; NovaDigm Therapeutics) [33], and secreted aspartic protease 2 (Sap2) embedded in a virosomal for adjuvanticity (PEV-7; Pevion Biotech) [34].

In this work, we assessed the effectiveness of the designed DODAB:MO liposomal nanoparticle associated with a *C. albicans* CWSP preparation in inducing protection in a mouse model of systemic candidiasis established by the hematogenous route. Our results showed that these liposomal systems induced strong opsonizing antibody responses and a cell-mediated immune response that allowed a significant protection of infected mice.

2. Material and methods

2.1. Materials

Dioctadecyldimethylammonium bromide (DODAB) was purchased from Tokyo Kasei (Japan). 1-monooleoyl-rac-glycerol (MO), Hanks' balanced salt solution (HBSS), glutaraldehyde, propidium iodide (PI) and DTT were supplied by Sigma–Aldrich (St. Louis, MO, USA). Tris–HCl Buffer was provided by Invitrogen/Molecular Probes (Eugene, OR, USA) and ethanol (high spectral purity) was purchased from Uvasol (Leicester, United Kingdom). Dulbecco's Modified Eagle's Medium (DMEM) was supplemented with 2 mM L-glutamine, (all from Sigma-Aldrich) and 10% heat-inactivated fetal bovine serum (FBS) provided by Lonz (Romania); HEPES-Buffer solution pH 7.5 was provided by VWR International (Radnor, PA, USA) and 1 mM sodium pyruvate by Merck (Frankfurt, Germany). Sytox Green was purchased from Thermo Fisher Scientific (Massachusetts, MA, USA) and propidium iodide (PI) was obtained form Sigma-Aldrich.

2.2. Culture conditions of C. albicans strains

C. albicans strain SC5314 was used for CWSP extraction while *C. albicans* 124A clinical isolate [35] was used for infection experiments. All strains were maintained as frozen stocks in 30% glycerol at −80 °C. When needed, yeasts were obtained from a 2 day YPD agar plate (2% D-glucose, 1% Difco yeast extract, 2% peptone and 2% agar) (w/v) incubated at 30 °C.

2.3. Extraction of yeast CWSP

All procedures used for CWSP extraction were performed in a sterile environment and using apyrogenic solutions. CWSP were released from intact yeast cells by DTT treatment as described previously [26]. The concentrated proteins obtained were stored at −80 °C in aliquots of 100 μg/ml.

2.4. Preparation and characterization of CWSP-loaded liposomes

DODAB:MO based liposomes were prepared using the lipid-film hydration method [36]. Briefly, DODAB and MO, at a DODAB molar fraction (χDODAB) of 0.33, were dissolved in ethanol and mixed in a round-bottom flask. The solvent was removed by rotary evaporation, at a temperature 10 °C above the main phase transition of DODAB (Tm ≈ 44 °C), and liposomes formed after hydration of

the lipid film with 25 mM HEPES pH 7.5 at 55 °C. The dispersion was then placed in a bath sonicator during 2 min.

Two liposomal stock dispersions were prepared, stock 1, used for ADS1, at a total lipid concentration of 1774 μg/ml and stock 2, used for ADS2, at a total lipid concentration of 266 μg/ml. For the ADSs assembling, equal volumes of CWSP were added to the respective stock dispersions post lipid-film hydration. For both ADS the final concentration of CWSP was 50 μg/ml. For ADS1, the final total lipid concentration was 888 μg/ml and for ADS2 was 133 μg/ml (Table 1). The empty liposomes (EL) were always used at a final total lipid concentration of 888 μg/ml. These formulations were then incubated for 1 h, at 55 °C, to ensure CWSP adsorption followed by a brief sonication step in a water-bath sonicator.

2.5. Quantification of protein retention

ADS1, ADS2 and empty liposomes were prepared as described in Section 2.4. Protein retention was evaluated at different time points, 0, 3, 24, 48 and 72 h after preparation. The prepared formulations were pelleted by ultracentrifugation (100,000*g* for 1 h), the pellet submitted to TCA protein precipitation (Thermo Scientific Pierce), and the proteins quantified with the BCA Protein Assay Kit (Thermo Scientific Pierce), according to the manufacturer's instructions. Empty liposomes were used as a negative control in order to exclude lipid interference in the protein quantification method.

2.6. Stimulation of bone marrow-derived DC (BMDC)

Bone marrow cells were collected from femurs and tibias of female BALB/c mice by flushing with cold RPMI 1640 (Sigma). BMDC were differentiated as described by Cerca et al. [37]. Briefly, cells (1×10^6/mL) were cultured in 6-well plates in RPMI supplemented with 15% (v/v) J558-cell supernatant, 10% FBS penicillin (100 U.I./mL)-streptomycin (100 μg/ml) (Sigma), and L-glutamine (2 mM) (Sigma) and incubated at 37 °C and 5% CO_2. Half of the medium was renewed every two days. At day 8, BMDCs were detached and distributed in 96-well round bottom plates adjusted at a concentration of 2×10^5 cells/well in supplemented RPMI medium. Differentiation of bone marrow precursors into BMDC was confirmed by flow cytometry assessing surface expression of CD11c, CD80, CD86 and MHC class II (Fig. S1). Immediately after being seeded, cells were stimulated with 10 μl of CWPS (0.5 μg), ADS1 (0.5 μg of CWSP loaded in 8.8 μg of total lipid), ADS2 (0.5 μg of CWSP loaded in 1.33 μg of total lipid) or EL (8.8 μg of total lipid) in a final volume of 200 μl. LPS (1 μg/ml) (Sigma) and un-stimulated cells were used as positive and negative controls of activation, respectively. After 6 or 24 h, the culture supernatants were removed and stored at -80 °C. For the assessment of cell surface markers, after stimulation, the BMDC were collected from the culture plates, washed twice in Hanks's Balanced Salt Solution (Sigma) and incubated with specific monoclonal antibodies (mAb). The following mAbs, along with their respective isotype controls were used (at previously determined optimal dilutions [38] for immunofluorescence cytometry: fluorescein isothiocyanate (FITC) hamster anti-mouse CD11c (HL3), phycoerythrin (PE) anti-mouse CD80 (B7-1) (16-10A1); phycoerythrin-cyanine 7 (PE-Cy7) anti-mouse CD86 (B7-2) (GL1); peridinin-chlorophyll protein (PerCp) anti-mouse I-Ad/I-Ed (clone 2G9); all from BD Biosciences Pharmingen, San Diego, CA. The analyzed cell samples were always pre-incubated with anti-FcγR mAb before the antibody incubation to prevent non-specific antibody binding. All cytometric measurements were performed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter). The collected data files were analyzed using FLOWJO X 10.0.7r2 software.

The concentrations of IL-23, IL-12 and TNF-α in cell culture supernatants were quantified with the respective Mouse enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go kit (eBioscience, San Diego, CA) while IL-10 was quantified using the Mouse IL-10 DuoSet ELISA development system (R&D Systems, Minneapolis, MN); according to the manufacturer's instructions.

Table 1
Composition, mean size and ζ-potential of ADS. DODAB:MO liposomes were mixed with CWSP for 1 h to prepare ADS1 and ADS2. Mean size and ζ-potential were measured by dynamic light scattering on a ZetaSizer NanoZS. PDI: polydispersity index.

	ADS1	ADS2	CWSP	Empty liposomes
Total lipid (μg/ml)	888	133	–	888
CWSP added (μg/ml)	50	50	50	–
Mean size (nm)	223 ± 37	255 ± 46	88.7 ± 5.1	176.8 ± 23
PDI	0.19 ± 0.015	0.25 ± 0.017	0.63 ± 0.01	0.24 ± 0.08
ζ-potential (mv)	−18.3 ± 1.5	−21.8 ± 1.4	−14 ± 0.7	54.6 ± 3.2

2.7. Cellular delivery of proteins via ADS

Confocal microscopy was performed as described before [26]. Briefly, macrophages (RAW 264.7 cell line) were plated in 6-well chamber plates (Ibidi) 3×10^5 cells/well and left to adhere overnight at 37 °C in a humidified atmosphere of 5% CO_2. Prior to ADS preparation, Rhodamine (Rho) DHPE (at a molar ratio of 1:200) was incorporated into DODAB:MO liposomes during the preparation phase, prior to solvent removal by rotary evaporation. Before incubation with Rho-DHPE labeled ADS, macrophages were labeled with Wheat Germ Agglutinin Alexa Fluor 633 Conjugated. Labeled macrophages were then incubated with labeled ADS, the microscopy chamber plate was placed in the integrated chamber (37 °C, 5% CO_2) of LSM 780 Carl Zeiss and a mid-point cell thickness view images and z-stack images were obtained after 60 min. Images were analyzed using ZEN 2012 lite software (ZEISS).

2.8. Immunization procedures

Female BALB/c mice, 8–10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific-pathogen-free conditions at the Animal Facility of the Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), the 86/609/EEC directive and Portuguese rules (DL 129/92). Twenty BALB/c mice per group, were injected subcutaneously three times with a 2-week intervening period, with 200 μl of one of the following preparations: CWSP alone (50 μg/ml); DODAB:MO EL (888 μg/ml), ADS1 (50:888 μg/ml; CWSP:Lipid) or ADS2 (50:133 μg/ml; CWSP:Lipid). Additionally, a group of six mice was injected with HEPES-Buffer (vehicle), following the same immunization procedure. The schematic representation of the immunization protocol is shown in Fig. S2. Blood samples were collected in the submandibular vein on day 35 of the immunization protocol to obtain serum samples to confirm immunization efficiency.

2.9. C. albicans hematogenously disseminated infections

BALB/c mice were infected intravenously (i.v.) with 1×10^5 *C. albicans* yeast-form cells, according to the schedule presented in Fig. S2. *C. albicans* inoculum for infection was grown in a shaking incubator for 14 h at 30 °C in Winge medium (0.2% glucose, 0.3%

yeast extract). Yeast cells were harvested, washed twice with sterile, apyrogenic phosphate-buffered saline (PBS), counted in a hemocytometer, and resuspended at the appropriate concentrations. Inocula were always confirmed by colony forming units (CFU) counts on YPD agar plates at 37 °C for up to 48 h. In order to assess survival, eight mice of each of the four immunized groups, and the mouse group treated with the vehicle alone, were infected, weighted and monitored twice daily to evaluate the progress of hematological disseminated candidiasis. Mice attaining established human end points were humanely sacrificed using isoflurane anesthesia followed by cervical dislocation. Their deaths were recorded as occurring on the following day. To determine immunological parameters, eight mice of each immunized group were also infected with 1×10^5 *C. albicans* cells and sacrificed three and seven days after infection to collect blood and spleens.

2.10. Intracytoplasmic cytokine quantification

At days 49 (before infection), 52 and 56 (three and seven days post-infection, respectively) four immunized mice per group were sacrificed and the spleens were aseptically removed, homogenized in Hanks' balanced salt solution (Sigma) and red blood cells lysed with 0.15 M ammonium chloride. The remaining cells were counted and plated in round-bottom 96-well plates (Nunc, 1×10^6 cells) in RPMI-1640 compete medium [RPMI-1640 (Sigma) supplemented with 10% FBS, HEPES (10 mM), penicillin (200 IU/ml), streptomycin (200 g/ml) (all from Sigma) and 2-mercaptoethanol (0.1 mM) (Merk)]. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37° for 5 h under stimulation with 20 ng/ml PMA (Sigma), 200 ng/ml ionomycin (Merk) and 10 ng/ml brefeldin A (Epicentre Biotechologies, Madison, WI, USA). Then, cells were recovered and non-specific antibody binding was prevented by the pre-incubation with anti-FcγR mAb followed by incubation with anti-CD4 peridinin-chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5)-conjugated (clone RM4-5). Following extracellular CD4 staining cells were washed, fixed in 2% formaldehyde, washed again and permeabilized with 0.05% saponin (Sigma) in PBS solution. Intracytoplasmic staining was carried out with anti-interferon-γ (IFN-γ) FITC-conjugated (clone XMG1.2) (Biolegend) and anti-IL-10 PE-conjugated (clone JES5-16E3) (BD Biosciences) or with anti-IL-4 PE-conjugated (clone BVD4-1D11) (BD Biosciences) and anti-IL-17A FITC conjugated (clone TC11-18H10.1) (Biolegend). Antibody-labeled cells were analyzed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter). At least 150 000 events were acquired per sample. The collected data files were analyzed using FLOWJO X 10.0.7r2 software.

2.11. In vitro antigen-recall stimulation assay and cytokine quantification

To assess cytokine production by CWSP-stimulated spleen cells, 5 ml aliquots of cell suspensions prepared as described above for intra-cytoplasmic staining, were layered onto 2.5 ml of a polysucrose-sodium ditrizoate solution (Histopaque 1083®, Sigma) and centrifuged at 800*g* for 20 min at room temperature. Mononuclear cells collected from the medium–Histopaque interface were collected, washed, suspended in RPMI-1640 complete medium, plated (5×10^5/well) in round-bottom 96-well plates, and stimulated with CWSP (final concentration of 20 μg/ml) for 3 days at 37° and 5% CO_2. The concentrations of IFN-γ, IL-4 and IL-17A in cell culture supernatants were quantified with the respective Mouse ELISA Ready-Set-Go kit (eBioscience, San Diego, CA) while IL-10 was quantified using the Mouse IL-10 DuoSet ELISA development system (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.12. Quantification of CWSP-specific antibodies

Specific anti-CWSP immunoglobulins in the collected serum were quantified by ELISA according to Ferreirinha et al. [39]. Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml CWSP and incubated overnight at 4 °C. Wells were then saturated for 1 h at room temperature with 1% BSA in Tris-saline Tween 20 (TST) (w/v) and serial dilutions of the serum samples were plated and incubated for 2 h at room temperature. After washing, alkaline phosphatase-coupled monoclonal goat anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL) were added and incubated for 1 h at room temperature. After washing, the specifically bound antibodies were detected by adding the p-nitrophenyl phosphate (Sigma) substrate solution and on development the reaction was stopped by the addition of 0.1 M EDTA, pH 8 solution. The absorbance was measured at 405 nm, subtracting for each well the value of the absorbance at 570 nm. The antibody titers were expressed as the reciprocal of the highest dilution with an absorbance 2 fold higher than the value of the control (no serum added).

2.13. Phagocytosis of opsonized C. albicans cells

Phagocytosis was assessed by flow cytometry following a previously described method [40]. Briefly, fixed *C. albicans* cells were labeled with Sytox Green and incubated in Dulbecco's modified Eagle medium (DMEM) during 30 min at 37 °C with 20% mouse serum collected from mice immunized thrice with CWSP, ADS1, ADS2 or EL. Serum from four independent mice was used, each one in triplicate. Then, cells were washed twice with PBS and resuspended in DMEM without serum. RAW 264.7 macrophages were incubated with labeled yeast suspensions at a multiplicity of infection (MOI) of 1 macrophage per 5 yeast cells, for 30 min, at 37 °C and 5% CO_2. After incubation, plates were kept on ice to stop phagocytosis, and wells rinsed twice with PBS to remove unbound yeasts. Macrophages and associated yeasts were then incubated with PI at a final concentration of 6 μg/ml, for 5 min at RT. The percentage of macrophages with internalized cells ($Sytox^+$ PI^- and $Sytox^+PI^+$), as well as the percentage of macrophages with adherent cells ($Sytox^-PI^+$), was determined as previously described [40]. Phagocytosis was confirmed by confocal microscopy (Leica SP2 AOBS SE) and images were analyzed using Fiji-ImageJ software 2.00 (NIH-USA).

2.14. Statistical analyses

Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni post-test to compare the mean values of the different groups, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Unless otherwise stated, results shown are from at least three independent experiments with three replicates. Differences were considered significant when the *P* value was lower than 0.05.

3. Results

3.1. Characterization of ADS, quantification of protein retention and delivery

ADS used here were prepared using preformed DODAB:MO liposomes loaded with CWSP (50 μg/ml) as previously described [26]. ADS1 presented a size around 223 ± 37 nm with a polydispersity index (PDI) of 0.19 ± 0.015, while ADS2 had a similar size, 255 ± 46 nm, but were more polydisperse, 0.25 ± 0.017 (Table 1). These ADS were negatively charged, with ADS1 presenting a

ζ-potential value of −18.3 ± 1.5 mV and ADS2 a ζ-potential value of −21.8 ± 1.5 mV, while DODAB:MO EL exhibited a ζ-potential value of 54.6 ± 3.2 mV, showing that antigenic proteins (CWSP) are surface adsorbed to liposomes. Owing to the complexity of *Candida* cell wall antigens and their surface localization, we thus focused on the stability over time of the proteins associated with the liposomes when stored at 4 °C. Immediately after formulation assembling (time 0), ADS1 presented 91 ± 12% of the proteins adsorbed while ADS2 showed only 25 ± 12% (Fig. 1). Although by 48 h approximately 12.6% of CWSP were released from the surface of ADS1 liposomes and 17.1% from the surface of ADS2, no significant changes were observed over 72 h after preparation. Thus, in this study, both ADS1 and ADS2 were used within 48 h after preparation to ensure the% of CWSP absorbed. In this way, the effective concentration calculated taking into consideration the 50 μg/ml of total protein initially added ranged from 45.5 ± 6–39.2 ± 3 μg/ml (91 ± 12–78.4 ± 5 of CWSP absorption) for ADS1 and from 12.5 ± 6–10.3 ± 1 μg/ml (25 ± 12–20.7 ± 3% of CWSP absorption) for ADS2. These results highlighted the fact that the interactions between DODAB:MO liposomes and CWSP proteins are strong and protein adsorption on to ADS1 or ADS2 is stable, at least over a range of 72 h after liposomes preparation and storage at 4 °C.

In a previous study we have shown that ADS1 and ADS2 were non-toxic to J774A.1 macrophages and were avidly internalized by these cells [26]. In this study we confirmed by confocal microscopy that after 60 min of incubation ADS1 also effectively delivered CWSP into RAW 264.7 macrophages (Fig. 2). The results obtained with these different cell lines, indicate that these ADS are able to efficiently deliver antigens into macrophages that may trigger T and B cells *in vivo*.

3.2. Immunostimulatory effect of ADS on dendritic cells

The activation of antigen presenting cells (APC), such as dendritic cells, is one of the fundamental steps in inducing an effective *in vivo* immune response [14]. Therefore, we evaluated the ability of the formulations to activate BMDC *in vitro* by measuring surface expression of T cell co-stimulatory (CD80 and CD86) and MHC class II molecules, and by quantifying the production of pro-inflammatory cytokines TNF-α, IL-12 and IL-23 and anti-inflammatory cytokine IL-10 in the culture supernatants.

Fig. 3a shows that upon 6 h stimulation with CWSP, ADS1 and ADS2, BMDC already presented a significantly higher expression of all the surface activation markers analyzed in comparison with unstimulated or EL stimulated cells. At this time point, no higher expression of the assessed BMDC cell surface markers was induced by any ADS, as compared to CWSP. Actually, ADS1-induced CD80 expression was lower than the one induced by CSWP. Contrastingly however, after 24 h incubation a markedly higher surface CD80, CD86 and MHC II expression was observed in the BMDC stimulated with ADS1 as compared to that induced by free CWSP (Fig. 3a). In contrast to ADS1, ADS2 did not promote an enhanced expression of those dendritic cell surface markers, comparatively to CWSP.

BMDC stimulated for 6 h with either CWSP or ADS2 produced similar levels of TNF-α and IL-12 as those stimulated with LPS, a strong activator of the innate immune system and a potent inducer of inflammation [41]. CWSP and ADS2 stimulated cells also produced significantly higher amounts of IL-23 and IL-10 when compared with stimulation by ADS1 or EL (Fig. 3b). After 24 h stimulation, the levels of TNF-α, IL-12 as well as of IL-10 were maintained or enhanced in the cell culture supernatants of CWSP- or ADS2-stimulated BMDC, while those of IL-23 were lower, as compared to the respective ones detected at 6 h. Contrastingly, stimulation with ADS1 led to intermediate levels of all cytokines, ranging between the ones detected when using CWSP or ADS2, and EL (Fig. 3b).

These results suggested that although both ADS1 and ADS2 activated BMDC, ADS2 and free CWSP appeared to be the most inflammatory stimuli. While ADS1 could stimulate BMDC, as assessed by significantly enhanced expression of surface co-stimulatory and MHC II proteins, the induced production of inflammatory cytokines as well as of IL-10 were significantly lower than the ones observed for ADS2 and CWSP.

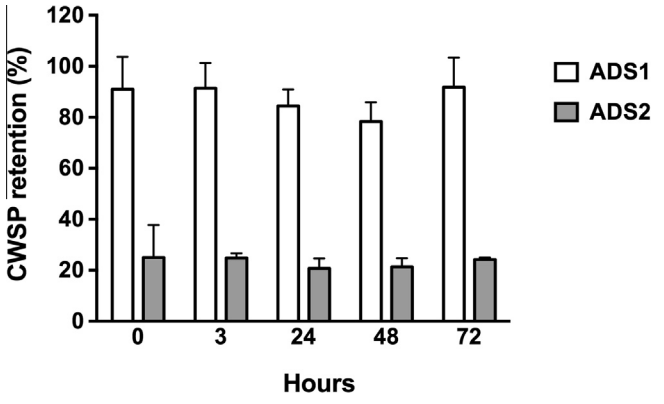

Fig. 1. Percentage of CWSP retention in ADS1 and ADS2 over time. ADS were prepared, stored at 4 °C, and retention of CWSP antigen was monitored during 72 h after preparation. Each bar represents mean + SD of the percentage retention of initial antigen added (50 μg/ml for both ADSs). Data shown are representative results of two independent experiments (N = 3 per experiment).

3.3. Production of antigen-specific antibodies in vivo

Having determined that ADS1 and ADS2 stimulated BMDC, we next immunized BALB/c mice with these formulations. The serum titers of CWSP-specific IgG1 and IgG2a antibodies were determined in mice immunized with free CWSP, ADS1 and ADS2 or sham-immunized with EL as controls. As previously reported, mice immunized with free CWSP or ADS raised antigen-specific IgG antibodies that were significantly higher in the ADS1 immunized group, as compared to the other assessed groups (Fig. 4a). Mice immunized with ADS2 or free CWSP presented similar levels of anti-CWSP IgG1 and IgG2a antibodies. The efficacy of serum from immunized mice in enhancing the phagocytosis of *C. albicans* cells was then tested *in vitro* and analyzed by flow cytometry using a previous described method that enables the discrimination of yeast cells that are internalized from yeast cells that are only adhered to phagocytes (fig. 4c) [40]. As shown in Fig. 4b, *C. albicans* cells pre-treated with serum from mice immunized with ADS1 and ADS2 were significantly more internalized by macrophages than non-opsonized yeast cells or cells opsonized with serum from mice sham-immunized with EL or immunized with CWSP alone. This indicates that the increased percentage of *C. albicans* yeasts internalized by macrophages was due to CWSP-specific IgG yeast opsonization. Although serum from CWSP-immunized mice contained CWSP-specific IgG, no differences in the percentage of internalized yeast were observed for yeast cells treated with this serum in comparison with cells treated with serum from mice immunized with EL. This result might be a consequence of low CWSP-specific IgG titles detected in serum of mice immunized with free antigen. According to all these results, mice immunized with ADS presented a significant increase in serum CWSP-specific antibody titers before infection that markedly enhanced *ex vivo* phagocytosis of *C. albicans* yeast cells.

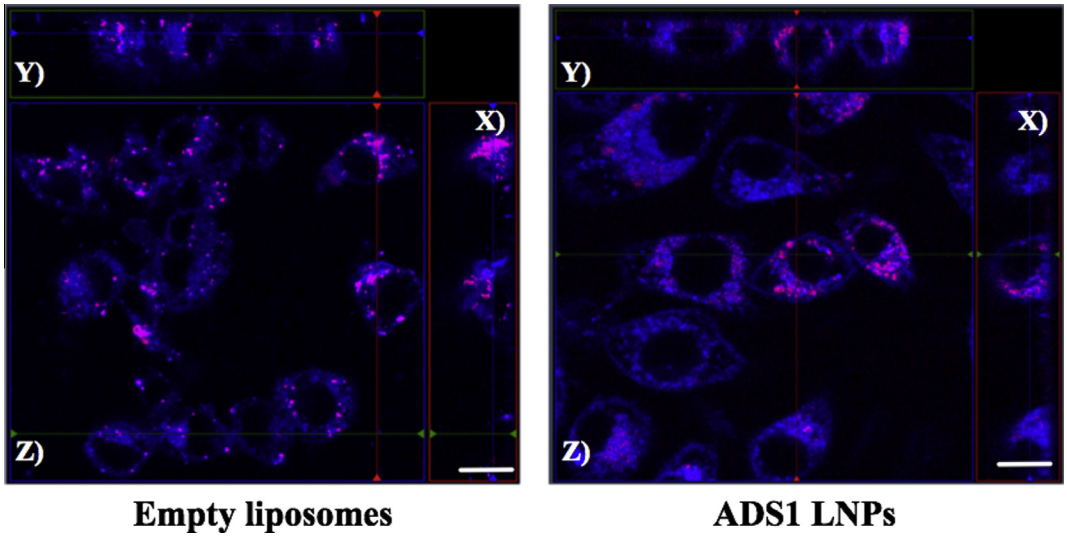

Fig. 2. Cellular uptake of empty liposomes and ADS1 after 1 h incubation. The membranes of macrophage cells were labeled with wheat germ agglutinin Alexa Fluor® 633 conjugate followed by incubation with rhodamine-labeled liposomes. Representation of a mid-point thickness view (X68, Y44, Z24). (z). Z-axis rotations of a single transverse slice through two sections of the cell: view in the x-0-z plane (x) and view in the y-0-z plane (y). The scale bar represents 10 nm.

3.4. ADS vaccination protects against hematogenously disseminated C. albicans infection

In order to evaluate the protective effect of ADS1 and ADS2 immunization against systemic candidiasis, s.c. vaccinated and control mice were challenged at day 49 with a clinical *C. albicans* isolate obtained from a patient with systemic infection (strain 124A [35]). The immunization protocol is schematized in Fig. S2 (supplementary data). Mice were weighted and visually monitored for signs of disease over 50 days. Mice sham-immunized with EL were the first attaining established humane endpoints, by day 8 upon infection and were closely followed by mice of the CWSP and ADS2 immunized groups. In the ADS1 immunized group, only by day 15 the first mouse death was recorded (Fig. 5). Moreover, only the ADS1 immunized group presented a significantly extended survival as compared to any of the other experimental groups. By the end of the experimental period, 62.5% of the mice immunized with ADS1 survived infection as compared to 12.5% in the EL treated group and 100% mortality in the CWSP and ADS2 immunized groups. These results show that ADS1, but not the other immunogenic preparations, conferred protection against *C. albicans* challenge. Moreover, as the *C. albicans* strain used to isolate CWSP antigens was different from the one used to infect the immunized mice it may indicate that achieved protection could be strain-independent.

3.5. Splenocytes stimulation and intracellular cytokine quantification

Cell-mediated immunity is essential for the control of *C. albicans* and Th1- and Th17-type cytokines are critical for coordinating protective immunity against the fungus [42]. Thus, to determine whether the immunization with either ADS could induce a particular type of T cell polarization, the expression of IFN-γ, IL-4, IL-10 and IL-17 was assessed by flow cytometry in splenic $CD4^+$ T cells collected from mice of the different assessed groups (Fig. 6). IFN-γ and IL-17 are main effectors in the protective immune response to *C. albicans* [43]. The detected proportions of IL-17^+CD4^+ T splenocytes were significantly and markedly higher in the ADS1-immunized mice, as particularly evident in 3-day infected mice. Contrastingly, the frequency of splenic $CD4^+$ T cells producing IFN-γ was not majorly altered among groups upon infection. Nevertheless, significantly lower frequency of IFN-γ^+CD4^+ cells was detected in the ADS1-immunized mice as compared with the frequency found for CWSP-immunized mice. Immunization with ADS1 or ADS2 also increased the proportions of splenic IL-4^+ and IL-10^+ $CD4^+$ T cells, as compared with mice immunized with EL or CWSP (Fig. 6a). The increase in $IL4^+CD4^+$ T cell proportions was higher only in ADS1 immunized mice, as compared to CWSP immunized counterparts. These results indicate that immunization with ADS, and more markedly with ADS1, induced a balanced immune response encompassing pro- and anti-inflammatory cytokine production.

Cytokine production was also assessed by ELISA in culture supernatants of CWSP-stimulated splenocytes obtained from the different used mouse groups, Before infection, a mild production of pro-inflammatory cytokines IFN-γ and IL-17 was detected in CWSP-stimulated splenocytes of mice immunized with ADS1. That was nevertheless higher than that detected in splenocytes of mice immunized with CWSP or EL in which no or very little production of these cytokines was detected (Fig. 7). At this time point, a mild production of IFN-γ was also detected in similarly stimulated splenocytes of ADS2 immunized mice. In the cultures of splenocytes collected at day 53 (3 days after *C. albicans* i.v. infection), the levels of IFN-γ were found significantly increased in culture supernatants of all assessed splenocyte groups. However, production of IL-17 and of IL-4 was markedly higher in the groups corresponding to ADS1 immunized mice, detected in cultures of splenocytes collected at days 52 and 56 (3 and 7 upon infection, respectively). Splenocytes of the ADS2 immunized group also produced these cytokines upon stimulation, although at lower levels than those detected in the ADS1 group. No IL-17 production was detected in the remaining splenocyte culture from other mouse groups. IL-4 production by splenocytes of the CWSP immunized mice was detected, although at very low levels (Fig. 7). Interestingly in the cultures of splenocytes collected from both ADS immunized groups 3 and 7 days upon infection, significantly higher levels of IL-10 were detected upon the antigenic stimulation than in the other two groups (Fig. 7).

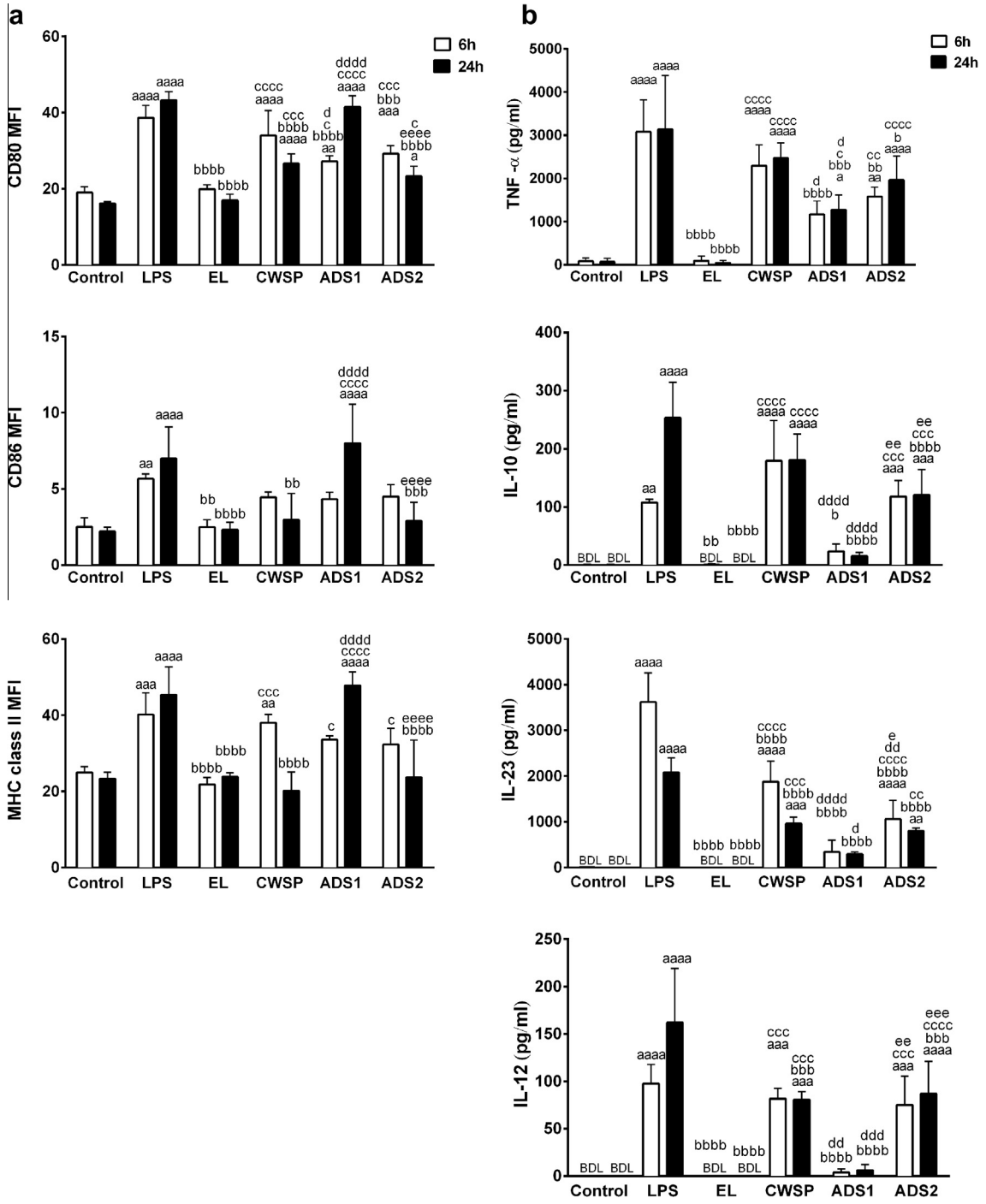

Fig. 3. BMDC activation by ADS. BMDC were incubated *in vitro* with medium alone (control) or stimulated with LPS, EL, CWSP, ADS1 or ADS2 as indicated, for 6 or 24 h. (a) Flow cytometric analysis of CD80, CD86 and MHC class II molecule expression on the surface of BMDC. Bars represent mean fluorescence intensities (MFI) + SD due to respective mAb staining of the indicated molecules. (b) Concentrations of the indicated cytokines detected in BMDC culture supernatants (bars represent mean + SD). Data shown in (a) and (b) are pooled results from four independent experiments (n = 3 per group per experiment). BDL: below detection limit. Statistical significant differences are represented above bars. Comparisons between control, LPS, EL, CWSP and ADS1 and the other groups are indicated by letters a, b, c, d and e, respectively (one, two, three and four letters correspond to $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively).

4. Discussion

The surge of *Candida* species infections coupled to higher resistance rates to antifungal drugs resulted in a tremendous increase in the prevalence of systemic candidiasis in hospitalized patients over the past two decades [30–33]. Hence, the development of novel immune-based approaches that could prevent *Candida* infections is of paramount interest.

In a previous study, we have shown that DODAB:MO liposomes loaded with *C. albicans* CWSP improved the immunogenicity of these antigens which suggested that these liposomes could be promising delivery systems for use in vaccination against

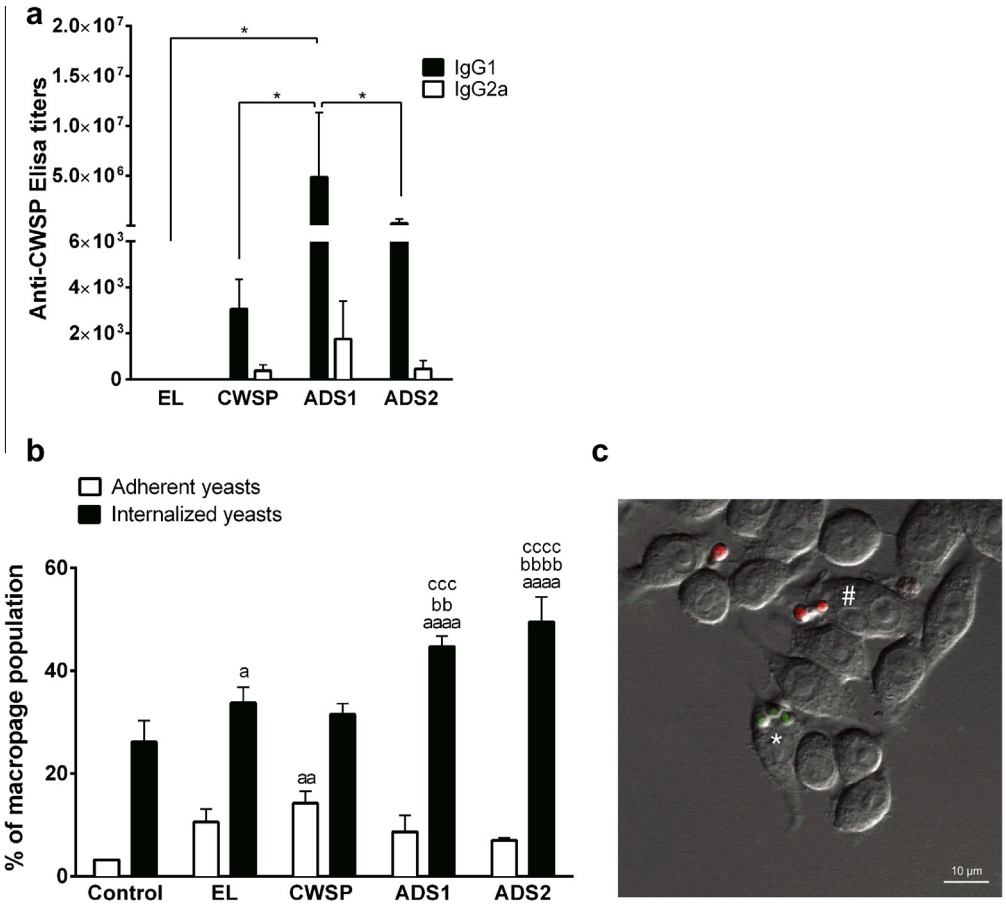

Fig. 4. CWSP-specific IgG antibody titers and opsonophagocytic assay. (a) Specific anti-CWSP IgG1 and IgG2a were quantified in mice immunized s.c. with CWSP, ADS1, ADS2 or EL. Antibody titers were measured by ELISA using serum collected at day 49 (n = 4 per mouse group). The statistical significance between the different groups is indicated above the lines: *P < 0.05. (b) Serum obtained from four mice immunized with CWSP, ADS1, ADS2 or EL were used independently to opsonize yeast cells before interaction with RAW264.7 cells. Three experimental replicates for each serum sample were performed. Phagocytosis was measured by flow cytometry after 30 min of incubation. Closed bars correspond to the mean percentage + SD of macrophage cells with internalized (Sytox Green positive fluorescence) and open bars correspond to the mean percentage + SD of macrophage cells with only adhered yeast cells (PI + staining). Non-opsonized yeast cells are also represented (Control). (c) Representative confocal microscopy image of internalized (Sytox Green$^+$, *) and adhered (PI$^+$, #) non-opsonized yeast cells by RAW264.7 macrophages. Statistical significant differences are represented above bars. Comparisons between control, EL and CWSP and the other groups are indicated by letters a, b, and c, respectively (one, two, three and four letters correspond to P < 0.05, P < 0.01, P < 0.001 and P < 0.0001, respectively).

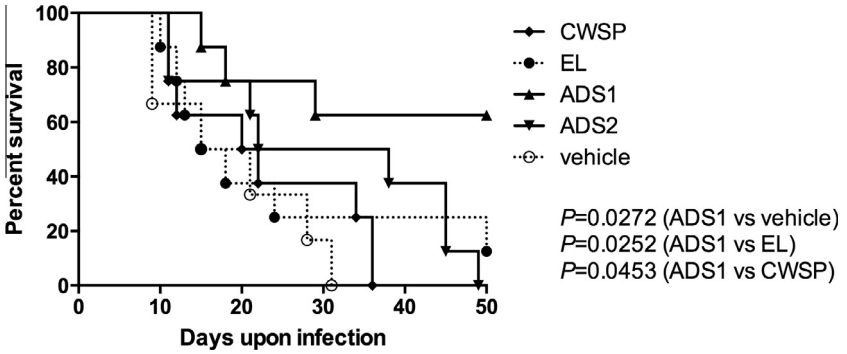

Fig. 5. Protection conferred by ADS vaccination. Naive BALB/c mice were immunized s.c. with buffer (vehicle), EL, CWSP, ADS1 or ADS2, boosted twice, with an intervening interval of 2 weeks, and infected intravenously (i.v.) with 1×10^5 *C. albicans* yeast-form cells, 21 days after the last s.c. immunization. Mice were sacrificed when reaching humane endpoints and deaths were recorded in the following day. Groups that showed statistical significant differences on survival, and significance, were indicated in the figure (n = 6 and n = 8 in control and immunized groups, respectively).

C. albicans [26]. The proteins present in the fungal cell wall are good candidates for vaccine development due to their exposed location [44]. In addition, the combination of antigens that are related to key *C. albicans* virulence attributes or biological functions may induce additive or synergistic immune responses and therefore reduce the probability of fungal immune evasion [45].

The ADS were prepared as two vaccine formulations, ADS1 and ADS2, differing only in the total lipid concentration used for CWSP adsorption. ADS1 is composed by approximately 7 times more total lipid than ADS2 and, as a consequence, it retains 91 ± 12% of the protein added, while ADS2 only retains 25 ± 12%, as previously reported [26]. Its characterization confirmed the ADS mean sizes to

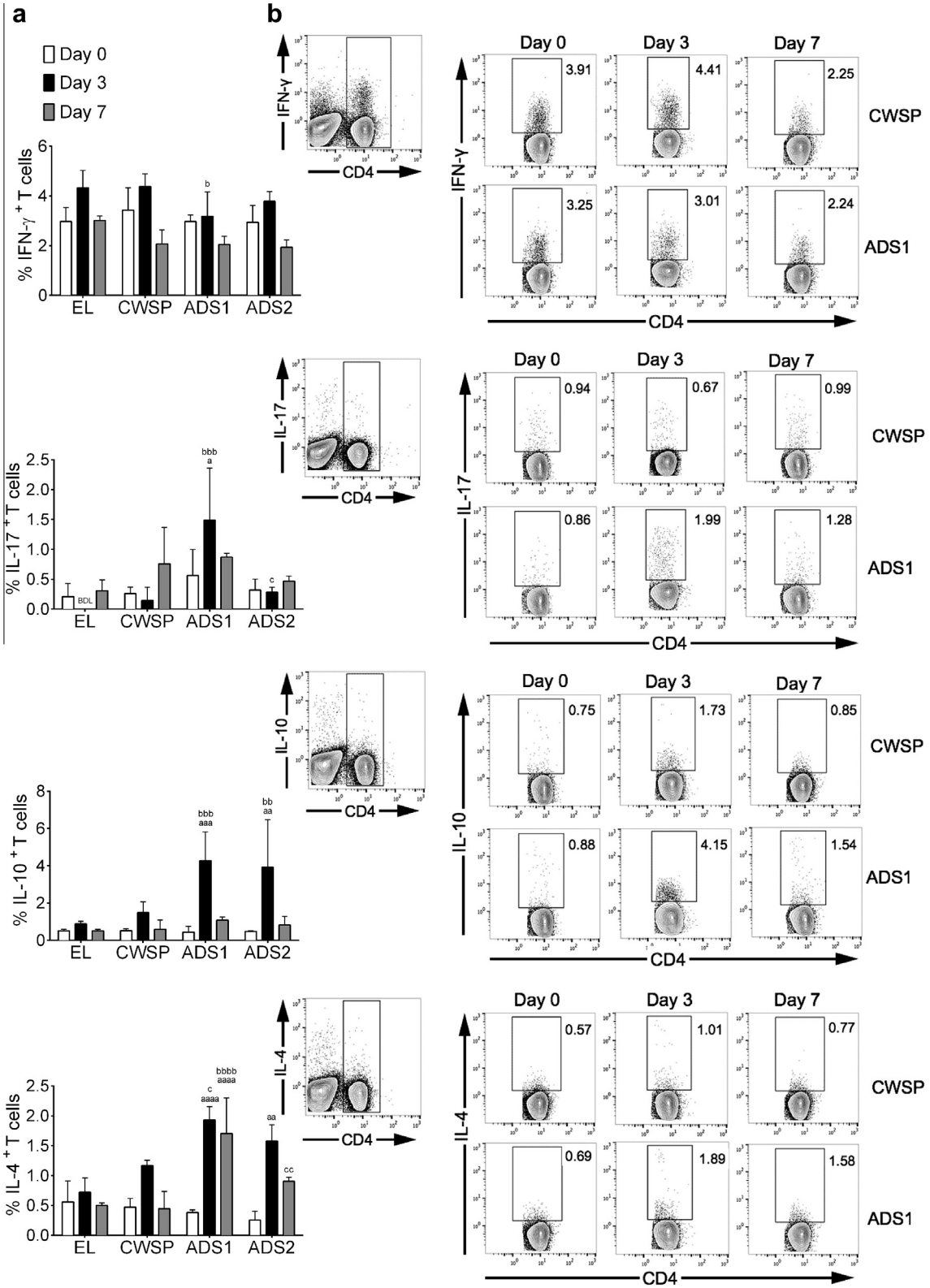

Fig. 6. a) Frequency of IFN-γ^+, IL-17^+, IL-10^+, or IL-4^+ cells in splenic $CD4^+$ T cells from mice s.c. immunized with CWSP, ADS1, ADS2 or liposomes, isolated 21 days after the last s.c. immunization (before infection) and 3 and 7 days post-intravenous (i.v.) infection with 1×10^5 *C. albicans* yeast-form cells. Bars represent mean + SD of four mice used per group. Statistical significant differences are represented above bars. Comparisons between EL, CWSP, ADS1 and the other groups are indicated by letters a, b, and c, respectively (one, two and three letters correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively). (b) Representative examples of flow cytometry analysis of intracellular IFN-γ^+, IL-17^+, IL-10^+, or IL-4^+ expression on gated splenic $CD4^+$ T cells before infection (day 0) or after 3 and 7 days of infection, as indicated, for mice immunized with CWSP or ADS1. BDL: below detection limit. Numbers shown inside dot plots correspond to the frequency of cells as indicated.

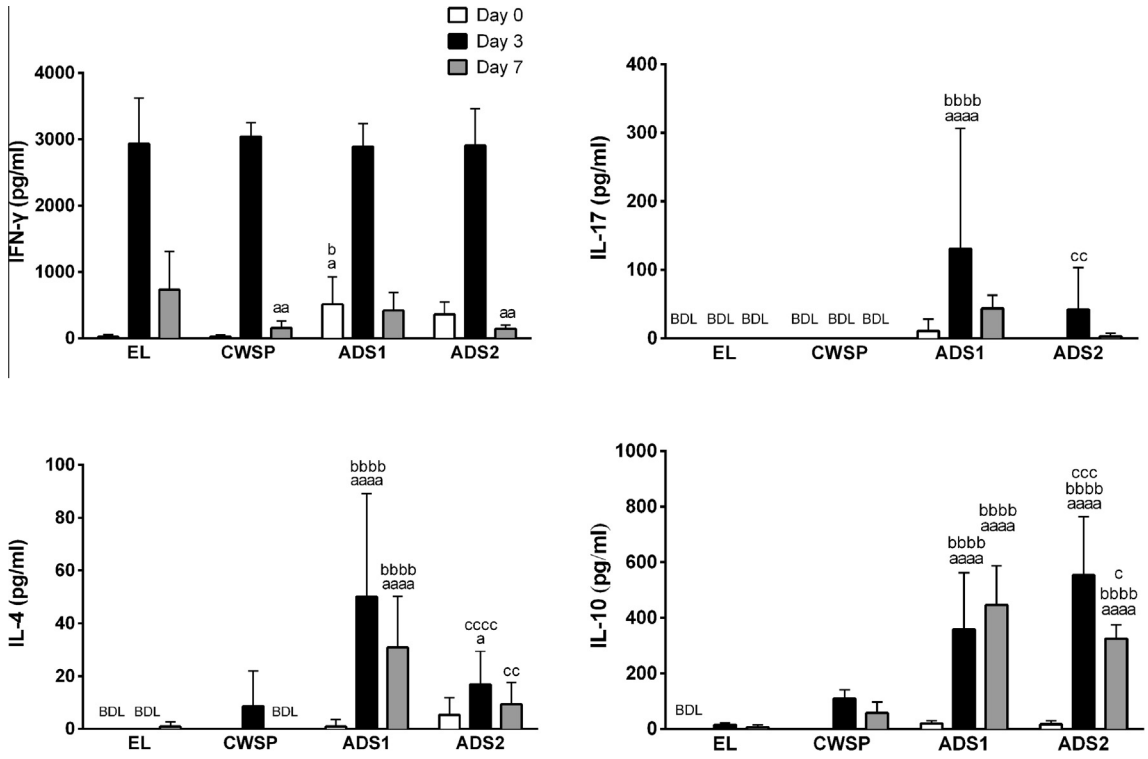

Fig. 7. Cytokine production by splenocytes stimulated with CWSP. Splenocytes from mice s.c. immunized with EL, CWSP, ADS1 or ADS2 were isolated 21 days after the last s. c. immunization before infection (Day 0) and 3 and 7 days post-intravenous (i.v.) infection with 1×10^5 *C. albicans* yeast-form cells. Recall stimulation with CWSP (20 μg/ml) was performed for 3 days and IFN- γ, IL-17, IL-4 and IL-10 quantified by ELISA in the cell culture supernatants. Bars represent mean + SD (n = 4 per group). BDL: below detection limit. Statistical significant differences are represented above bars. Comparisons between EL, CWSP, ADS1 and the other groups are indicated by letters a, b, and c, respectively (one, two, three and four letters correspond to $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively).

be approximately 223 and 255 nm for ADS1 and ADS2 respectively, and that the antigenic proteins are adsorbed to the cationic liposomes, as shown by ζ-potential values. Furthermore, the electrostatic interactions between the cationic liposomes and the anionic proteins are strong enough to retain the antigens at the ADS surface for at least 3 days after preparation. Importantly, the quantity of antigens loaded in the ADS is dependent of the total lipid concentration, but the stability of the core proteins at their surface is not.

DC are important antigen presenting cells (APC) as they are very effective in antigen capturing, processing and subsequent presentation to antigen-specific T cells [41,46]. DC maturation *in vitro* is characterized by up-regulation of MHC II and the co-stimulatory molecules CD80 and CD86 [47]. Un-stimulated DC express moderate levels of CD80, CD86 and MHC-II molecules on their surface. Here we show that stimulation with ADS1, but not ADS2, significantly elevated the expression of these three markers, in comparison to cells exposed to the same concentration of free CWSP. Importantly, the activation promoted by CWSP was more transient than the one obtained with ADS1. This may indicate that delivery of CWSP using the DODAB:MO liposomes resulted in more sustained release of the target antigen or that the particulate nature of the ADS1 improved the antigen uptake by DC. Accordingly, the ability of cationic lipids to improve antigen immunogenicity was also observed in previous reports [48–50]. In contrast to ADS1, ADS2 was not effective in promoting significant DC activation. The lower lipid concentration used in this system, which results in just about 20% of CSWP entrapment, may explain the observed lack of adjuvant effect and the response similar to the one of free CWSP.

IL-12 and IL-23 are members of a family of heterodimeric pro-inflammatory cytokines that can respectively promote Th1 and Th17 responses [51]. IL-12 induces IFN-γ expression in $CD4^+$ T cells [52], and IL-23 maintains Th17 effector function [53]. In contrast, IL-10 might be required to limit host damage under circumstances of strong inflammation [54]. The cytokine profile obtained by stimulating DC with ADS2 and free CWSP, encompassing TNF-α, IL-12 and IL-23 production, revealed a response similar to that elicited by LPS, which could be considered as pro-inflammatory. On the contrary, ADS1 seemed to be less inflammatory since these cytokines were present but in significantly reduced levels. In accordance with a previous report [41], incubation of DC with EL didn't lead to production of significant levels of all cytokines, which indicates that the marked pro-inflammatory response induced by CWSP and ADS2 stimuli was likely due to the presence of high concentrations of free proteins in the incubation solution. In accordance, *C. albicans* cell wall proteins have been previously considered pro-inflammatory [55,56].

Although ADS2 and CWSP induced a more marked pro-inflammatory response by BMDC, ADS1 was the only system conferring significant protection to *C. albicans* challenge. Cytokine production analysis showed that mice immunized with ADS1 presented the highest frequency of splenic IL-17-producing $CD4^+$ T cells. The bias towards IL-17 production in the ADS1 immunized mice was confirmed by *in vitro* recall stimulation of splenocytes with CWSP antigen. Contrastingly, no major differences among groups were noticed in what concerned production of IFN-γ. IL-17 was shown to be a particularly important cytokine in mediating protection against *C. albicans* infections in both humans and mice [57]. Therefore, the detected higher production of IL-17 may have contributed for the protective effect conferred by ADS1, as shown by the extended survival of the mice immunized with this formulation. IFN-γ is also a recognized cytokine in mediating host protection against *C. albicans* [43]. However, as its production did

not markedly vary upon the fungal challenge in the different assessed mouse groups it is unlikely that it may determine the higher protective effect conferred by ADS1. ADS1 was the antigen-delivery system inducing the highest production of CWSP-specific antibodies, which led to, along with ADS2, to highest yeast opsonophagocytosis by macrophage cells. Immunization with ADS1 resulted in higher protection, indicating that protection could also be due to opsonizing antibodies raised by immunization before infection. The host protective role of *C. albicans* cell-wall specific antibodies has been previously documented [58] and might be expected to also contribute for the immunoprotective effect of the assessed ADS.

Although Th17 and Th1 cells, respective producers of IL-17 and IFN- γ, have been shown to be important in the development of protective host responses against *C. albicans* infection [42,59], it is the balance between pro- and anti-inflammatory signaling that defines a successful fungal control [60,61]. In *C. albicans* infected hosts Th1 cells secrete IFN-γ favouring phagocyte cells activity [43] while Th17 cells enhance neutrophil recruitment through secretion of IL-17 [59,62]. Despite Th17 cells through IL-17 production undoubtedly contribute to fungal elimination in infected hosts, the pro-inflammatory effect of these cells may be deleterious to the host [63]. Here, at day 3 post-infection, the pro-inflammatory response was marked in all immunized mice. However, and importantly, also at this time point, the ADS immunized mice showed the highest production of anti-inflammatory cytokine IL-10. As previously remarked, a vaccine that could elicit by itself an inflammatory reaction might end damaging the host when the infectious agent is encountered. In such case, IL-10 production may act as a homeostatic response to keep inflammation under control [54,64]. Indeed, a previous reported experimental vaccine that elicited a mixed response involving pro-inflammatory and anti-inflammatory cytokines could provide host protection in the absence of significant tissue pathology [65]. In mice immunized with ADS1, the production of IFN-γ and IL-17, Th1- and Th17-type cytokines, respectively, as well as IL-4 and IL-10, Th2-type and anti-inflammatory cytokines, respectively, supports the idea that DODAB:MO liposomes contribute to a balanced immune response against *C. albicans* infection, that ultimately culminated in host extended survival, when compared with free CWSP immunization. These results highlight once more the adjuvant potential of these DODAB:MO liposomes, suggesting that a decrease in lipid concentration could be fatal once ADS2 failed in inducing protection against systemic candidiasis.

Currently, scientific interest has grown towards the development of nanoparticles for use as adjuvants and delivery of antigens for vaccine development [66–70]. Other studies, using DODAB liposomes with monophosphoryl lipid A (MPLA) or with trehalose 6,6′-dibehenate (TDB) as adjuvants, also resulted in a protective immune response towards *Chlamydia* infection [71]. Similarly, in another study, the combination of DODAB with a major antigen of *Paracoccidioides brasiliensis* resulted in the lowest numbers of viable yeast cells in mice infected with this fungus in comparison with the use of other adjuvants such as aluminum hydroxide, Freund's Complete Adjuvant (CFA) or flagellin [72]. Other authors have described enhanced protection provided by liposomes with entrapped *Candida* antigens. In one of the studies the authors used a double immunization strategy with priming antigen dose of 50 μg and boosted with 25 μg [61]. Chauhan and co-authors using escheriosomes with entrapped *Candida* antigen were able to protect mice against *C. albicans* infection upon two immunizing administrations using 100 μg of antigen per immunization [73]. Of note, in the present study only a total 30 μg of antigen were used divided in three immunizing administrations, which is a small amount in comparison to that used in other studies, supporting the high adjuvant potential of DODAB:MO liposomes. This was the first time that this novel DODAB:MO liposomal nanoparticle associated with *C. albicans* CWSP was tested in a systemic *C. albicans* infection reaching 62.5% protection. This reveals a good potential for future vaccine design. Nevertheless, the major goal of any vaccine development is to achieve 100% protection. The administration route is a key issue in vaccination by affecting the type and strength of the elicited immune response [74]. Therefore the intradermal and intramuscular routes may be worth to explore in further studies as possible ways to achieve higher protection.

5. Conclusion

This study demonstrated that DODAB:MO-based ADS efficiently enhanced the stimulatory effect of CWSP and furthers the evidence suggesting that it is a promising new vaccine antigen delivery system. The *in vivo* results showed that by using DODAB:MO in the ADS1 formulation, an immunizing dose as low as 10 μg of antigen administered three times elicited significant humoral and cellular immune responses in BALB/c mice, conferring significant protection against *C. albicans* systemic infection. Thus, the use of this novel ADS1 liposomal nanoparticle revealed good potential for future vaccine design.

Disclosures

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.05.001.

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