Rodrigues, TC, Figueiredo, DB, Gonçalves, VM, Kaneko, K, Saleem, IY and Miyaji, EN

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Liposome-based dry powder vaccine immunization targeting the lungs induces broad protection against pneumococcus

T.C. Rodrigues [a,b], D.B. Figueiredo [b,c], V.M. Gonçalves [b,c], K. Kaneko [d], I.Y. Saleem [d,*], E. N. Miyaji [a,b,**]

[a] Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil
[b] Programa de Pós-Graduação Interunidades em Biotecnologia, Universidade de São Paulo, São Paulo, Brazil
[c] Laboratório de Desenvolvimento de Vacinas, Instituto Butantan, São Paulo, Brazil
[d] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, Merseyside, United Kingdom

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ABSTRACT

Streptococcus pneumoniae is an important human pathogen. Currently used conjugate vaccines are effective against invasive disease, but protection is restricted to serotypes included in the formulation, leading to serotype replacement. Furthermore, protection against non-invasive disease is reported to be considerably lower. The development of a serotype-independent vaccine is thus important and Pneumococcal surface protein A (PspA) is a promising vaccine candidate. PspA shows some diversity and can be classified in 6 clades and 3 families, with families 1 and 2 being the most frequent in clinical isolates. The ideal vaccine should thus induce protection against the two most common families of PspA. The aim of this work was to develop a liposome-based vaccine containing PspAs from family 1 and 2 and to characterize its immune response. Liposomes (LP) composed of dipalmitoylphosphatidylcholine (DPPC) and 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) with or without α-galactosylceramide (α-GalCer) were produced by microfluidics, encapsulating PspA from clade 1 (PspA1, family 1) and/or clade 4 (PspA4Pro, family 2) followed by spray-drying with trehalose to form nanocomposite microparticles carriers (NCMP). LP/NCMPs showed good stability and preservation of protein activity. LP/NCMPs containing PspA1 and/or PspA4Pro were used for immunization of mice targeting the lungs. High serum IgG antibody titers against both PspA1 and PspA4Pro were detected in animals immunized with LP/NCMPs containing α-GalCer, with a balance of IgG1 and IgG2a titers. IgG in sera from immunized mice bound to pneumococcal strains from different serotypes and expressing different PspA clades, indicating broad recognition. Mucosal IgG and IgA were also detected. Importantly, immunization with LP/NCMPs induced full protection against strains expressing PspAs from family 1 and 2. Furthermore, $CD4^+$ resident memory T cells were detected in the lungs of the immunized animals that survived the challenge.

1. Introduction

Streptococcus pneumoniae, commonly known as pneumococcus, can colonize the human nasopharynx without symptoms. In some cases, it can cause severe diseases with high mortality and morbidity, especially in the population under 5 years of age and the elderly [1,2]. In 2017, pneumococcal pneumonia was responsible for 55.4% of all deaths related to lower respiratory tract infections (LRTI), and in 2019, the pneumococcus caused 829,000 deaths worldwide [3,4]. The pneumococcus has a capsule composed of polysaccharide (PS), its major virulence factor. The PS is highly variable and pneumococcal strains are classified in >100 serotypes (ST) [5,6].

Currently licensed vaccines against pneumococcal disease are based on the induction of antibodies against PS. After introduction of the pneumococcal conjugate vaccines (PCV), numbers of cases of invasive pneumococcal disease (IPD) reduced drastically, especially in children under five years and in IPD caused by vaccine serotypes (VT) [2,7]. However, ST replacement by non-vaccine serotypes (NVT) was observed [8,9]. Furthermore, the degree of protection against non-invasive disease induced by PCVs is still debated [10–12].

* Corresponding author.
** Corresponding author at: Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil.
E-mail addresses: I.Saleem@ljmu.ac.uk (I.Y. Saleem), eliane.miyaji@butantan.gov.br (E.N. Miyaji).

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To overcome the limitations of PCVs, studies have focused on the development of vaccines that induce ST-independent protection [13]. One of the most important antigens studied is Pneumococcal surface protein A (PspA), which was shown to induce protection against both colonization and invasive disease in mice [14,15]. PspA is an important virulence factor, avoiding complement deposition on the surface of the bacteria and inhibiting the bactericidal activity of apolactoferrin in the mucosa [16–18]. PspA is present in all clinical isolates. The mature molecule can be divided in an α-helical N-terminal domain, a proline rich domain, one domain with a repetition of 20 amino acids, and the C-terminal domain. The C-terminal region has a choline-binding domain, which is responsible for anchoring the protein to the cell surface. The N-terminal domain is exposed outside the capsule and is responsible for the antigenic activity [19,20]. The final portion of the N-terminal domain, right before the proline rich domain, was used to classify PspA. Family 1 includes clades 1 and 2, family 2 includes clades 3, 4 and 5, and family 3 includes clade 6 [21]. The distribution analysis of the families indicates that family 1 and 2 are the most frequent in clinical isolates, present in 80.0 to 99.7% of isolates [22–24].

We have previously developed polymer-based nanoparticles (NPs) with adsorbed or encapsulated recombinant PspA from clade 4 (PspA4Pro, family 2). Immunization targeting the lungs induced both mucosal and serum antibodies, which led to protection of mice against a strain expressing PspA from family 2, but not against a strain expressing PspA from family 1 [25,26].

In this work, we now test the combination of PspA4Pro and recombinant PspA from clade 1 (PspA1), delivered by liposomes (LPs) produced by microfluidic mixing method, and formulated as nanocomposite microparticles carriers (NCMP) by spray-drying. Besides increased stability, LP/NCMPs can enhance deposition of the formulation in the deep bronchoalveolar region of the lungs [27,28]. In addition to inducing local immune responses, a dry powder formulation for mucosal delivery would avoid the use of needles and syringes and would also eliminate cold-chain transportation and storage. LPs have been applied in vaccine formulations approved for human use, such as the Epaxal® and Inflexal® V that use virosomes, and Shingrix® and Mosquirix® that use adjuvant systems containing LPs [29–32]. For our formulations, we used LPs composed of dipalmitoylphosphatidylcholine (DPPC), found in lung surfactant [33], and 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), a synthetic cationic derivate of natural cholesterol, that was developed for cell transfection but that has also been used in LP formulations [34,35]. In addition, we have tested the inclusion of α-galactosylceramide (α-GalCer) as an adjuvant of the LP formulation. This lipid belongs to the class of sphingolipids, found in many species [36–38]. The sphingolipids can also be synthetically produced and modified for modulation of Th1 and Th2 response. α-GalCer was shown to induce both Th1 and Th2 cytokine release by activated natural killer T (NKT) cells [39,40]. The objective of this work was to develop a ST-independent nanovaccine for pulmonary immunization and show its potential to protect against pneumococcal infections.

2. Materials and methods

2.1. Recombinant proteins

The untagged PspA4Pro with low endotoxin content was obtained as described by Figueredo et al. [41]. The untagged PspA1 with low endotoxin content was obtained using a similar method as described by Rodrigues et al. (manuscript in preparation) with some modifications. Briefly, cells were disrupted and the homogenate was treated with the cationic detergent cetyltrimethylammonium bromide (CTAB). After clarification, the supernatant followed to anion exchange chromatography, cryoprecipitation at pH 4.0, cation exchange and multimodal chromatography. The recombinant proteins include the mature N-terminal region of PspA till the proline-rich region.

2.2. Liposome preparation

LP formulations were prepared by microfluidic mixing method using NanoAssemblr Benchtop (Precision Nanosystems, Canada). Initially, one design of experiment (DoE) was performed to choose the flow rate ratio (FRR) and the total flow rate (TFR) using different concentrations of DPPC (Croda/Avanti Polar Lipids, USA). The parameters for LP preparation were chosen based on the results of a 2^3 full factorial design with 3 center points (Table S1). After that, a mixture DoE of extreme vertices for 3 components with 3 center points was performed to choose the lipid percentage of DPPC, DC-Chol (Croda/Avanti Polar Lipids, USA) and α-GalCer (Abcam, UK) (Table S2), using bovine serum albumin (BSA) at the fixed concentration of 0.3 mg/mL, with a proportion of 0.2 mg of protein to 1.0 mg of lipid mixture. For LPs without α-GalCer, its fraction was replaced with DPPC. Initially for LP preparation, syringes containing 5.0 mL of the aqueous phase containing one or two proteins at 0.3 mg/mL total concentration and 1.0 mL of the resuspension of the lipids in ethanol at 15.0 mg/mL total concentration (Merck, Germany), as the organic phase, were coupled to the system. The two solutions were warmed up to 55 °C for 5 min and plugged in the inlet position of the NanoAssemblr Microfluidic Cartridge (Precision Nanosystems, Canada), previously attached into the NanoAssemblr Benchtop. At the outlet position, one waste and one sample collection tube were positioned. The NanoAssemblr Benchtop software (Precision Nanosystems, Canada) was set up according to the defined FRR and TFR. Different experiments were then performed using 4.52 mL of aqueous phase (1.35 mg of total protein) and 0.48 mL of organic phase (5.57 mg DPPC; 1.58 mg DC-Chol; 0.06 μg α-GalCer) to obtain 5.00 mL of LP suspension, or 9.14 mL of aqueous phase (2.74 mg of total protein) and 0.87 mL of organic phase (10.18 mg DPPC; 2.87 mg DC-Chol; 0.11 μg α-GalCer) to obtain 10 mL of LP suspension. The obtained formulations were centrifuged at 84,035 ×*g* for 35 min at 20 °C. The supernatants were recovered and stored at 2–8 °C. The pellets were resuspended in deionised water or other solution for further steps.

2.3. Characterization of liposomes

The size, polydispersity index (PdI) and the surface charge were assessed using Zetasizer Nano ZS (Malvern Panalytical Ltd., UK). For the analysis, LP samples were diluted 1:50 in deionised water and measurements were performed in triplicate at 20 °C.

2.4. Protein quantification and encapsulation efficiency

For determination of protein concentration in the supernatant, the HPLC method described by Kunda et al. [28] was used with some minor modifications. Briefly, HPLC system series 1100 (Agilent Technologies, USA) connected to Aeris™ column (3.6 μm widepore C4 200Å, 150 × 4.6 mm) (Phenomenex Inc., USA) was used. For each run, 100 μL of the sample was applied to a linear gradient started with 80% (*v*/v) trifluoroacetic acid (TFA) (Merck/Sigma-Aldrich, Germany) in water (0.1% (*w*/*v*)) and 20% (v/v) TFA in acetonitrile (Merck, Germany) (0.1% (w/v)). Each run performed a 20 min separation at 40 °C and flow of 0.8 mL/min. Samples with known concentrations (0.0010–1.0000 mg/mL) in water were used for the standard curve (R^2 PspA1: 0.9997; R^2 PspA4Pro: 0.9999). Samples with known concentrations in the supernatant of blank LP were used to determine the error factor of the method and to correct the final protein concentration.

The encapsulation efficiency (EE(%)) was determined by the initial protein content minus the protein quantified in the supernatant, divided by the initial protein content and multiplied by 100, as expressed in Eq. 1.

$$EE\ (\%) = \frac{\left(Protein\ (mg)_{initial} - Protein\ (mg)_{supernatant}\right)}{Protein\ (mg)_{initial}} x\ 100 \quad (1)$$

2.5. Production of the nanocomposite microparticles carriers

The production of the NCMP followed the steps of spray-dry process used by Kunda et al. [28] with some modifications. Briefly, LPs recovered after centrifugation were resuspended in an aqueous solution with sucrose, mannitol, lactose or trehalose supplemented with or without L-leucine. The proportion of mass of LP:Sugar:L-leucine was kept unaltered at 1:20:0.5 mg. For the spray-dry process, the mini spray-dryer Büchi B-290 (Büchi Labortechnik, Switzerland) operated at 90 °C in the inlet position and 45–50 °C in the outlet position. The air flow was set at 670 L/h, aspirator rate at 40 L/h and feed rate at 1.5 mL/min. The dry powder was recovered in tubes and stored in desiccator at room temperature.

2.6. Characterization of the nanocomposite microparticles carriers

The yield (Y (%)) of the spray dry process was calculated as the difference in the weight of the tube after dry powder collection compared to the total dry mass of components added during the process for each formulation, as shown in Eq. 2.

$$Y\ (\%) = \frac{Dry\ powder\ (mg)}{Lipid_{added}\ (mg) + Protein_{added}\ (mg) + Trehalose_{added}\ (mg)}\ x\ 100 \quad (2)$$

For the measurement of the size, PdI and charge of the LP released from the NCMP, 5 mg of the dry powder were resuspended in 1 mL deionised water and characterized as described in item 2.3.

The amount of LPs inside the NCMPs was estimated. First, we estimated the mass of LP obtained using microfluidics by multiplying the concentration of the components by the volume used for the formulation. The Y(%) of the spray-dry process was then determined. The final amount of LP inside the NCMPs was determined by multiplying the Y(%) and the estimated amount of LP produced, then divided by 100.

The final protein concentration in mass inside the LP/NCMP (PC LP/NCMP) was calculated by the difference between the amount of protein added and the amount of protein remaining in the supernatant, divided by the amount of lipids added to the formulation. The result was divided by the dilution factor (DF) with excipients used in the spray dry. The concentration was expressed as mg of protein per mg of LP/NCMP, as shown in Eq. 3.

$$PC\ LP \Big/ NCMP\ \left(mg_{Protein} \Big/ mg_{LP/NCMP}\right) = \frac{\left(Protein\ (mg)_{initial} - Protein\ (mg)_{supernatant}\right)}{Lipid_{mass}\ (mg)} : DF \quad (3)$$

The moisture content (M (%)) was determined by thermogravimetric analysis (TGA). For the measurement, samples of at least 10 mg of dry powder were transferred to a platin plate and attached to the TGA Q50 (Waters-TA Instruments, USA). The software connected to the machine monitored the change in the weight of the samples when they were submitted to heat from 20 to 160 °C, at 20 °C/min rate. The percentage of weight loss was calculated by TA Universal Analysis 2000 (Waters/TA Instruments, USA) for each sample and defined as the moisture content after spray-dry.

The NCMP morphology was observed by scanning electron microscopy (SEM) using the FEI Quanta™ 200 ESEM (Thermo Scientific/FEI Company, USA). For the analysis, a small amount of the samples was sprinkled on the surface of aluminium pins covered with conductive carbon tape. Before the SEM, the pins with the samples were coated with palladium, using the X Gold Sputter Coater (Quorum Technologies Ltd., UK).

2.7. Recognition of of the proteins released from the LP/NCMP by polyclonal sera

The recognition of the proteins after LP/NCMP production by polyclonal sera was assessed by western blot. For the release of the protein from the LP/NCMP, 1 mg of the dry powder was resuspended in 200 μL of SDS-PAGE buffer with 2-mercaptoethanol, sonicated for 6 min and boiled at 95 °C for 5 min. As controls, purified PspA1 and PspA4Pro were diluted in SDS-PAGE buffer and boiled. The purified proteins were separated by SDS-PAGE in a range from 10 to 500 ng. LP/NCMP samples containing an estimated amount of 250 ng protein were also separated by SDS-PAGE. Samples were transferred to nitrocellulose membranes and incubated with tris buffered saline and 0.1% (*v*/v) Tween® 20 (TBS-T) containing 5% (*w/v*) skimmed milk. Membranes were incubated with 1:5000 dilution of polyclonal anti-PspA1 or anti-PspA4Pro serum, followed by incubation with anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:1000, Merck/Sigma-Aldrich, Germany) and by incubation with ECL™ Prime kit (Cytiva/GE Healthcare, UK).

2.8. Biological activity of the proteins released from the LP/NCMPs

The preservation of the capacity of PspA to bind to lactoferrin was also assessed with the proteins released from the LP/NCMPs. LP/NCMPs samples containing 2 μg protein were separated by SDS-PAGE. Samples with 2 μg BSA (Merck/Sigma-Aldrich, Germany), PspA1 and PspA4Pro were also loaded to the gel. Samples were transferred to nitrocellulose membranes and incubated with TBS-T containing 5% (*w/v*) BSA. Membranes were incubated with 4 μg/mL of human lactoferrin (Merck/Sigma-Aldrich, Germany) conjugated to biotin, using the Biotin Labelling kit (Roche, Switzerland). Membranes were then incubated with streptavidin conjugate with HRP (1:100, R&D Systems, USA), and with ECL™ Prime kit (Cytiva/GE Healthcare, UK).

2.9. Ethics statement

Animal studies were performed according to the guidelines outlined by the Brazilian National Council for Control of Animal Experimentation (CONCEA), which follows international guidelines for animal welfare and the principles of the 3Rs. Experimental protocols were approved by the Ethic Committee on Animal Use of the Butantan Institute (CEUAIB) under protocols number 7667100317 and 6905050820. Animals were obtained from the Medical School of the University of São Paulo (São Paulo, Brazil) and housed in a BSL2 animal facility, in micro isolators with individual ventilation and temperature and light cycle control. Animals received food and water *ad libitum* and manipulation was performed by trained personnel.

2.10. Pneumococcal strains and growth conditions

S. pneumoniae strains EF3030 (ST 19F, PspA clade 1), A66.1 (ST 3, PspA clade 2), and 3JYP2670 (ST 3, PspA clade 4) were kindly provided by Dr. David Briles (University of Alabama at Birmingham, USA). Strain M10 (ST 11A, PspA clade 3) was kindly provided by Dr. Ana Lúcia S. S. de Andrade (Federal University of Goias, Goiania, Brazil). Strain ATCC6303 (ST 3, PspA clade 5) was kindly provided by Dr. Maria Cristina Brandileone (Instituto Adolfo Lutz, São Paulo, Brazil). For the challenge, bacteria were plated on blood agar and incubated overnight at 37 °C. Bacteria were then inoculated in Todd-Hewitt broth (Becton, Dickinson & Company/Difco, USA) supplemented with 0.5% (*w/v*) yeast extract (THY) and incubated at 37 °C without shaking till OD_{600nm} 0.4. Aliquots of stocks used for challenge were maintained at −80 °C in THY containing 20% (*v*/v) glycerol.

2.11. Immunization of mice and serum collection

Five- to seven-week-old female specific-pathogen-free (SPF) BALB/c

mice were anesthetized through the intraperitoneal (ip) route with 20 mg/Kg of xylazine chloride and 100 mg/Kg of ketamine chloride. Mice were immunized by nasal instillation using a micropipette with 50 μL of a resuspension of the LP/NCMPs containing 6 μg of total protein. This protocol has previously been shown to deliver the instilled liquid to the lungs of mice [25]. As controls, animals were immunized subcutaneously (sc) with 100 μL of saline or solution of PspA1, PspA4Pro or a mixture of both proteins (6 μg of total protein). Mice received 2 doses with a 15-days interval. After 14 days of each dose, blood samples were collected to recover serum. The amount of protein used for immunization was determined by previous work from our group using polymeric nanoparticles showing that NP/NCMPs containing 2 μg PspA4Pro induced suboptimal response, whereas formulations containing 6 μg PspA4Pro displayed increased response [26].

2.12. Recovery of vaginal washes

Vaginal washes were performed 14 days after the second immunization and repeated for 4 more days. In each animal, 25 μL of saline was placed over the vaginal orifice using a micropipette. The volume was homogenized 5 to 6 times with the micropipette softly touching the vaginal orifice. The volume was placed in a microtube and the process was repeated once. After each day of collection, individual samples were stored at −20 °C. In the 5th day of collection, samples from the same animal were pooled and stored at −20 °C.

2.13. Measurement of antibodies

Enzyme-linked immunosorbent assay (ELISA) was carried out for antibody detection as described previously [42]. Briefly, for the detection of IgG in the serum, plates were coated with 5 μg/mL PspA1 or PspA4Pro. Plates were incubated with serial dilutions of individual sera and serum IgG antibodies were detected using goat anti-mouse IgG conjugated with HRP (1:10,000, Merck/Sigma-Aldrich, Germany). For the detection of IgG1 and IgG2a, sheep anti-mouse IgG1 (1:1000, SouthernBiotech, USA) or sheep anti-mouse IgG2a (SouthernBiotech, USA) was used, followed by rabbit anti-sheep IgG conjugated with HRP (1:10,000, SouthernBiotech, USA). *o*-Phenylenediamine dihydrochloride (OPD) (Merck/Sigma-Aldrich, Germany) was used as substrate and titer was defined as the reciprocal of the highest dilution with $Abs_{492nm} \geq 0.1$. For the detection of IgG antibodies in vaginal washes, goat anti-mouse IgG conjugated with alkaline phosphatase (AP) (1:5000, SouthernBiotech, USA) was used. For the detection of IgA in vaginal washes, sheep anti-mouse IgA (1:4000, SouthernBiotech, USA) followed by anti-sheep IgG conjugate with AP (1:5000, SouthernBiotech, USA) was used. The *p*-nitrophenyl phosphate (p-NPP) (Merck/Sigma-Aldrich, Germany) substrate was used and Abs_{405nm} was determined at dilution 1:2 of the samples.

2.14. Binding of antibodies to the surface of bacteria

Binding of antibodies to the surface of intact bacteria was performed according to the method described by Ren et al. [16]. Bacteria were plated on blood agar, grown in THY until OD_{600nm} 0.4 and washed with PBS twice. 100 μL of the bacteria, containing approximately 1×10^6 CFU from strains EF3030, A66.1, M10, 3JYP2670 and ATCC6303 were incubated with 1% (*v*/v) pooled sera from each group. Samples were then incubated with anti-mouse IgG conjugated with FITC (1:100, Merck/Sigma-Aldrich, Germany). Samples were analysed by flow cytometry using FACS Canto II (BD Biosciences, USA), recording at least 10,000 events for each sample. The mean fluorescence intensity (MFI) and percentage of positive bacteria were determined for each sample using the FlowJo v10.8.1 (Becton, Dickinson & Company/FlowJo, LLC, USA).

2.15. Pneumococcal challenge

Twenty-one days after the last immunization, mice were anesthetized through the ip route with 20 mg/Kg of xylazine chloride and 100 mg/Kg of ketamine chloride and challenged with 3×10^5 CFU of pneumococcal strain ATCC6303 (ST 3, PspA5) or 1×10^6 CFU of A66.1 (ST 3, PspA2). A volume of 50 μL was inoculated into the nostrils of the mice and animals were evaluated for 10 days. Mice that presented signs of disease with piloerection, hunched posture and reduction in activity, were euthanized immediately with a lethal dose of anesthetics (60 mg/Kg of xylazine chloride and 300 mg/Kg of ketamine chloride, ip). Mice that remained until the end of the experiment were healthy and active; no animals died before meeting the criteria for euthanasia.

2.16. Assessment of lung $CD4^+$ resident memory T cells

Recovery of lung cells, labelling and analysis followed the method described by Smith et al. [43]. Briefly, animals were euthanized with a lethal dose of anesthetics and lungs were collected. Lungs were digested with a mixture of 1.0 mg/mL colagenase type II (Merck/Sigma-Aldrich, Germany), 150 μg/mL DNase I (Merck/Sigma-Aldrich, Germany) and 0.28 mg/mL $CaCl_2$ (Merck, Germany) for 1 h at 37 °C with smooth shaking (100 to 120 rpm). The cell suspension was obtained after passage through a cell strainer (Corning, USA) and red blood cells lysis with ACK buffer (Becton, Dickinson & Company/Gibco, USA). Samples containing 10^6 cells were incubated with FVS510 (1:500, BD Biosciences, USA), followed by incubation with anti-mouse CD45 PE (clone 30-F11); anti-mouse CD4 APC-Cy7 (clone GK1.5); anti-mouse CD11a BV421 (clone M17/4); anti-mouse CD69 APC (clone H1.2F3); anti-mouse CD62L FITC (clone MEL-14); anti-mouse CD44 PE-Cy7 (clone IM7) (1:100, BD Biosciences, USA). Samples were analysed by flow cytometry using the LSRFortessa X-20 (BD Biosciences, USA), recording at least 500,000 events for each sample. CompBeads (BD Biosciences, USA) stained with single fluorochrome were prepared for compensation. Unstained cells were used for the adjustment of voltage and initial gates. Fluorescence minus one was also prepared for each fluorochrome and used for the delimitation of the gates. Data collected were analysed with FlowJo v10.8.1 (Becton, Dickinson & Company/FlowJo, LLC, USA) to identify the population of cells defined as $CD45^+CD4^+CD11a^{High}CD69^+CD44^{High}CD62L^{Low}$. Fig. S1 shows the gating strategy for the identification of $CD4^+$ lung resident memory T cells.

2.17. Statistical analysis

For the optimization of the LPs, Minitab Statistical Software 18 (Minitab,LCC, USA) was used. The formulation optimizer tool in the software was used for predicting the LP composition based on the mathematical model developed with the responses from each combination of components. One-way ANOVA with Tukey's multicomparison post-test was used for comparisons of antibodies titers and the percentage of the $CD4^+$ resident memory T cells. Log-rank (Mantel-Cox) test and Fisher's exact test were used for the analysis of the difference between survival curves and the final survival rate (GraphPad Prism 8, Dotmatics, USA).

3. Results

3.1. Production and characterization of LP/NCMPs containing PspA

Parameters to produce LPs were defined using DPPC alone with different combinations of TFR, FRR, and lipid concentration (Table S1). The following parameters for production of LPs were chosen based on the lowest PdI and LP size closer to 100 nm: 15.00 mg/mL DPPC, 10.5:1 (aqueous:organic) as the FRR and 9.5 mL/min as the TFR. For determination of LP composition, a mixture DoE was performed to choose the ideal combination of DPPC, DC-Chol and α-GalCer using a constant

concentration of 0.3 mg/mL BSA (Table S2). The lipid composition was selected using the formulation optimizer tool which analysed the responses produced for each run of the mixture DoE and elaborated a mathematical model to predict the optimal LP composition. The mathematical model was built focusing on maximizing encapsulation efficiency and minimizing the size, PdI and charge. The final formulation containing α-GalCer (Formulation B) was composed of 69.999% mols of DPPC (equivalent to 11.60 mg/mL DPPC), 30.000% mols of DC-Chol (equivalent to 3.30 mg/mL DC-Chol), and 0.001% mols of α-GalCer (equivalent to 0.13 μg/mL α-GalCer), with 15 mg/mL of total lipids. For this formulation, encapsulation efficiency was 34.08%, mean size was 458.34 nm, PdI was 0.512 and charge was +32.00 mV. As a comparison, one formulation without α-GalCer was also produced, replacing the fraction of the α-GalCer with DPPC. The formulation without α-GalCer (Formulation A) was composed of 70.00% mols of DPPC (equivalent to 11.70 mg/mL DPPC) and 30.00% mols of DC-Chol (equivalent to 3.30 mg/mL DC-Chol). The mean size and PdI of the LPs without α-GalCer were 390.99 nm and 0.346, respectively.

The test of excipients for production of the NCMPs was performed using LPs composed of DPPC:DC-Chol and containing BSA. Sucrose, mannitol, lactose and trehalose with or without L-leucine were tested. After resuspension in duplicate of the LPs with the specific excipient or combination of excipients, samples were spray-dried for the formation of NCMP. The LPs released from NCMPs were characterized (Table S3). The excipients were chosen based on the lowest PdI and size of the LPs released from the NCMPs. Trehalose alone showed the best results, with 480.28 ± 11.8 nm in size and 0.474 ± 0.085 of PdI. The yield of the drying process for this excipient was 68.84 ± 4.5%.

After definition of the parameters for production of the LP/NCMPs, incorporation of PspA1 and PspA4Pro was tested. LPs composed of DPPC:DC-Chol:α-GalCer (69.999:30.000:0.001% mols - Formulation B) and DPPC:DC-Chol (70.000:30.000% mols - Formulation A) were produced with the aqueous phase containing 0.3 mg/mL PspA1, 0.3 mg/mL PspA4Pro, or a mixture of 0.15 mg/mL PspA1 and 0.15 mg/mL PspA4Pro (Fig. 1, Table S4 and S6). The size and PdI of the particles containing protein varied from 113.46 ± 20.20 nm to 160.02 ± 64.12 nm and from 0.170 ± 0.026 to 0.261 ± 0.103, respectively. Empty LPs displayed higher size and PdI. The LPs were positively charged above +30 mV for all formulations. The encapsulation efficiency was close to 100%, except for LPs produced with PspA4Pro, which showed encapsulation efficiency of 88.74 ± 17.85% and 75.04 ± 19.34% for Formulation A and Formulation B, respectively. LPs encapsulating PspA1 and/or PspA4Pro thus displayed better results in terms of size, PdI and encapsulation efficiency when compared to BSA.

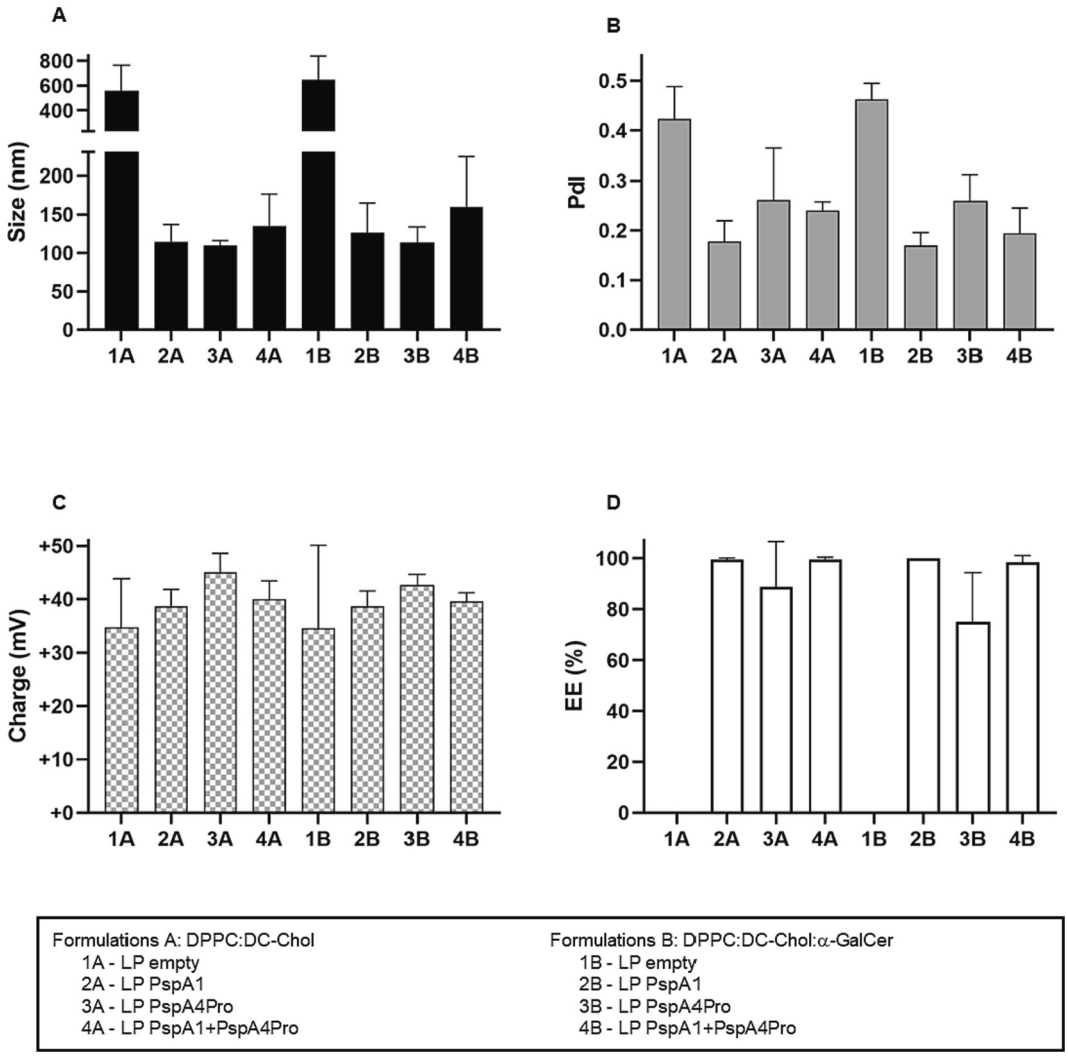

Fig. 1. Characterization of LPs encapsulating PspA1 and PspA4Pro. LPs composed by DPPC:DC-Chol (Formulation A) or DPPC:DC-Chol:α-GalCer (Formulation B) were produced using microfluidics mixing method. Empty LPs (1 A and 1B), LPs encapsulating PspA1 (2 A and 2B), PspA4Pro (3 A and 3B), and the combination PspA1 + PspA4Pro (4 A and 4B) were characterized. Size (A), PdI (B) and Charge on the surface (C) of the LPs were determined by DLS and the encapsulation efficiency (EE (%)) (D) was determined by HPLC. Graphs are shown as mean with SD and are representative of two independent experiments.

NCMPs were then produced by spray-drying after diluting the LPs in 30 mg/mL trehalose solution in a mass proportion of 1:20 mg (lipid: trehalose). LPs released from the NCMPs were characterized (Fig. 2, Table S5 and S6). LPs encapsulating protein displayed mean size of 181.24 ± 13.79 nm and mean PdI of 0.233 ± 0.041, with values similar to those obtained before spray-drying. Empty LPs displayed again higher size and PdI. The charge of the LPs containing protein did not alter after spray dry, but charge of empty LPs was lower. The mean yield of the spray-dry process was 72.94 ± 4.51%, and it reduced the moisture content to <5.00% in almost all the formulations, except for Formulation 3 A - LP/NCMP PspA4Pro, which showed moisture content of 6.21 ± 2.39%. The estimated amount of protein in the LP/NCMPs was between 0.007 and 0.008 mg per mg of NCMP and the estimated amount of α-GalCer was around 0.3 ng per mg of NCMP (Table S6).

Morphological characterization of the LP/NCMPs using SEM showed homogeneity between formulations, with particle size around 5 μm (Fig. 3A). Recognition by polyclonal sera and biological activity of the proteins recovered from formulations were also assessed. Proteins released from the LP/NCMPs were analysed by western blot and were recognized by polyclonal antibodies anti-PspA1 and anti-PspA4Pro with the expected molecular weight (Fig. 3B). Furthermore, PspA1 and PspA4Pro maintained their lactoferrin binding activity, as observed by probing proteins released from the LP/NCMP with biotinylated lactoferrin (Fig. 3C). These findings demonstrated that the LP/NCMP formulation efficiently encapsulated PspA1 and PspA4Pro and maintained their properties.

3.2. *Antibody response after immunization with LP/NCMPs containing PspAs*

Formulation A (DDPP:DC-Chol) and Formulation B (DPPC:DC-Chol:α-GalCer) encapsulating PspA4Pro were used to immunize female

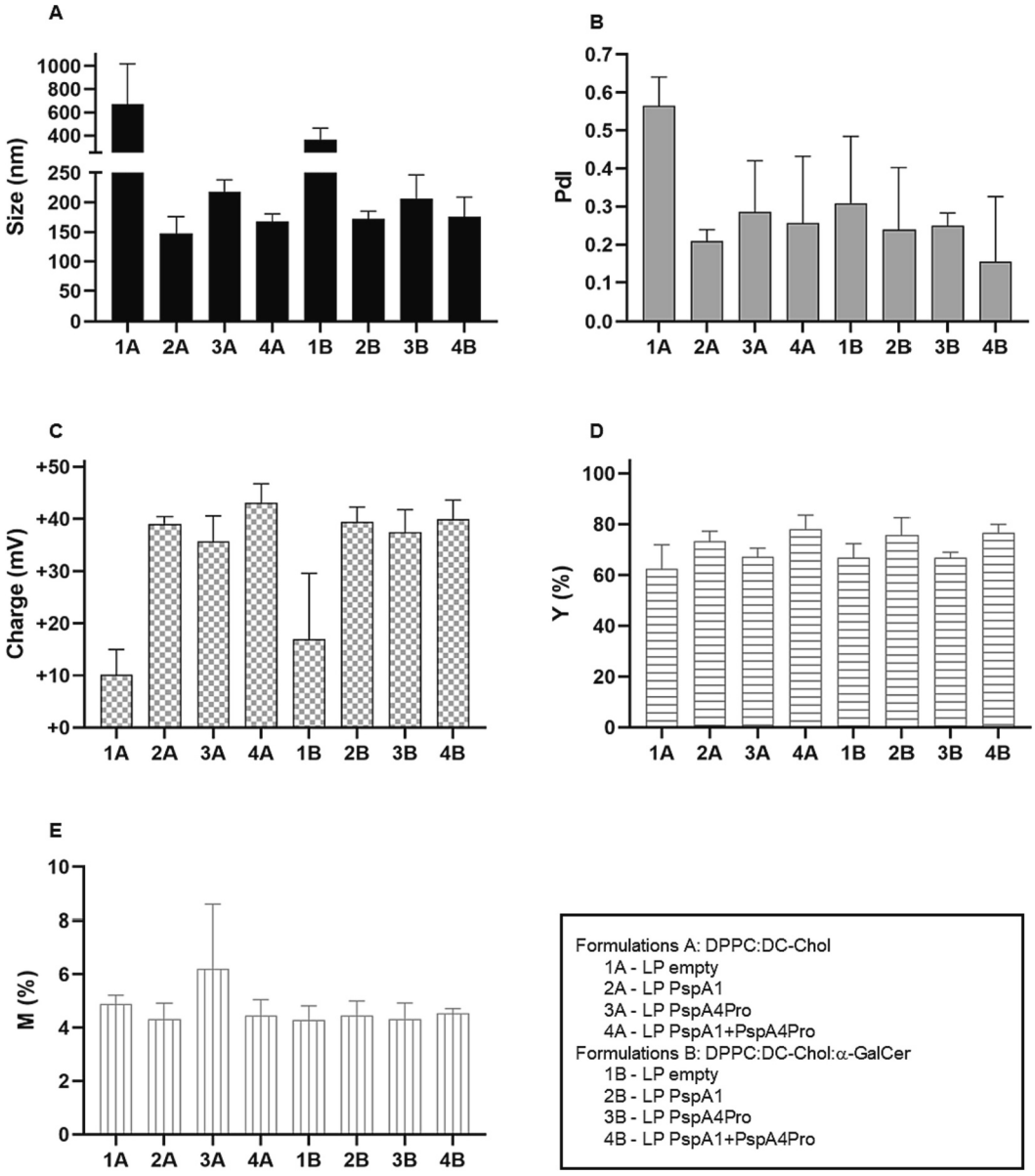

Fig. 2. Characterization of LPs encapsulating PspA1 and PspA4Pro released from NCMPs. LPs composed by DPPC:DC-Chol (Formulation A) or DPPC:DC-Chol:α-GalCer (Formulation B) were produced as empty LPs (1 A and 1B) or encapsulating PspA1 (2 A and 2B), PspA4Pro (3 A and 3B), and the combination PspA1 + PspA4Pro (4 A and 4B) and submitted to spray-dry with trehalose to form NCMPs. The Size (A), PdI (B) and Charge on the surface (C) of the LPs released from the NCMPs were determined by DLS. The yield (Y (%)) (D) of the spray-drying process and the moisture content (M (%)) (E) of the powder were also determined. Graphs are shown as mean with SD and are representative of two independent experiments.

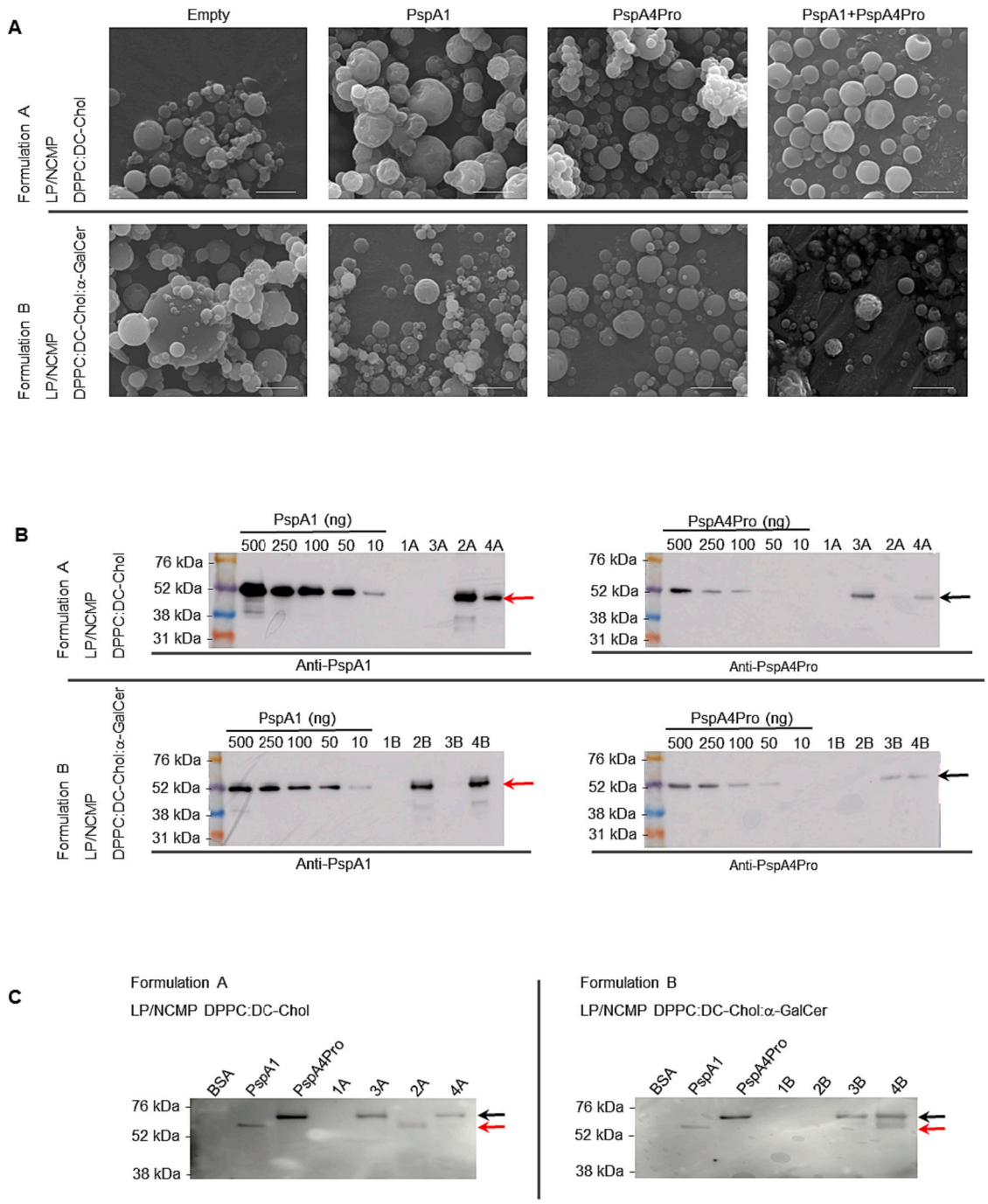

Fig. 3. Characterization of LP/NCMPs. (A) Images of LP/NCMPs composed of DPPC:DC-Chol and DPPC:DC-Chol:α-GalCer encapsulating PspA1, PspA4Pro, PspA1 + PspA4Pro or without protein (empty) were captured by SEM using 10,000× magnification. The white bars indicate the size of 5 μm. (B) Western blot analysis of protein released from the indicated LP/NCMPs with anti-PspA1 or anti-PspA4Pro polyclonal serum. Samples containing 250 ng were analysed and 10 to 500 ng of purified PspA1 or PspA4Pro were used as controls. (C) Analysis of lactoferrin binding of protein released from the indicated LP/NCMPs using biotinylated lactoferrin. Samples containing 2 μg PspA1 or PspA4Pro were analysed and 2 μg BSA, purified PspA1 and purified PspA4Pro were used as controls. The red and black arrows indicate the predicted size of PspA1 and PspA4Pro, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BALB/c mice. For immunization targeting the lungs, mice were anesthetized and immunized with a LP/NCMP suspension containing 6.0 μg PspA4Pro. The estimated amount of α-GalCer in each dose of LP/NCMP in Formulation B is between 0.20 and 0.26 ng. Mice were immunized with 3 A - LP/NCMP PspA4Pro or 3B - LP/NCMP PspA4Pro. As negative controls, mice were immunized with the formulations without protein 1 A - LP/NCMP empty and 1B - LP/NCMP empty. Animals were also immunized sc with saline and 6.0 μg PspA4Pro. Immunization *via* sc route with purified PspA4Pro alone was added as control, since it was previously shown to induce serum IgG and partial protection against pneumococcal challenge. Lung immunization with the purified protein only was not included in the experiment, since it was previously shown that it does not induce any antibody response or protection [25,26]. The formulations were administered in two doses and serum anti-PspA4Pro IgG titers were assessed by ELISA after each dose (Fig. 4). PspA4Pro sc and 3B - LP/NCMP PspA4Pro induced anti-PspA4Pro IgG with statistical

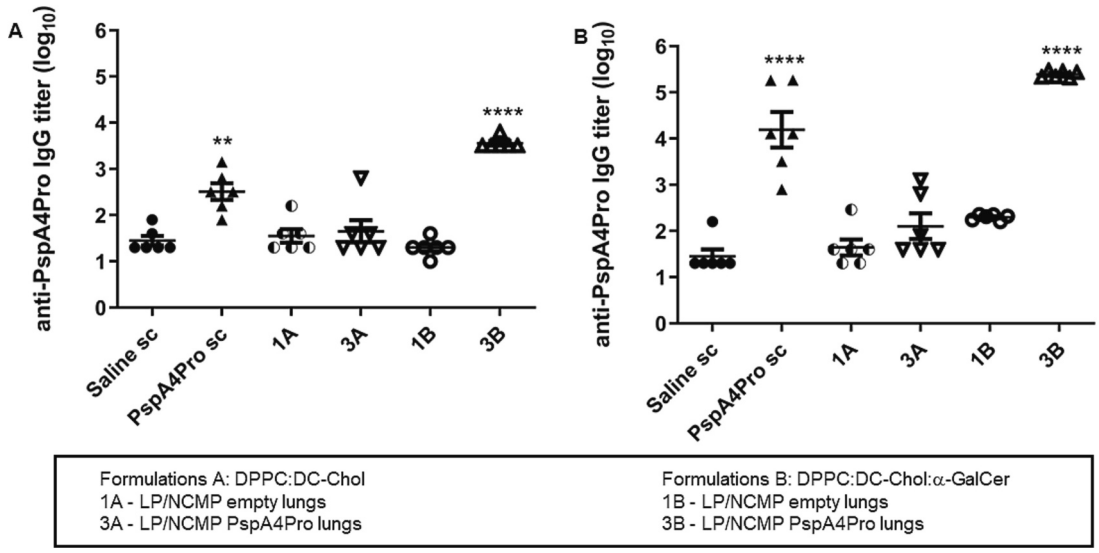

Fig. 4. Induction of serum anti-PspA4Pro IgG after immunization with LP/NCMPs formulated with or without α-GalCer and containing PspA4Pro. Mice were immunized with the indicated formulations and serum anti-PspA4Pro IgG titers were evaluated 14 days after the first (A) and second (B) doses. * Indicates significant statistical difference compared with saline (One-way ANOVA with Tukey's post-test).

difference with the saline group, after the first and second doses, with higher titers observed for 3B - LP/NCMP PspA4Pro. The anti-PspA4Pro IgG titers for 3B - LP/NCMP PspA4Pro were also significantly higher than PspA4Pro sc, 1 A - LP/NCMP empty, 3 A - LP/NCMP PspA4Pro, and 1B - LP/NCMP empty after both first and second doses (Table S7). In contrast, the group immunized with 3 A - LP/NCMP PspA4Pro, as well as control groups 1 A - LP/NCMP empty and 1B - LP/NCMP empty, did not induce statistically significant increase in serum IgG. These results indicate that the inclusion of α-GalCer as adjuvant of the LPs is crucial for the induction of humoral immune response and Formulation B was thus selected for further immunizations.

Immunization with LP/NCMPs containing α-GalCer and both PspA1 and PspA4Pro was assessed. Female BALB/c mice were immunized sc with saline, 6.0 μg PspA4Pro, 6.0 μg PspA1 or a mixture of 3.0 μg PspA4Pro and 3.0 μg PspA1. For immunization targeting the lungs, mice received an LP/NCMP suspension containing 6.0 μg of total PspA: 2B - LP/NCMP PspA1, 3B - LP/NCMP PspA4Pro, 4B - LP/NCMP PspA1 + PspA4Pro and the mixture 2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro. As negative control, mice received 1B - LP/NCMP empty. Mice were immunized with two doses and blood samples were collected after the second dose for detection of serum anti-PspA1 and anti-PspA4Pro IgG. All LP/NCMP formulations containing protein induced significant higher IgG titers against PspA1 compared with saline and 1B - LP/NCMP empty, with slightly lower titers for 3B - LP/NCMP PspA4Pro. Subcutaneous immunizations with the proteins did not induce significant anti-PspA1 response (Fig. 5A, Table S8). In addition, subcutaneous immunization with PspA4Pro and with PspA1 + PspA4Pro induced anti-PspA4Pro IgG titers with statistical difference with the saline group. The groups immunized with the 3B - LP/NCMP PspA4Pro, 4B - LP/NCMP PspA1 + PspA4Pro and the combination 2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro showed induction of the highest anti-PspA4Pro IgG titers, with statistical difference with saline, 1B - LP/NCMP empty and purified proteins (Fig. 5B, Table S8). Isotyping of anti-PspA4Pro IgG showed a balanced induction of anti-PspA4Pro IgG1 and IgG2a in the groups immunized with the LP/NCMPs, with statistical difference in the titer ratios with the groups immunized subcutaneously with PspA4Pro and PspA1 + PspA4Pro (Fig. 5C).

Sera from the groups immunized with the LP/NCMP were also tested for the capacity to bind to the surface of pneumococcal strains of different STs and expressing PspAs from clades 1 to 5 (Fig. 6, Fig. S2 and Table S9). Serum from the group immunized with 4B - LP/NCMP PspA1 + PspA4Pro and the mixture 2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro showed antibodies that recognized all the strains tested with high MFI values. Overall, sera from the groups immunized with 2B - LP/NCMP PspA1 and 3B - LP/NCMP PspA4Pro bound more efficiently to the strains which express PspA from the same family. For immunization with protein sc, only sera from the groups immunized with PspA4Pro sc and PspA1 + PspA4Pro sc bound to strains 3JYP2670 (PspA clade 4) and ATCC6303 (PspA clade 5), but the MFIs were much lower than the LP/NCMPs groups. These results indicate that immunization with LP/NCMPs containing PspA1 and PspA4Pro or LP/NCMP PspA1 combined with LP/NCMP PspA4Pro could induce broader coverage of pneumococcal strains.

The induction of mucosal antibodies was also assessed in vaginal washes from each animal (Fig. 7). Induction of vaginal antibodies can be used as a proxy for respiratory tract mucosa due to the common mucosal immune system [44]. Subcutaneous immunization with PspA1, PspA4Pro and the mixture PspA1 + PspA4Pro did not induce any mucosal antibodies. Formulation 2B - LP/NCMP PspA1 induced higher anti-PspA1 IgG levels with statistical difference with saline (Fig. 7A) and also showed a trend towards higher anti-PspA1 IgA levels (Fig. 7C). For the immunization with 3B - LP/NCMP PspA4Pro, statistical difference was observed in the anti-PspA4Pro IgG levels (Fig. 7B) and again a trend towards higher anti-PspA4Pro IgA levels was observed (Fig. 7D). Immunization with the mixture 2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro induced IgA and IgG both anti-PspA1 and anti-PspA4Pro, but statistical difference was only observed for anti-PspA1 IgG and anti-PspA4Pro IgA. Formulation 4B - LP/NCMP PspA1 + PspA4Pro induced only IgG against both PspA1 and PspA4Pro, but no statistical difference was observed. These results indicate that mucosal antibodies were induced by the LP/NCMPs, albeit at low levels.

3.3. *Protection against pneumococcal challenge and induction of memory cells*

Animals were then challenged with strains ATCC6303 (ST 3, PspA5) or A66.1 (ST 3, PspA2) and followed for 10 days. The survival curves are presented in Fig. 8A and Fig. 8B, and the summary of the final survival rates analysis are presented in Table S10. Immunization with 4B - LP/NCMP PspA1 + PspA4Pro induced protection of 100% against both strains with statistical significance in the analysis of the survival curves and in the final survival rate compared to the saline group. The mixture

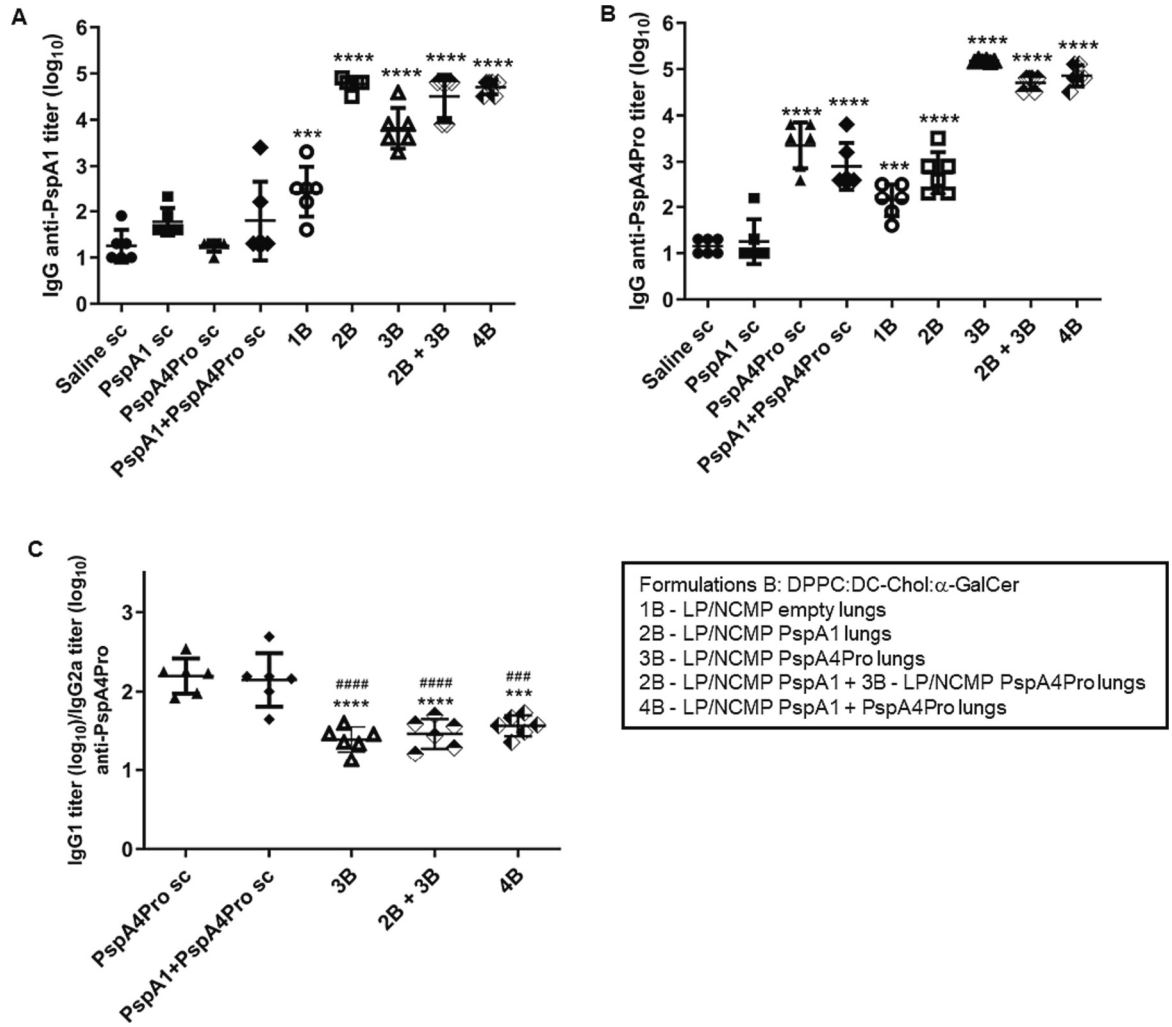

Fig. 5. Induction of serum anti-PspA1 and anti-PspA4Pro IgG by immunization with LP/NCMPs with α-GalCer containing PspA1 and PspA4Pro. Mice were immunized with two doses of the indicated formulations and serum anti-PspA1 (A) and anti-PspA4Pro (B) IgG titers were evaluated after 14 days. Anti-PspA4Pro IgG1:IgG2a titer ratios were also determined (C). * Indicates significant statistical difference compared with saline (A and B) and with PspA4Pro sc (C) and # indicates significant statistical difference compared with PspA1 + PspA4Pro sc (C) (One-way ANOVA with Tukey's post-test).

2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro induced protection of 100% against the A66.1 and 83% against the ATCC6303, with statistical significance for both survival curves and final survival rate analysis. Immunization with 3B - LP/NCMP PspA4Pro induced protection of 100% against the ATCC6303 and 63% against the A66.1, with statistical difference in the survival curves analysis for both strains, but for the final survival rate the statistical difference was only observed against ATCC6303. Formulation 2B - LP/NCMP PspA1 induced protection of 33% against ATCC6303 and 100% against A66.1, which resulted in statistical difference for the survival curve analysis for both strains but only against A66.1 in the survival rate analysis. For the groups immunized with purified protein, only PspA4Pro sc and the mixture PspA1 + PspA4Pro sc showed statistical difference in the survival curve analysis after challenge with ATCC6303. These results confirm that the immunization with LP/NCMPs containing PspA1 and PspA4Pro or LP/NCMP PspA1 combined with LP/NCMP PspA4Pro have the potential to induce protection against a broader range of pneumococcal strains.

Lungs of animals that survived the challenge against A66.1 were analysed for the presence of $CD4^+$ resident memory T cells in the lungs (Fig. 8C). An increase in the memory cell population was observed in mice immunized with formulations 2B - LP/NCMP PspA1, 3B - LP/NCMP PspA4Pro and the mixture 2B - LP/NCMP PspA1 + 3B - LP/NCMP PspA4Pro with statistical difference with the naïve mice. Animals immunized with 4B - LP/NCMP PspA1 + PspA4Pro also showed higher percentage of $CD4^+$ resident memory T cells, but without statistical difference.

4. Discussion

We have developed a dry powder LP-based formulation containing protein antigens as a mucosal vaccine against pneumococcal infections. For the development of the formulations, the parameters for microfluidics mixing method for production of LPs were first defined using BSA, and then the protein was replaced with PspA1 and/or PspA4Pro. Interestingly, particle size and PdI was lower for the formulations using PspA compared to BSA, with size around 100 nm and PdI lower than 0.300. This phenomenon may be explained by differences in molecular weight (36.6 KDa PspA1, 47.7 KDa PspA4Pro, 66.5 KDa BSA) as well as in spatial structure (BSA is a globular protein, whereas both PspA1 and PspA4Pro produced as recombinant proteins encompass an α-helical region followed by a short proline-rich region). The lower molecular weight and the elongated α-helical structure of PspA could promote better compartmentalization of the protein inside the LPs, leading to lower size and PdI compared to BSA [28,45]. Another important factor is the difference in the surface charge of the proteins (the net charge at pH 7.4 of BSA is −17.254, of PspA1 is −26.477, and of PspA4Pro is

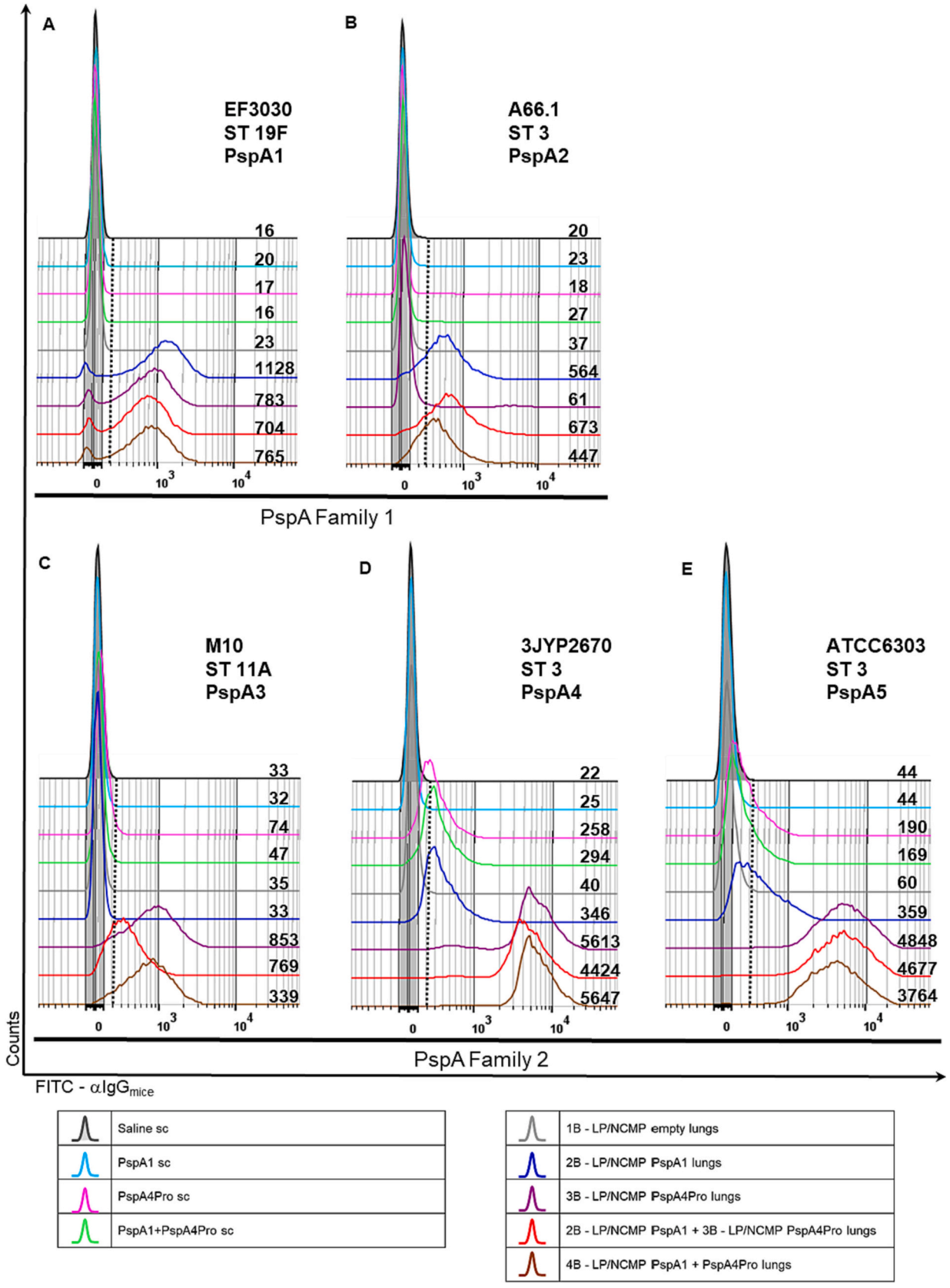

Fig. 6. Binding of serum IgG induced by the LP/NCMPs with α-GalCer containing PspA1 and PspA4Pro to the surface of different pneumococcal strains. Pneumococcal strains EF3030 (A), A66.1 (B), M10 (C), 3JYP2670 (D) and ATCC6303 (E) were incubated with sera from the indicated groups and binding of IgG was evaluated by flow cytometry. Data are shown as fluorescence intensity histograms with respective MFI values on the right side of the graphs. The dashed lines represent the end of the peak of fluorescence intensity of the negative control.

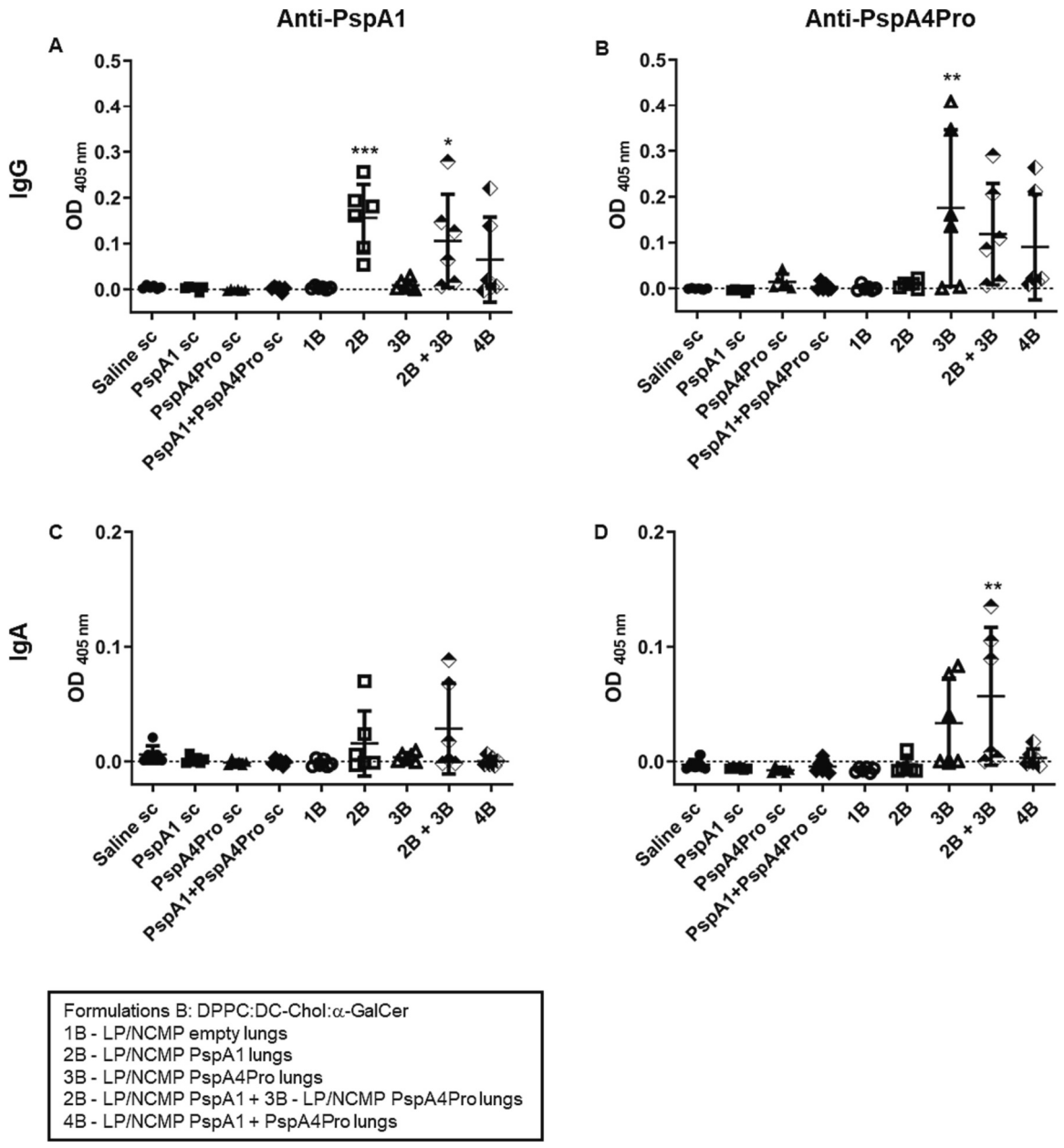

Fig. 7. Induction of mucosal antibodies after immunization with LP/NCMPs with α-GalCer containing PspA1 and PspA4Pro. Vaginal washes were collected after immunization with two doses of the indicated formulations for the evaluation of anti-PspA1 IgG (A), anti-PspA4Pro IgG (B), anti-PspA1 IgA (C) and anti-PspA4Pro IgA (D). * Indicates significant statistical difference compared with saline group (One-way ANOVA with Tukey's post-test).

−23.909), indicating that PspA could interact better with the positive head of DPPC present in the core of the LPs. Colletier et al. showed that the encapsulation efficiency is dependent on the electrostatic interactions between the surface charge of the protein and the lipid bilayer. Also, using BSA as a stabilizer of acetylcholinesterase, they observed reduction in the encapsulation efficiency [46]. The LP formulations also showed high encapsulation efficiency with values above 90%, except for the LPs containing only PspA4Pro. This high encapsulation efficiency can be explained by the combination of high concentration of lipids, high percentage of DC-Chol in the formulation (30% mols) and the major lipid being DPPC. The high lipid concentration in the formulation can lead to increase in total internal volume, facilitating encapsulation of proteins [47–49]. The difference of encapsulation efficiency between PspA1 and Psp4Pro could be explained by their slightly different surface charge. The established spray-drying process for production of LP/NCMPs containing PspA using trehalose showed preservation of particle size, PdI and charge. The method also showed yield and moisture content of 66.44 to 78.13% and 4.29 to 6.21%, respectively. Obtaining a dry powder with low moisture content is important to prevent agglomeration, to preserve the aerodynamic performance of the particles, and to stabilize proteins [50,51]. Trehalose has been previously shown to lead to better preservation of biological activity of proteins after spray-drying [52]. PspAs released from the LP/NCMPs were still recognized by polyclonal sera with the expected molecular weight in western blot and maintained capacity to bind to lactoferrin, indicating preservation of the encapsulated proteins. Similar results were obtained in previous work using dry powder vaccines composed of adipate-*co*-ω-pentadecalactone (PGA-co-PDL) and Poly (lactic-*co*-glycolic acid) (PLGA) produced by solvent evaporation [26,28]. LPs and LP/NCMPs composed by DPPC:DC-Chol and DPPC:DC-Chol:α-GalCer showed similar characteristics. We did not directly measure incorporation of α-GalCer into the LPs, but we expect most α-GalCer

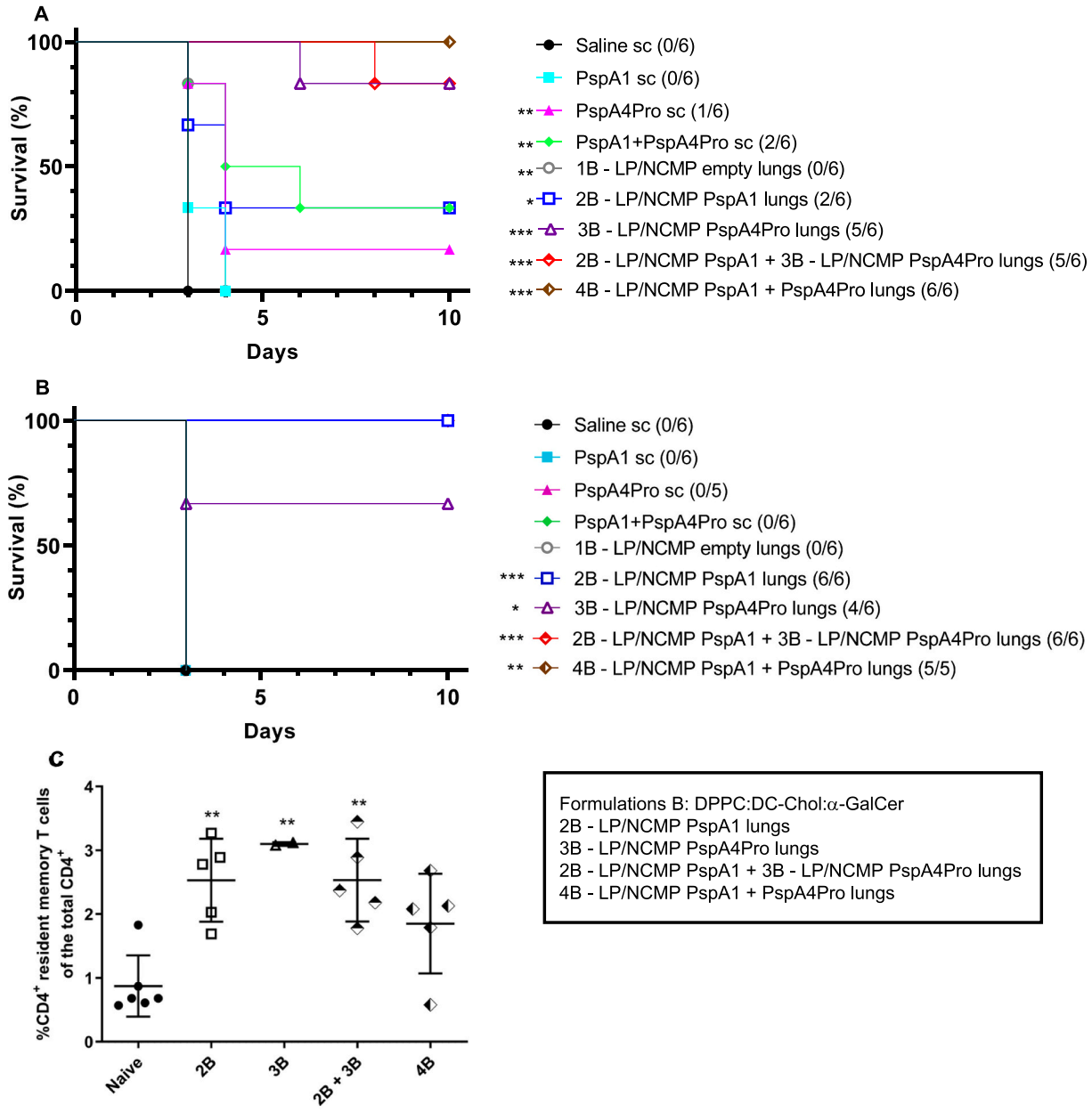

Fig. 8. Survival of mice immunized with LP/NCMPs with α-GalCer containing PspA1 and PspA4Pro after pneumococcal challenge and induction of lung $CD4^+$ resident memory T cells. Mice immunized with two doses of the indicated formulations were challenged with the strain ATCC6303 (ST 3, PspA5) (A) or A66.1 (ST 3, PspA2) (B) and survival was monitored for 10 days. Percentage of lung $CD4^+$ resident memory T cells among $CD4^+$ cells was assessed in the lungs of mice surving challenge with A66.1. Naive mice that were not immunized nor challenged were used as control (C) * Indicates significant statistical difference in the survival time compared with saline (Log-rank test (Mantel-Cox)) (A and B) and in the percentage of lung $CD4^+$ resident memory T cells compared with naive animals (C).

used for the preparation of the formulation to be incorporated into the LPs, since evaluation of similar LPs composed by DPPC:Chol by mass spectrometry showed incorporation higher than 90% of α-GalCer used in the preparation of the formulation [53].

For the analysis of the immune response of the LP/NCMPs, mice were immunized through nasal instillation of LP/NCMPs resuspended in 50 μL saline. We have previously shown that this method delivers the volume to the lungs of anesthetized mice [25]. We have also shown that purified protein inoculated into the lungs does not elicit any antibody response [25,26]. Furthermore, we have not tested immunization with purified protein or LPs without α-GalCer in the composition combined with soluble α-GalCer because it has been shown that free soluble α-GalCer can induce anergy in NKT cells after the first inoculation [54]. Mice were first immunized with the LP formulations with or without α-GalCer and encapsulating PspA4Pro. The evaluation of the humoral immune response showed that α-GalCer exerted excellent adjuvant activity, with the induction of high serum anti-PspA4Pro IgG. The formulation without α-GalCer did not elicit any antibody response. IgG titers were much higher than what we had previously observed for polymeric NPs containing PspA4Pro [25,26]. In fact, the adjuvant activity of α-GalCer is well known and has been explored against different pathogens for induction of both local and systemic responses. α-GalCer was shown to increase cytotoxic T lymphocyte response of spleen cells recovered from mice immunized with a synthetic peptide from the HIV envelope protein gp120 by both nasal and oral immunization [55]. This adjuvant also enhanced the response induced by the oral vaccine against *Vibrio cholerae* Dukoral®, with high antibody titers in both faecal and serum samples. The encapsulation by minispheres of a killed whole-cell

vaccine, together with cholera toxin B subunit and α-GalCer for oral immunization of mice induced higher response than the licensed formulation [56,57]. Furthermore, addition of α-GalCer to a whole-cell killed *Helicobacter pylori* antigen induced specific intestinal IgA and protection [58].

The LPs composed of DPPC:DC-Chol:α-GalCer containing PspA1 and/or PspA4Pro were then used to further characterize the humoral response in mice. The best results were obtained with LP/NCMPs containing both proteins or with the mixture of LP/NCMP containing PspA1 and LP/NCMP containing PspA4Pro. Importantly, the group immunized with purified proteins subcutaneously induced much lower IgG responses. Furthermore, the groups immunized with the LP/NCMPs induced a more balanced IgG1/IgG2a response, whereas sc immunization showed preferential induction of IgG1. It was previously shown that a balanced IgG1/IgG2a response correlates with better protection against pneumococcus due to the higher capacity of mouse IgG2a to fix complement [59]. These results are in contrast with those obtained using polymeric NPs, which showed preferential induction of IgG1 [25,26]. The change in the IgG1/IgG2a ratio may be due to the presence of α-GalCer in the formulation. α-GalCer has been shown to induce a balanced production of Th1/Th2 cytokines *in vivo*, and chemical alterations can enhance its activity [60]. Sera from mice immunized with LP/NCMPs combining PspA1 and PspA4Pro also showed binding to pneumococcal strains from different STs and expressing PspAs from different clades, indicating broad coverage. Moreover, immunization with the LP/NCMPs with α-GalCer targeting the lungs induced anti-PspA IgA and IgG in the vaginal mucosa, albeit at low levels. Immunization with the purified proteins did not elicit vaginal antibodies. The induction of mucosal antibodies can act as an important barrier against the pathogen in the main entrance site of the body. Holmgren et al. had shown that nasal immunization can induce humoral response in the airways and in the cervicovaginal mucosa [44]. So, the presence of mucosal antibodies in the vaginal wash can be used as an indicator of antibody response in the lungs [15,61].

Protection against lethal challenge was then assessed in immunized mice and showed that LP/NCMPs with α-GalCer encapsulating both PspA1 and PspA4Pro induced complete protection against challenge with pneumococcal strains expressing PspA from family 1 (A66.1) and family 2 (ATCC6303). Similar protection levels were obtained with the mixture of LP/NCMP encapsulating PspA1 and LP/NCMP encapsulating PspA4Pro. Inclusion of two PspA variants can thus overcome the restricted coverage of strains observed with the polymeric NPs produced by our group containing only PspA4Pro [25,26]. Tada et al. produced LPs as adjuvant of a PspA from clade 2 (PspA2) vaccine for nasal immunization of mice, which induced antibodies in the serum, lungs, and in both nasal and vaginal mucosa. Protection was observed, but differently from our work, they only tested protection against Xen10 (fluorescent strain derived from A66.1 - ST 3, PspA2) [61]. Kye et al. produced NPs and used them to form complexes with PspA2 prior to nasal immunization of mice. The NPs induced high mucosal and serum antibodies titers, but the serum isotype was predominantly IgG1, which is in contrast with what we observed for LP/NCMPs containing α-GalCer. Furthermore, mucosal and serum antibody titers remained high for a long period post-immunization and protection against WU2 (ST 3, PspA2) was observed. Once again, protection against different strains was not tested though [62]. Lyophilized vaccines composed of NPs encapsulating PspA2 and administered subcutaneously in mice in a single dose induced high serum antibody response and partial protection against A66.1. Storage at room temperature did not affect protection [63,64]. Our formulation is also a dry-powder vaccine to be stored at room temperature that induced high antibody response even after a single dose delivered to the lungs and led to complete protection of mice against challenge with pneumococcal strains expressing different PspAs. Kong et al. also showed that nanometer-sized hydrogels containing PspA2 for nasal immunization protected mice against lethal challenge with Xen10 and 3JYP2670 (ST 3, PspA clade 4) strains [65]. The vaccine was then tested in macaques and sera from these animals passively protected mice against Xen10 and 3JYP2670 [66]. The same nanogels were used for subcutaneous immunization of mice with a fusion protein of PspA from clades 1, 2 and 3 and showed induction of mucosal IgA [67]. In the macaque model, the nanogels containing the PspA fusion protein induced lung and serum antibodies that bound to the pneumococcal surface and induced complement deposition *in vitro*. The vaccine also reduced bacterial carriage in the lungs of immunized animals after intratracheal challenge [68]. It is important to note that the immunization was performed with 3 doses in mice and with 5 doses in macaques. Our work showed similar protection in mice after 2 doses only and with a slighlty lower amount of protein.

Animals that survived the challenge were evaluated for the presence of $CD4^+$ resident memory T cells in the lungs and an increase in this cell population was observed. The induction of memory cells can represent an important way to fight new infections. Resolution of pneumococcal pneumonia in mice was shown to provide heterotypic protection against a lethal pneumococcal challenge. Protection was attributed to the induction of $CD4^+$ resident memory T cells, leading to rapid neutrophil recruitment and cytokine production after a new infection [43]. The induction of this cell population in the nasal mucosa was also shown to protect against pneumococcal colonization [43,69]. Our results thus indicate that the formulation composed of DPPC:DC-Chol:α-GalCer encapsulating PspA can induce an increase in the $CD4^+$ resident memory T cells that could induce long-lasting protection against pneumococcal infections. We have compared naïve mice with immunized mice surviving the challenge, because no control animals inoculated with saline or protein survived. Though pneumococcal challenge by itself could induce the observed increase in $CD4^+$ resident memory T cells, it is important to note that the heterotypic protection observed by Smith and collaborators was only achieved after 2 or 3 previous pneumococcal self-limiting infections [43]. Lyon De Ana et al. also demonstrated reduction in the percentage of $CD4^+$ resident memory T cells subpopulations in the lungs of mice after a single self-limiting infection. They only observed accumulation of $CD4^+$ resident memory T cells and significant increase of percentage of this cell population in mice that received consecutive infections [70]. These findings suggest that the increase in the percentage of $CD4^+$ resident memory T cells in the lungs of immunized mice that we observed in this work is not solely due to the challenge, but also depends on prior lung immunization with LP/NCMPs.

In conclusion, we have produced a dry powder vaccine formulation for lung immunization composed of LP/NCMPs containing the adjuvant α-GalCer and encapsulating PspA1 and PspA4Pro. One strength of this work is the use of the microfluidics system, a process that can be scaled up for production. The formulation showed potential as a broad coverage vaccine against pneumococcal infections, with the induction of high antibodies titers, broad recognition of pneumococcal strains and protection against strains expressing PspAs from the most prevalent families. The immunization also induced increase in the memory cell population in the lungs, indicating potential for long-lasting protection.

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CRediT authorship contribution statement

T.C. Rodrigues: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **D.B. Figueiredo:** Methodology, Investigation. **V.M. Gonçalves:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **K. Kaneko:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **I.Y. Saleem:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **E.N. Miyaji:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have a patent deposited on the production of a vaccine against pneumococcal infection using LP/NCMPs containing PspA.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.02.028.

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