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Exploring yeast glucans for vaccine enhancement: Sustainable strategies for overcoming adjuvant challenges in a SARS-CoV-2 model

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ABSTRACT

Vaccine adjuvants are important for enhancing vaccine efficacy, and although aluminium salts (Alum) are the most used, their limited ability to induce specific immune responses has spurred the search for new adjuvants. However, many adjuvants fail during product development due to manufacturability, supply, stability, or safety concerns. This work hypothesizes that protein-free yeast glucans can be used as vaccine adjuvants due to their known immunostimulatory activity and high abundancy. Thus, high molecular weight glucans with over 99% purity, comprising 64–70% β-glucans and 29–35% α-glucans, were extracted from a wild-type yeast and an engineered yeast to produce a steviol glycoside. These glucans underwent carboxymethylation to enhance solubility. Both water-dispersible and particulate glucans were evaluated as adjuvants, either alone or in combination with Alum or squalene stable emulsion (SE), for a SARS-CoV-2 vaccine. The study demonstrated that glucans triggered a robust immune response and enhanced the effects of Alum and SE when used in combination, both *in vitro* and *in vivo*. Water-dispersible glucans combined with Alum, and particulate glucans combined with SE, increased the production of specific antibodies against SARS-CoV-2 spike protein and enhanced serum neutralization titers against SARS-CoV-2 pseudovirus. Furthermore, the results indicated that larger molecular weight glucans from engineered yeast exhibited stronger immunogenic activity in comparison to wild-type yeast glucans. In conclusion, appropriately formulated glucans have the potential to be scalable, low-cost vaccine adjuvants, potentially overcoming the limitations of current adjuvants.

1. Introduction

Vaccine adjuvants have been shown to play a key role in vaccines' efficacy by boosting and shaping antigen-specific immune responses. These often-overlooked vaccine components are molecules or compounds that have intrinsic immunomodulatory properties and, when administered in conjunction with an antigen, effectively potentiate the host's antigen-specific immune responses compared to responses raised when the antigen is given alone [1]. Substantial effort has been focused on adjuvant selection for SARS-CoV-2 vaccine candidates since 2020 [2,3].

Despite the acknowledged need for novel adjuvants, there are still few adjuvants in licensed human vaccines [4]. After almost a century, aluminium salts (Alum) remain the predominant human adjuvants,

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appearing in over twenty marketed vaccine products. The mechanism of action of Alum, like for most adjuvants, is poorly understood, yet it is believed to exert its adjuvant effects by stimulating Th2-type responses and antibody production through activation of B cells. On the contrary, it seems to be less effective against pathogens that require Th1-cell-mediated immunity. Despite the incomplete understanding of its effects, the repeated use of Alum in vaccines is justified by its apparent safety profile, ease of preparation, stability, potent immunostimulatory ability, and importantly, due to the lack of suitable alternatives [5]. Aluminium salts' limitations on inducing and modulating specific arms of the immune response (humoral and cellular) led to a search for new adjuvants and combinations thereof with specific immune effects critical for successful vaccines against challenging new targets.

Vaccines containing novel adjuvant formulations are increasingly reaching advanced development and licensing stages, providing new tools to fill previously unmet clinical needs. However, many adjuvants fail during product development owing to factors such as manufacturability, stability, lack of effectiveness, unacceptable levels of tolerability or safety concerns [6]. Moreover, the adjuvants in approved products have major supply issues. For instance, squalene oil-in-water (o/w) emulsions are the most widely used class of vaccine adjuvants next to Alum and are often employed in pandemic settings to promote vaccine antigen dose sparing (e.g., MF59® or MF59C.1 used in influenza vaccines FLUAD® and Foclivia®; AS03 used in pandemic H5N1 and H1N1 influenza vaccines Adjupanrix™, Arepannix™ and Pandemrix™). However, the squalene in these adjuvant emulsions is currently derived from shark liver, which raises sustainability and scalability questions [7,8], though recently semi-synthetic squalene analogues which impart vaccine adjuvant activity when formulated into o/w emulsions have been described [9]. Another example of adjuvant supply issues concerns the blockbuster shingles vaccine Shingrix®, which contains a potent adjuvant called AS01. Despite high demand for the vaccine, year-long wait times were not uncommon during initial vaccine roll-out, possibly related in part to the difficult-to-source naturally derived components (e.g., RP-HPLC purified saponin QS-21 from the bark of the Chilean tree, *Quillaja saponaria*). As there is a high demand for this adjuvant and its production is limited due to long processing times and limited availability of natural resources, it is crucial to research more sustainable alternatives from both environmental and commercial points of view [10].

The COVID-19 pandemic illustrates the need for sustainable, scalable, cost-effective adjuvants with established mechanisms of action that can be rapidly deployed to generate effective vaccines and promote antigen dose sparing in pandemic settings. Discovering new adjuvant candidates and learning more about how they work, as well as the best way to formulate them to stimulate specific immune responses, is critical for the development of advanced adjuvants for next-generation vaccines. The availability of new, sustainable, and scalable adjuvants, along with an understanding of their mode of action, will facilitate and accelerate their development and manufacture.

Glucans are naturally occurring glucose polymers present in abundance in plants, bacteria, and fungi. The immunomodulatory properties of glucans are influenced by differences in the type of linkage and branching, solubility, molecular weight, tertiary structure, and polymer charge. Compared to glucans from other origins, fungal β-glucans reveal increased immunogenic properties due to a higher degree of structural complexity – the presence of β(1,6)-side branches [11]. The complexity of β-glucan in yeast is higher than in mushrooms as the latter only possess short β(1,6)-linked branches [12]. Besides, β-glucans, as the most abundant fungal cell wall polysaccharide, are also a key pathogen-associated molecular pattern (PAMP) that is detected upon fungal infection to trigger the host immune responses. Depending on the structure and nature of glucans, they seem to be recognized as PAMPs by different receptors on the surface of neutrophils, monocytes, macrophages, lymphocytes, and dendritic cells, such as toll-like receptors (e.g., TLR-2, 4, 6) and lectin-type C (e.g., dectin-1) receptors [13,14]. Other pattern-recognition receptors are also described as being associated with glucan recognition, including complement (CR2 and CR3) and scavenger (CD5) receptors. Recently, β-glucans have also been shown to induce epigenetic modifications on monocytes and macrophages, which are maintained up to several weeks after the initial stimulus, resulting in an enhanced trained immune memory [15]. These interactions and the capacity to stimulate an immune response are important features in the development of new vaccine adjuvants [16–18]. The use of glucans as vaccine adjuvants has already been tested in the context of cancer treatment, and infectious diseases [19–22]. Furthermore, it is suggested that ingestion of glucans can function either as an adjuvant or as a prophylactic measure [23]. However, the purity of glucan extracts has been pointed out as a limitation for further application in the medical field [13]. Thus, the difficulty in generating pure glucans without the presence of proteinaceous content (glucans' major contaminant) and other impurities has been hampering studies necessary for a better understanding of the complete potential of glucans as lead adjuvants (and as co-adjuvants) in the development of prophylactic vaccines. Indeed, purity is a critical feature for compounds extracted from microbial origin, since less pure adjuvant will increase the risk of toxicity or loss of effect due to the presence of contaminants. In particular, the presence of residual proteinaceous material may distort the metabolic effects evaluation and may lead to unexpected effects when testing different batches of the same adjuvant [6,24]. Overcoming these issues is critical for the potential use of glucans in vaccine adjuvants, especially pandemic vaccines which demands high quantities.

In this work, we hypothesize that protein-free glucans from yeast can be a good adjuvant candidate for SARS-CoV-2 vaccines. We have explored different purification methodologies aiming to obtain protein-free glucans from two *Saccharomyces cerevisiae* strains – parental CEN.PK (subsequently referred to as 'WT') and an engineered steviol glycoside producing derivative (subsequently referred to as 'SG') – suitable for pharmaceutical applications. The highly pure glucans were also chemically modified by carboxymethylation to increase solubility. The glucans were then used to develop a preliminary SARS-CoV-2 vaccine formulation, alone or serving as a co-adjuvant together with Alum and squalene emulsion (SE), using the monomeric Spike S1 protein as an antigen. The vaccines were tested *in vitro* and *in vivo*, and the immunological response was further scrutinized.

2. Material and methods

2.1. Yeast strains

Two strains of *S. cerevisiae* were utilized in this study: a parental (WT) CEN.PK strain and an engineered strain capable of producing a steviol glycoside (SG), which was provided by Amyris, Inc. (Emeryville, CA, USA). The WT strain was cultured in yeast extract peptone dextrose medium (Grisp, Portugal) using a 2L Bioreactor (Eppendorf) at a temperature of 30 °C, maintaining pH at 5, and controlling agitation and air supply to maintain dissolved oxygen at 30 %. Fermentations were terminated once the glucose content was depleted, which was indicated by the increase in dissolved oxygen levels. The yeast was then collected by centrifugation and stored at −20 °C until sufficient quantities were attained for glucan extraction. The SG strain was a by-product of the steviol glycoside fermentation process (i.e., spent yeast), which was removed at the end of a bulk commercial fermentation.

2.2. Glucans extraction

The pipeline for extraction and purification of glucans (Figure S1, supplementary material) was identical for both *S. cerevisiae* strains. Initially, the yeasts underwent a pressure and heat treatment in an autoclave at 121 °C for 20 min. After cooling down to room temperature, the samples were centrifuged at 11,000 g for 10 min at 4 °C, and the supernatant was discarded. The resulting pellet was then used for glucan extraction from WT and SG yeast (WT-Glu and SG-Glu, respectively).

2.2.1. Chemical extraction

The yeast pellets were resuspended at 20 % (w/v) in NaOH 1 M and incubated at 90 °C for 4 h. After incubation, the samples were centrifuged at 11,000 g for 10 min at 4 °C, and the supernatant was discarded. The resulting pellet, with the insoluble yeast components was further washed with deionized water and the pH was adjusted to 7 using 3 M HCl. The material was then dried using a Mini spray dryer (Buchi B-290) with an inlet temperature of 130 °C and an outlet temperature of 60 °C. The resulting dried alkali glucans were further purified using an acid/alcohol solution. Specifically, 1g of the dried material was resuspended in a mixture of ethanol (99 %; v/v) and HCl (32 %; w/w) at an 80:20 ratio (EtOH:HCl; v/v) and incubated at 50 °C for 4 h in an orbital shaker. The suspension was then centrifuged under the same conditions as before, and the resulting pellet was washed twice, once with ethanol and once acetone. The glucans were dried overnight in a vacuum oven at 50 °C.

2.2.2. Protein removal

To remove the remaining protein, 1 % (w/v) glucan aqueous solutions were prepared with 2 % (w/v) sodium dodecyl sulphate (SDS) and boiled at 100 °C for 15 min. The suspensions were then centrifuged, and the resulting pellet was resuspended in SDS solution and boiled again. This step was repeated three times. After the third boiling step, the pellet was washed twice with deionized water and twice with acetone. The supernatants were discarded in each wash by centrifugation. The purified glucans were dried overnight at 50 °C in a vacuum oven.

2.3. Glucans carboxymethylation

The purified glucans from both strains were functionalized by the addition of carboxymethyl groups (CM) to enhance water solubility. Thus, 1 g of purified glucans was suspended in 25 mL of 80 % (v/v) ethanol and 2.5 mL of 50 % (w/v) NaOH, and then incubated for 1 h in a water bath at 35 °C with agitation at 160 rpm. Subsequently, 2.5 mL of 50 % (w/v) NaOH and 35 mL of monochloroacetic acid (0.35 M in ethanol) were slowly added under stirring at room temperature and incubated for 2 h at 50 °C. Once the reaction time had elapsed, the mixture was neutralized with glacial acetic acid and washed via centrifugation with 80 % (v/v) ethanol to eliminate undesired salts. The supernatant was discarded, and the resulting pellet was dried in a vacuum oven at 40 °C. The degree of substitution (DS) of this functionalization was performed according to Ding and co-workers [25].

2.4. Glucans extracts characterization

2.4.1. Chemical composition determination

The protein content of the samples was determined by quantifying the total amino acids using iodoacetic acid derivatization and o-phthaldialdehyde methodology [26]. For sugar analysis, the samples were hydrolyzed to monosaccharides, and the resulting alditol acetates were analyzed by gas chromatography flame ionization detection (GC-FID) [27]. The moisture content was determined by drying the samples for 24 h at 105 °C to remove any moisture, while the total ash content was analyzed by incinerating the extracts at 550 °C for 24 h. Total lipid was analyzed according to Breil et al [28]. The β- and α-glucan content in the samples was quantified using an enzymatic yeast β-Glucan assay kit (K-EBHLG, Megazyme kit) and an α-glucan assay kit (K-YBGL, Megazyme kit), respectively. The molecular weights of the glucans were estimated using high performance size exclusion chromatography (HPLC-SEC) with evaporative light scattering detection (Agilent). The water-insoluble glucans were dissolved in dimethyl sulfoxide (DMSO) with 0.25 M lithium chloride, and the water-dispersable glucans (carboxymethylated ones) were dissolved in ultra-pure water, both at a concentration of 1 mg/mL. The HPLC separations of the water-insoluble glucans were performed on an Agilent PLgel column with DMSO as the mobile phase, while an Agilent PL aquagel-OH MIXED-H column with ammonium acetate 10 mM as the mobile phase was used for the water-dispersable glucans. Both columns were operated at room temperature with a flow rate of 0.7 mL/min, and the injection volume was 20 μL. The evaporative light scattering detector (ELSD) was operated at evaporation temperatures of 70 °C and 80 °C and nebulization temperatures of 65 °C and 85 °C for the water-insoluble and water-dispersable glucans, respectively. The nitrogen flow rate was 1.10 SLM (standard litter per minute) for the water-insoluble glucans and 1.24 for the water-dispersable glucans. MW measurements were made using polystyrene and pullulan standards.

2.4.2. Chemical structure characterization

Fourier-transform infrared spectroscopy (ATR-FTIR) analysis was conducted using a Spectrum 100 FTIR spectrometer (Perkin Elmer, Massachusetts, USA) equipped with an attenuated total reflection (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate. The analysis utilized 20 scans and spectra were collected in the absorbance range of 3600 to 600 cm^{-1} at a resolution of 1 cm^{-1}. For nuclear magnetic resonance (NMR) analysis, 20 mg of glucan samples were solubilized in deuterated dimethyl sulfoxide (DMSO-d_6) to obtain the NMR spectra of 13C, 1H, and the heteronuclear single quantum coherence (HSQC) ^1H–^{13}C correlation spectra. The spectra were acquired on a Bruker Avance III 600 HD spectrometer and processed using Bruker TopSpin 4.1.3 software (Bruker, Massachusetts, United States). Chemical shifts were reported in ppm (δ units) from internal tetramethylsilane (TMS).

2.5. Antigens and adjuvants

The extracted glucans were formulated with aluminium hydroxide (Alhydrogel, Croda) or squalene emulsion (SE, AAHI). The antigen used in this work was the monomeric Spike S1 protein from SARS-CoV-2 (Wuhan-Hu-1 variant) (Genescript, USA) and was added to the adjuvant formulation prior to the immunization. Saline solution was purchased to Ricca Chemical (USA). The adjuvant formulations were obtained by mixing glucans at 0.5 mg/ml with Alhydrogel (Alum) at 2 mg/ml aluminium or SE at 5 % (v/v) squalene.

2.6. Characterization of adjuvant formulations

A compatibility study was performed to assess the compatibility of spike protein and adjuvant combinations. Formulations were visually analyzed for potential flocculation/aggregation and also characterized for particle size and polydispersity index (PDI) by using the photon correlation spectroscopy technique of dynamic light scattering. The surface charge of the particles was analysed by measuring the zeta potential using laser-Doppler electrophoresis. Adjuvants alone or mixed with equal volumes of spike protein solutions were mixed and allowed to equilibrate for at least 10 min prior to the size and zeta potential measurements. Measurements were repeated after 4 h, with mixtures stored at 5 °C or 25 °C and after 1 year for mixtures stored at 5 °C. For the size measurements, the samples were diluted 100 times in milli-Q water. The measurements were performed at ambient temperature using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. Data acquisition and analysis were performed using Malvern DTS v.7 or later software. Particle size distribution was reflected in the PDI, which ranges from 0 for a monodisperse to 1.0 for a heterodisperse formulation.

2.7. Whole-blood cytokine secretion assay

Fresh heparinized whole blood from 8 human volunteers (equal numbers of male and female) was obtained from Bloodworks Northwest. Biological sample collections were approved by WCG IRB. All participants reviewed and signed informed consent forms. Glucan formulations were diluted 1:2.5, 5, 10, and 50 in 50 µl of saline, followed by the addition of 200 µl whole blood, resulting in final glucan concentrations of 40, 20, 10, and 5 µg/ml (plus 0.4, 0.2, 0.1, and 0.05 % v/v oil in the emulsion formulations and 160, 80, 40, and 20 µg/ml aluminium in the Alum formulations. The sample preparations were incubated for 18–24 h at 37 °C with 5 % CO_2. After incubation, supernatants were aspirated and assessed by ELISA kits for production of IL-6, IL-8, CCL2 (MCP-1) (Life Technologies), and CCL4 (MIP-1β) (R&D Systems) cytokines according to manufacturer's instructions.

2.8. Animal immunization

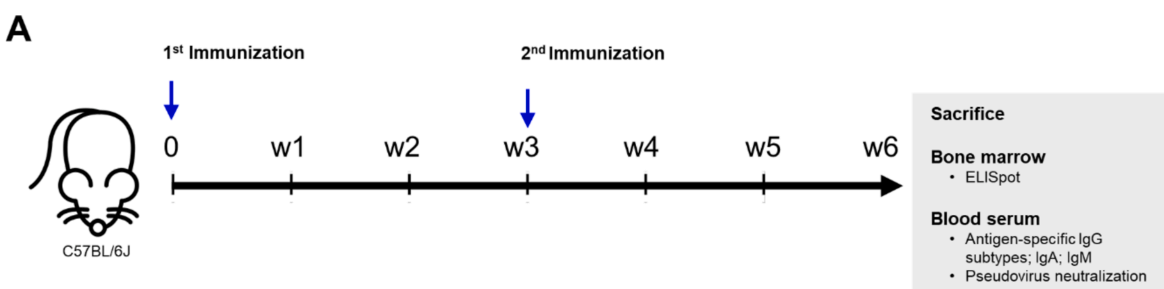
All animal experiments were conducted in accordance with the guidelines and regulations of the competent Portuguese institutions, including the *Direção Geral da Alimentação e Veterinária* and *Orgão Responsável pelo Bem-estar dos Animais*. C57BL/6J mice were purchased from Charles Rivers and 120 mice were distributed across 12 groups, consisting of 5 male and 5 female mice per group (Fig. 1). Mice were immunized intramuscularly twice (25 µl per injection), at day 0 and day 21 (3 weeks apart). Three weeks after the second immunization, animals were sacrificed, and serum, and splenocytes from both femurs were collected for further evaluation.

2.9. Enzyme-linked immunospot (ELISpot) assay

Bone marrow was harvested from both femurs by flushing snipped femurs with 1 mL of RPMI (Gibco) supplemented with 10 % FBS (Gibco) and 1 % penicillin/streptomycin (Gibco), and placed in a 0.5 ml tube with a snipped end coupled to a 2 ml tube. Samples were centrifuged for 5 min at 1800 rpm, the supernatant was removed, and the cell pellet was resuspended and incubated in RBC lysis buffer (BioLegend) for 5 min at room temperature. Samples were then centrifuged, resuspended in media, and filtered in 24 well filter-plates (PALL) with a short spin. Cell density was adjusted to $1x10^7$ viable cells/ml. ELISpot filter plates (Mabtech) were prepared according to the manufacturer's instructions using SARS-CoV-2 spike protein (Genscript) as the antigen. Samples were seeded in the filter plates in 4 serial dilutions (1:3) and then incubated overnight at 37 °C with 5 % CO_2. Afterwards, plates were washed 5 times with PBS and incubated with anti-mouse IgG-biotin antibody in PBS-0.5 % FBS at 100 ng/well for 2 h at room temperature. Plates were washed and incubated for 1 h at room temperature with streptavidin-ALP (1:1000). After washing the plates, 100 µl of freshly filtered substrate solution was added to each well for 15 min. Color development was stopped by extensive washing with tap water. Plates were air-dried and shipped to Mabtech facilities (Stockholm, Sweden) for ELISPOT plate scanning.

2.10. SARS-CoV-2 specific IgG titers

The level of specific IgG in the subjects' serum was evaluated. 96-well plates were coated overnight at 4 °C with SARS-CoV-2 spike protein diluted in PBS at 300 ng/well. The plates were washed five times with 0.1 % PBS Tween-20 and then blocked with 4 % powdered milk for



B

Group			Food	Dosage
1	Saline		Food and water ad libitum	Saline solution (0.9% NaCl)
2	Antigen			Spike S1 Protein (10µg total)
3	Alum			Alum at 2 mg/mL + Antigen
4	SE			SE at 5% + Antigen
5	WT-Glu	-		Glucans at 0.5 mg/mL + Antigen
6		w/Alum		Glucans at 0.5 mg/mL + Alum at 2 mg/mL + Antigen
7		w/SE		Glucans at 0.5 mg/mL + SE at 5% + Antigen
8	WT-Glu-CM	-		Glucans at 0.5 mg/mL + Antigen
9		w/Alum		Glucans at 0.5 mg/mL + Alum at 2 mg/mL + Antigen
10		w/SE		Glucans at 0.5 mg/mL + SE at 5% + Antigen
11	SG-Glu	-		Glucans at 0.5 mg/mL + Antigen
12		w/SE		Glucans at 0.5 mg/mL + SE at 5% + Antigen

Fig. 1. Immunization of mice with the developed vaccine formulations. A – Schematic representation of the immunization plan and the samples and data to be collected from the experiment. B – Distribution of the animals in 10 groups with each group representing each one a different treatment. WT-Glu: Glucans from parental CEN.PK yeast. SG-Glu: Glucans from engineered steviol glycoside-producing yeast.

2 h at room temperature. Serum samples were added at a 5-fold dilution and then serially diluted at 1:3. After 1.5 h of incubation at room temperature, plates were washed and anti-IgG (Sigma), anti-IgG1 (Invitrogen) and anti-IgG2a (Invitrogen) HRP conjugated antibodies were added at a 1:2500, 1:5000 and 1:2000 dilutions respectively, and incubated for 1 h at room temperature. Plates were washed and TMB substrate was added, and after 15 min, the colour development was stopped by adding 2 M H_2SO_4. The absorbance at 450 nm was read using a Synergy H2 microplate reader (Biotek). IgG titers were determined as the highest serum dilution providing an optical density twice that of the control group (saline group).

2.11. Immunoglobulin isotyping

The levels of immunoglobulin isotypes (IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM) in mouse serum were evaluated using a multiplex kit (Legendplex, Biolegend) and flow cytometry (Accuri C6plus, BD Biosciences) following the manufacturer's instructions.

2.12. Pseudovirus neutralization assay

To evaluate the ability of immunized mice to neutralize the interaction between SARS-CoV-2 and its receptor ACE-2 on the surface of human cells, a pseudovirus assay was performed, following the methodology described by [29]. A non-replicative pseudovirus expressing the SARS-CoV-2 spike protein with luciferase (provided by AAHI) or green fluorescent protein (GFP) reporters (BPS Bioscience) and a HEK-293 cell line modified to express the ACE2 receptor (BPS Bioscience) were used. Blood serum from immunized mice was serially diluted at 1:2, starting with a dilution of 1:5 in DMEM 10 % FBS, 1 % penicillin/streptomycin, and sodium pyruvate, and incubated with pseudovirus at a concentration of $4x10^6$ RLU/ml and polybrene (12.5 μg/ml, Sigma) for 1 h at 37 °C and 5 % CO_2. The neutralization mixtures were then added to HEK-293-ACE2 cells previously seeded in 96-well plates at $2x10^5$ cells/ml. The plates were incubated for 72 h, after which the Steady-Glo solution (Promega) was added, and luminescence was measured in a microplate reader. EC_{50} values were determined using the Quest Graph™ IC_{50} calculator.

2.13. Statistical analysis

Data analysis and comparisons between groups were calculated as follows: for whole blood chemokines and cytokines expression, a two-way ANOVA with Tukey's multiple comparisons as a post-test was performed. For immunoglobulin isotyping, specific IgG titers, and pseudovirus neutralization, a non-parametric Wilcoxon matched pairs test was performed, as the normality assumption was not met for any dataset according to the Shapiro–Wilk test. Data from the ELISpot assay were evaluated using a one-way ANOVA with Dunnett's multiple comparison test. Statistical analysis was performed using GraphPad Prism v9.4.0 and Statistica™ 14.0. Graphical representations were generated using GrapPad Prism v9.4.0 and the results are expressed in average ± standard error of the mean for each group.

3. Results

3.1. Chemical and structural characterization

Glucans extracted and purified from both yeasts have a similar composition, with over 99.0 % carbohydrates, where 64–70 % are β-glucans and 29–36 % α-glucans (Table 1).

The ^{13}C NMR spectrum or the glucans purified and treated with SDS (Fig. 2) showed the main carbon signal peaks with high resolution related with glucose structure were observed, and the chemical shifts were easily assigned, corresponding to a main chain of glycosidic units linked in β(1 → 3), with branching points [30]. The spectra for WT-Glu and SG-Glu were overlapped and no differences were observed in chemical shifts. The ^{1}H resonance spectra also revealed the same pattern between both glucan's sources (supplementary table S1).

To accurately assign the proton chemical shifts, ^{1}H–^{13}C NMR heteronuclear single quantum correlation (HSQC) spectrum was performed Fig. 2 A-B. With the peak areas of protons' assignments of H1 backbone (BC) and H1 of the branched single or terminal C1 in the side chain (TSC) (summarized in supplementary table S2), the degree of branching and α/β ratio was calculated according to Kim *et al* [31]. The α/β linkage ratio obtained was 1:2, which is similar to those obtained by enzymatic quantification, and further revealed a similar branching degree for both extracts, 0.06–0.07 (i.e., the main chain of β-(1,3)-glucopyranosyl units contains β-D-glucopyranosyl branch units linked 1,6 at an average interval of fourteen to sixteen main chain units [31]), These data demonstrate that both WT-Glu and SG-Glu present similar α/β glucans complexes (Fig. 2C).

Monosaccharide composition analysis confirmed the yeast glucans' characteristic homopolymer structure, exclusively composed of glucose monomers. After SDS treatment, the presence of proteins, lipids, minerals, and other carbohydrates could not be detected in the glucan samples, or were insignificant, and about 0.5–1 % was moisture. ATR-FTIR spectra showed no peaks in the region from 1650 to 1550 cm^{-1} (amide C = O stretching, a characteristic absorption of proteins), further confirming the absence of proteinaceous residues in both samples (Figure S2, supplementary material). On the contrary, two peaks were present at 1040 cm^{-1} and 1080 cm^{-1} (related to the stretching of C-O and C–C), which have been previously attributed to β-(1,3)- and β-(1,6)-glucans, respectively [30]. HPLC-SEC results showed that WT-Glu possessed a lower average molecular weight than SG-Glu, with values of 387 KDa and 451 KDa, respectively (Table S3, supplementary material).

In order to enhance the water solubility of the glucan extracts, a carboxymethylation step was performed. The results indicated that both WT-Glu-CM and SG-Glu-CM exhibited similar degrees of substitution, approximately 0.30 for the wild type and 0.33 for the SG strain. Consequently, the average molecular weight of both carboxymethylated glucans increased compared to the non-carboxymethylated forms, with values of 494 KDa for WT-Glu-CM and 489 KDa for SG-Glu-CM (Table S3, supplementary material). ATR-FTIR results confirmed the successful addition of carboxymethyl groups, revealing new strong absorption peaks at 1595 and 1421 cm^{-1}, which are attributed to COO^- stretching due to the introduction of carboxymethyl anions in the structure, as previously reported by [32].

Table 1
Chemical composition of the extracted glucans after alkali and organic/acid treatment and after SDS treatment. Results are presented as a percentage of the dry weight. (n.d. – not detected).

Extraction process	Strain	% dw						
		Moisture	Minerals	Protein	Lipids	Carbohydrates	β-Glucan	α-Glucan
Glucan Alkali + Organic/acid treatment	**SG**	2.0 ± 0.1	1.1 ± 0.3	3.9 ± 0.1	1.0 ± 0.1	90.9 ± 3.8	59.0 ± 0.2	31.8 ± 1.2
	WT	1.0 ± 0.1	0.8 ± 0.3	2.0 ± 0.1	0.5 ± 0.1	94.9 ± 3.8	64.6 ± 0.4	30.3 ± 0.2
Glucan Alkali + Organic/acid + SDS treatment	**SG**	1.0 ± 0.1	< 0.01	< 0.01	n.d	99.0 ± 1.1	64.0 ± 2.2	35.6 ± 2.2
	WT	0.5 ± 0.3	< 0.01	< 0.01	n.d	99.6 ± 3.8	70.0 ± 0.4	29.3 ± 1.0

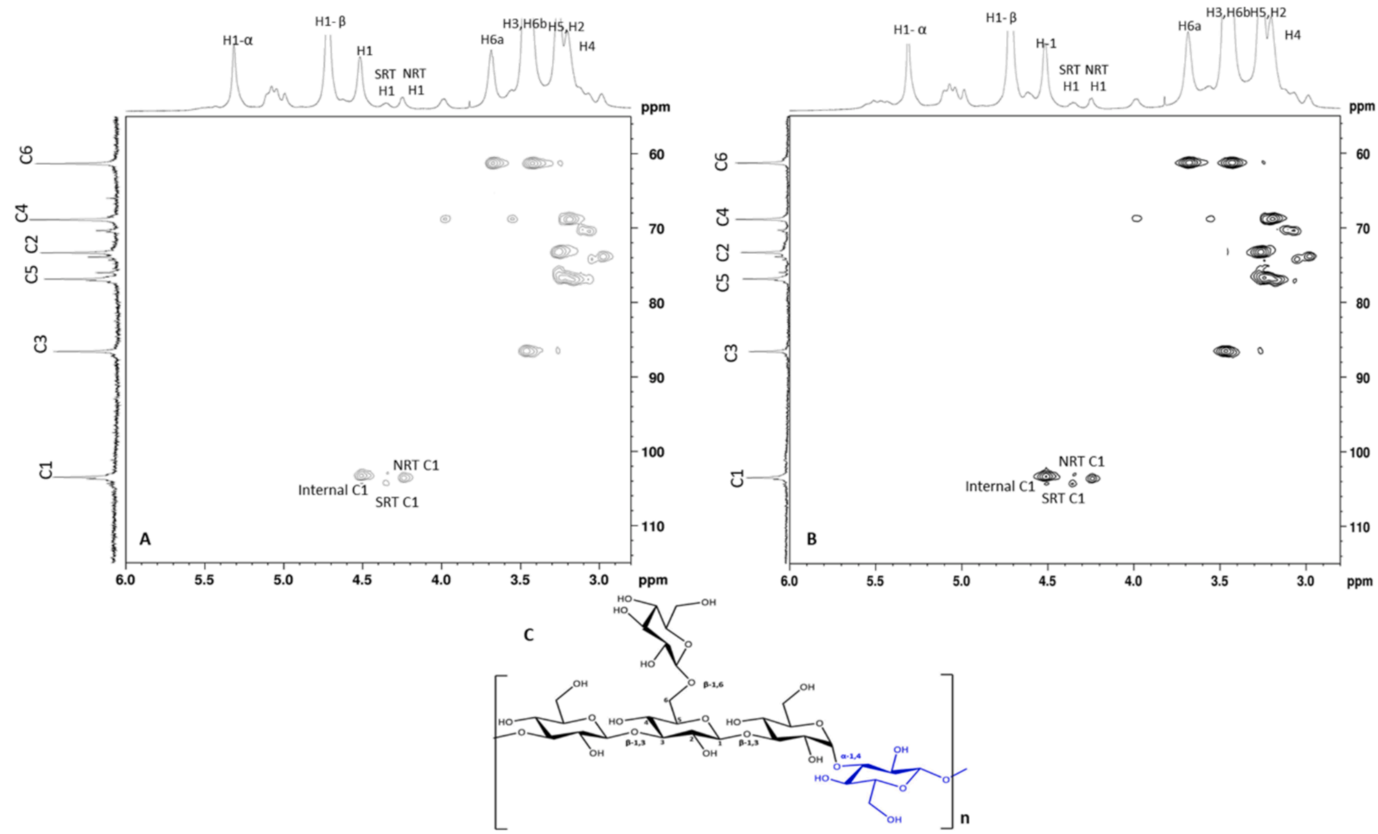


Fig. 2. [1]H – 13C correlation spectrum (HSQC) of glucan measured in DMSO-d_6. The measured 13C spectrum is shown on the vertical axis and the 1H projection from the two-dimensional-spectrum is shown on the top. (A) WT-Glu (B) SG-Glu (C) Structure of mixed linked glucans.

3.2. Glucans increase the innate immune activity of Alum and SE in human whole blood

The different adjuvant combinations prepared showed compatibility and stability over time as glucans did not alter the major physical chemical features of alum and SE (see supplementary tables S4, S5 and S6). The various adjuvant formulations (without antigen) were evaluated for their capacity to stimulate the secretion of chemokines (CCL2 and CCL4) and cytokines (IL-6 and IL-8) in whole blood, aiming to understand if glucans have the potential to enhance the innate immune response induced by the appropriately formulated classical adjuvants, Alum and SE (Fig. 3). The levels of CCL2 were significantly increased by formulations combining Alum with WT-Glu, WT-Glu-CM, SG-Glu, and SG-Glu-CM (Fig. 3A), while only formulations combining Alum with CM-glucans were capable of significantly enhancing CCL4 levels (Fig. 3B). Following the same trend, IL-6 (Fig. 3C) and IL-8 (Fig. 3D) levels were drastically increased by the addition of CM-glucans to Alum. Regarding SE-based formulations, only the combination with SG-Glu showed capability in significantly increasing the levels of the chemokines and cytokines being screened. Overall, the combination of Alum with CM-glucans (i.e., modified water-dispersable glucans) seems to perform better than with insoluble glucans, which is most probably related to Alum's compatibility with water dispersable components, in theory impacting on the formation of antigen-adjuvant complexes. Nevertheless, the evaluation of the stability of the different Alum formulations did not indicate major physicochemical differences between the formulations (Table S2 and S4, supplementary material), or even on spike protein adsorption to Alum (no non-adsorbed protein was detected for any of the Alum-containing formulations evaluated). It should also be mentioned that in many instances, the use of glucans alone (0.005 % w/v) showed equivalent or even better performance than Alum (0.002 % w/v) or SE (0.05 % w/v) alone. The capacity of glucans to independently enhance whole blood immune response was further increased when glucans concentrations were increased 2- to 8-fold, from 0.005 % w/v to 0.04 % w/v (Figure S3, supplementary material). Such effect is suggestive of the potential of the different glucans as vaccine adjuvants.

Taking into consideration the results obtained *in vitro* with whole blood, the adjuvant combinations that revealed better performance were selected to proceed to *in vivo* studies. Since water-dispersable glucans showed similar results regarding the stimulation of chemokines and cytokines production, we chose to proceed with formulations combining only the parental version, WT-Glu-CM, with Alum and SE. Glucans extracted from the SG strain were selected to be combined with squalene-based adjuvant (SE), as they were the only ones showing promising *in vitro* results. Additionally, WT-Glu was also used in combination with Alum due to the CCL2 and CCL4 stimulus shown *in vitro* and with SE in order to compare the results between glucans extracted from wild type and SG strains.

3.3. Glucans adjuvant potential: In vivo studies

3.3.1. Formulations of Alum with glucans

3.3.1.1. Serum immunoglobulins. The immunized animals remained healthy throughout the experiment, with no significant decrease in body weight compared to the control group (Fig. 4A). The evaluation of immunoglobulin classes and subclasses in blood serum was performed using multiplex bead-based flow cytometry. Differences in IgG1 levels were observed between formulations combining Alum with WT-Glu and the formulation containing the antigen alone or with Alum (Fig. 4B). Regarding IgG2a, only the formulation combining Alum with WT-Glu was able to significantly elevate the levels of this immunoglobulin (Fig. 4C). No significant variations were observed concerning IgG2b,

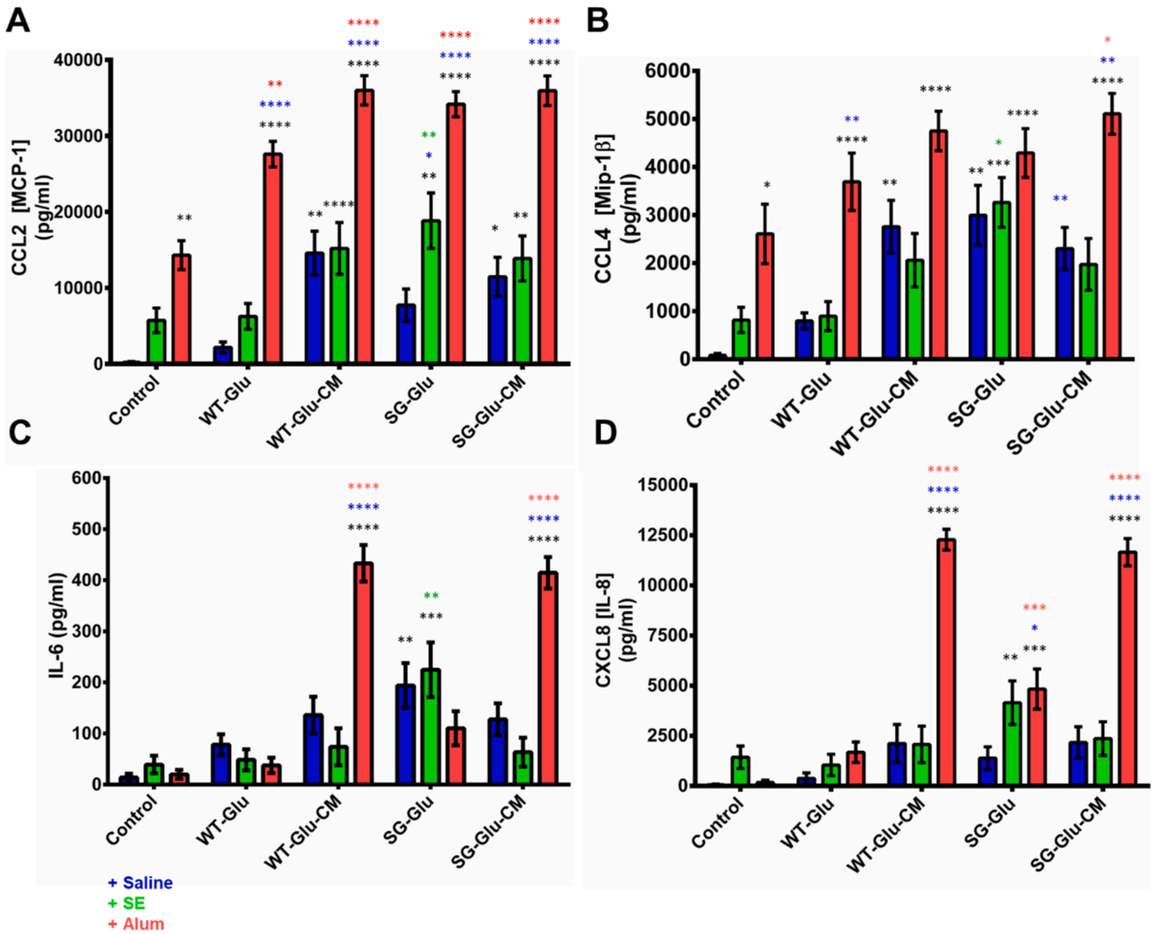


Fig. 3. Graphic representation of the cytokine and chemokine levels present in human whole blood exposed to different glucan formulations. The levels of CCL2 [MCP-1], CCL4 [Mip-1β], IL-6 and CXCL8 [IL-8] (A to D, respectively) were evaluated in whole blood exposed for 24 h to formulations with saline buffer (blue), 0.05 % SE (green) or 0.002 % alum (red). Indicated glucans were added at 0.005 % as shown. Results are expressed in pg/ml corresponding to the average ± standard error of the mean for each group. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the two-way ANOVA with Tukey's posttest; black asterisks indicate significative differences to control, blue asterisks to the glucans alone, green asterisks to SE and red asterisks to alum). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IgG3, or IgM serum levels (Fig. 4D, E, and G, respectively). Combinations of Alum with WT-Glu or with WT-Glu-CM produced a significant increase in serum IgA levels (Fig. 4F).

3.3.1.2. Stimulus of spike S1 specific IgG antibodies. Bone marrow samples were taken post-boost and used to measure spike protein S1-specific IgG-secreting cells by ELISpot. Higher levels of bone marrow-resident IgG-secreting cells were detected in groups immunized with formulations combining Alum with WT-Glu or with WT-Glu-CM than in the group immunized with Alum alone (Fig. 5A). These results suggest the establishment of robust, long-lived IgG-secreting plasma cell populations. However, the serum levels of IgG anti-SARS-CoV-2 spike protein were only significantly higher in the group immunized with a combination of Alum plus WT-Glu-CM (Fig. 5B). The ratio of antigen-specific IgG1 and IgG2a also elicits a favouring of a Th2 response being the combination of Alum with WT-Glu-CM significantly higher than antigen and alum (Fig. 5C). While an anti-IgG2a antibody was used in this study, it is recognized that IgG2c is a more accurate marker for Th1/Th2 responses in C57BL/6J mice. This distinction should be considered in future studies to improve the precision of immune response evaluations in this strain.

3.3.1.3. Capacity to neutralize SARS-CoV-2 pseudovirus. The capacity of blood serum antibodies to neutralize the activity of SARS-CoV-2 was evaluated using a luciferase-mediated pseudovirus neutralization assay. The neutralization of SARS-CoV-2 pseudotyped lentiviral particles was significantly higher in mice immunized with Alum plus WT-Glu or WT-Glu-CM than in the group immunized with Alum alone (Fig. 5D). The stronger neutralization capacity of SARS-CoV-2 pseudovirus by serum antibodies from mice immunized with the WT-Glu-CM formulation was further confirmed via fluorescence measurement using GFP as a reporter protein (Figure S4, supplementary material).

3.3.2. Formulations of SE with glucans

3.3.2.1. Serum immunoglobulins. Mice immunized with SE formulations were healthy during the experiment with no significant alterations in body weight, compared to the control group (Fig. 6A). The evaluation of immunoglobulin classes and subclasses found in blood serum was performed as previously mentioned for Alum formulations. Differences in IgG1 levels were observed between formulations combining SE with water-insoluble glucans (WT-Glu and SG-Glu) and the formulation containing the antigen alone (Fig. 6B). Additionally, the formulation combining SE with SG-Glu induced significantly higher IgG1 levels than the SE formulation. No significant differences were observed among the different immunized groups regarding other IgG subclasses, namely IgG2a, IgG2b, and IgG3 (Fig. 6 C, D and E, respectively). Additionally, the SE plus SG-Glu formulation also boosted IgA and IgM levels (Fig. 6 F and G, respectively).

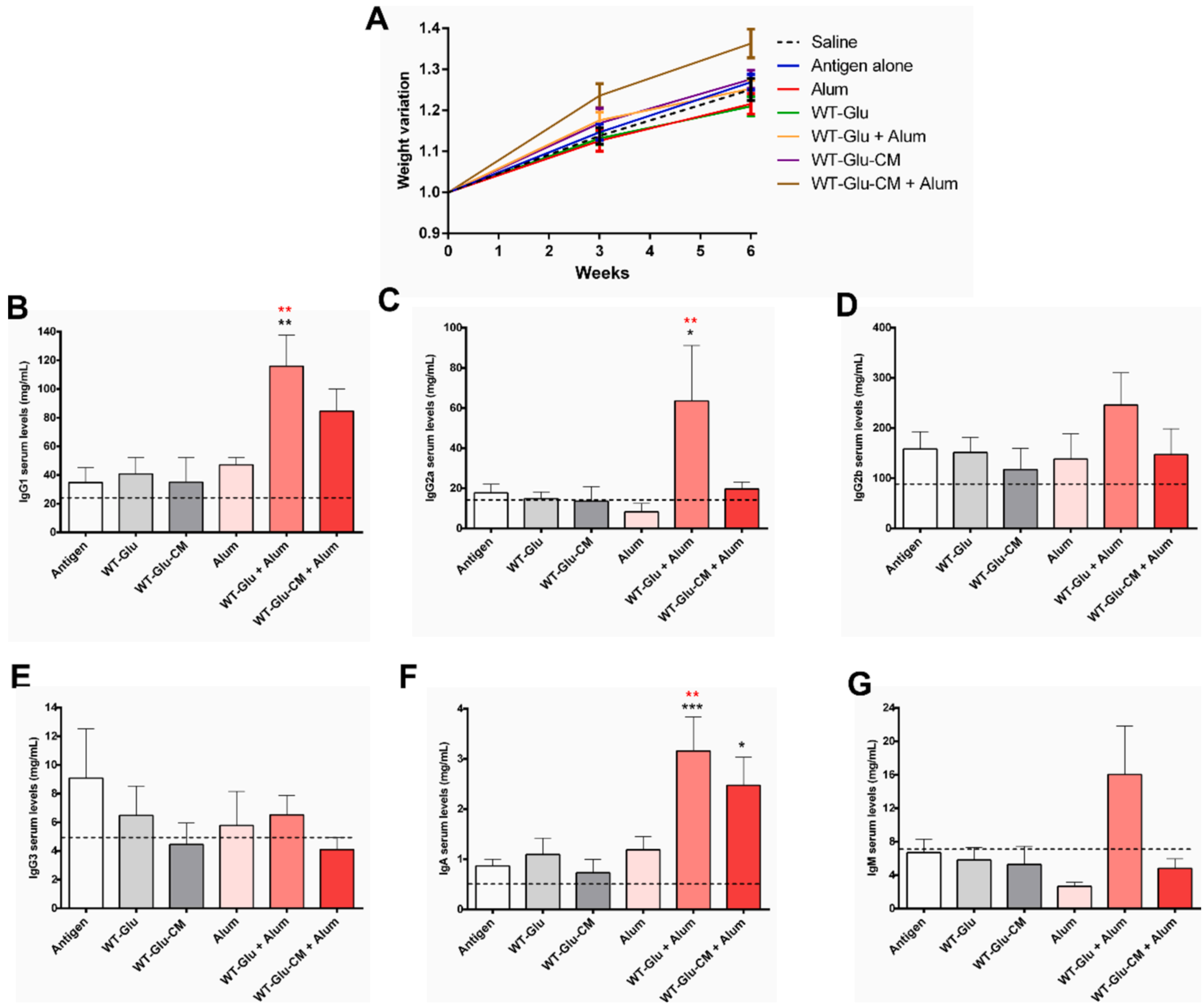


Fig. 4. General immunoglobulins levels upon immunization for SARS-CoV-2 spike protein using alum and glucans as adjuvants. A – Weight variation of the animals during the immunization period; B to G – levels of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM present in the blood serum of immunized mice. Results are expressed in mg/ml corresponding to the average ± standard error of the mean for each group. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test; black asterisks indicate significant differences to antigen and red asterisks to alum). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2.2. Stimulus of spike S1 specific IgG antibodies. Overall, higher levels of bone marrow-resident IgG-secreting cells were detected in all groups immunized with formulations combining SE with glucans than in the group immunized with the antigen alone (Fig. 7A). Moreover, SE combined with SG-Glu significantly stimulated the number of spike protein S1-specific IgG secreting cells, compared to the group immunized with SE alone. Following the same trend, this same formulation also induced significantly higher serum levels of IgG anti-SARS-CoV-2 spike protein than the group immunized with SE alone (Fig. 7B). In general, all groups immunized with SE (with or without glucans) showed higher levels of anti-spike protein IgGs than the group immunized with the antigen alone. The IgG1:IgG2a ratio for antigen-specific response support a Th2 response which is significantly higher than the antigen for the combination of SE with SG-Glu.

3.3.2.3. Capacity to neutralize SARS-CoV-2 pseudovirus. As with Alum formulations, we assessed the capacity of blood serum antibodies to neutralize the activity of SARS-CoV-2 through a luciferase-mediated pseudovirus neutralization assay and further confirmed the results via a second measurement using GFP as a reporter protein. Both methods revealed that the addition of water-insoluble glucans (WT-Glu or SG-Glu) to SE led to a significant increase in the capacity of serum antibodies to neutralize the SARS-CoV-2 pseudovirus, compared to the antibodies from the group immunized with SE alone (Fig. 7C; Figure S5, supplementary material).

4. Discussion

This study focuses on the obtention and use of highly purified yeast-derived glucans as adjuvants in vaccines. We present a method to obtain high MW protein-free glucans from *Saccharomyces cerevisiae* strains, which includes a purification step using the anionic surfactant SDS. This step was proven to effectively remove remaining proteinaceous residues thus, preventing cross-reactivity issues. However, high MW protein-free glucans are water insoluble which might present an issue for the development of adjuvant formulations. To address this, we chemically

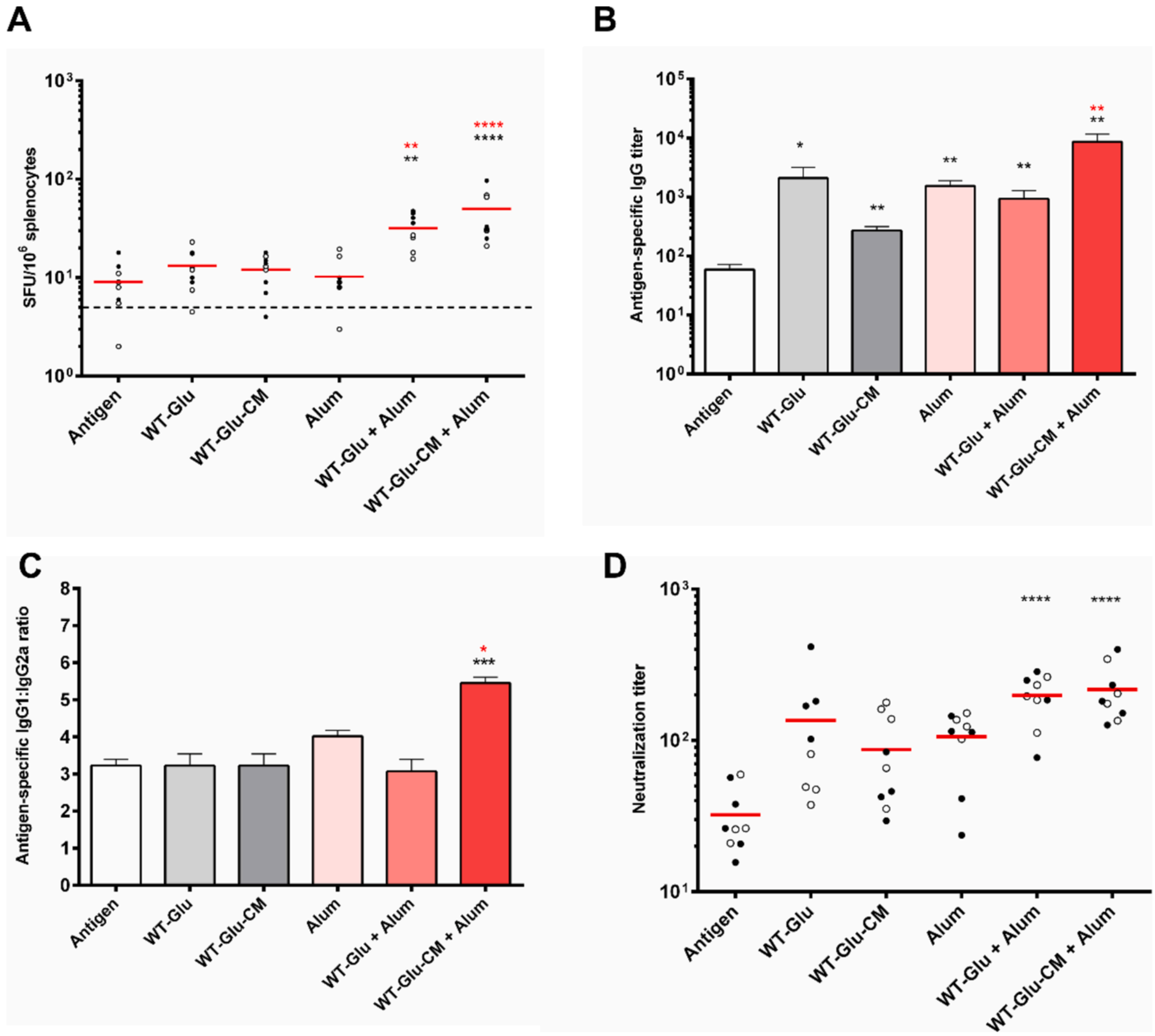


Fig. 5. Specific response against SARS-CoV-2 spike protein in mice immunized using alum and glucans as adjuvants. A – Levels of Antigen-specific IgG producing splenocytes in mice immunized with Alum and glucans formulations. Black dots correspond to females and white dots to males. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by one-way ANOVA); B – Titer of spike protein S1 specific IgG present in the blood serum of mice immunized with glucans and alum vaccine formulations. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test); C – Antigen-specific IgG1:IgG2a ratio calculated using IgG1 and IgG2a titers. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test); D – Pseudovirus Spike protein neutralization assay for mice immunized with different formulations of glucans and alum. Black dots correspond to females and white dots to males. (The presented values correspond to each animal in its respective group and the group average. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by Wilcoxon non-parametric test; black asterisks indicate significant differences to antigen and red asterisks to alum). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

modified the glucans by attaching carboxymethyl groups to their polysaccharide chain. This modification made the glucans more hydrophilic, and thus more dispersable.

The analysis of the structure of the purified glucans revealed no significant differences besides the molecular weight, between the glucans obtained from the wild type CEN.PK and from the SG *S. cerevisiae* strain. It is unclear whether the difference in MW of the glucans isolated from the two strains is due to the strain differences or in the different cultivation conditions of the yeast from which the glucans were isolated. The glucans were found to be a mixture of α/β-(1,4) and (1,3)-β-linked backbones with small numbers of (1,6)-β-linked side chains, which has been recently described in the literature [33], but their biological impact has not been fully explored yet. This provides a new perspective on mixed-linked glucans, which goes beyond β-glucans that are traditionally known as immunomodulators.

Despite the remaining uncertainties regarding the precise impact of glucan physicochemical characteristics on the immune system, water solubility (native glucans *vs.* water-dispersable CM-glucans) and molecular weight (wild-type *vs.* SG) have been strongly associated with distinct immune-modulating properties, even when comparing glucans obtained from the same source [11]. Studies have indicated that low molecular weight water-soluble β-glucans derived from yeast have a high affinity for binding to complement receptor 3 expressed by innate immune cells such as macrophages, dendritic cells, natural killer cells, and neutrophils [34,35]. However, these glucans do not appear to stimulate dectin-1 and lack the ability to promote an effective innate immune memory response [34]. Conversely, high molecular weight (particulate) β-glucans have been found to have a greater ability to modulate immune responses through dectin-1 activation on macrophages, leading to phagocytosis, ROS generation, microbial killing, and

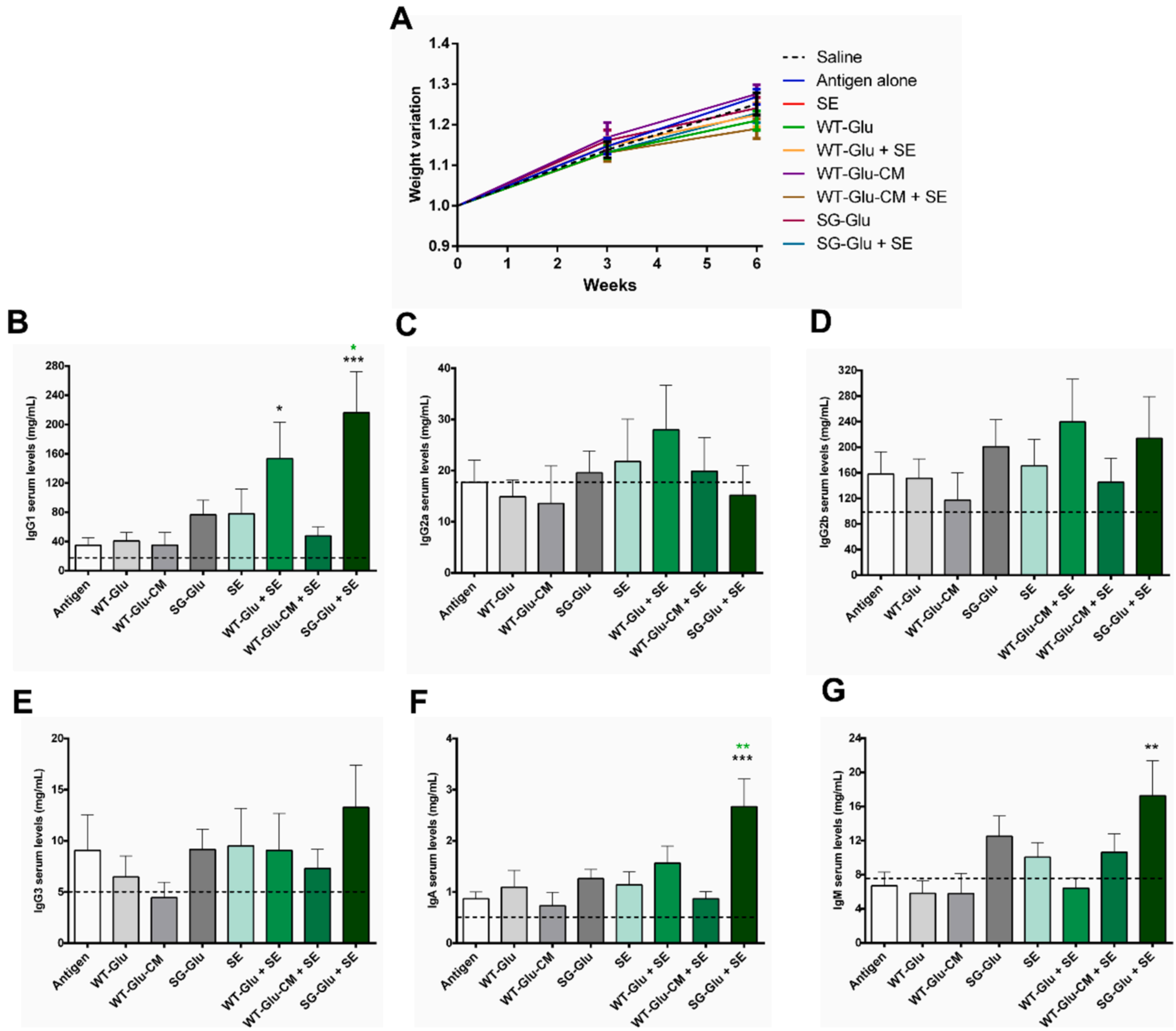


Fig. 6. General immunoglobulins levels upon immunization for SARS-CoV-2 spike protein using SE and glucans as adjuvants. A – Weight variation of the animals during the immunization period; B to G – levels of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM present in the blood serum of immunized mice. Results are expressed in mg/ml corresponding to the average ± ± standard error of the mean for each group. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test; black asterisks indicate significant differences to antigen and green asterisks to SE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cytokine/chemokine production [36]. In addition, higher molecular weight glucans demonstrate a higher affinity for complement receptor 3 expressed by granulocytes and may exert a more profound influence on the immune system, possibly due to their more stable structure [11]. This aligns with the general principle that larger immunogens are usually more immunogenic and provide a possible explanation for the observed differences in subsequent biological evaluations [37]. It is therefore essential to explore how glucans can function as vaccine adjuvants and interact with state-of-the-art adjuvants, such as Alum salts or SE, which trigger different immune pathways [17].

We developed glucan formulations along with Alum and SE, which demonstrated adequate physicochemical properties and stability. The cytokine and chemokine production by whole blood exposed to glucan formulations with Alum or SE indicated additive and synergistic effects between glucans and these classic adjuvants. Combining Alum with glucans resulted in a significant stimulation of CCL2 for all tested samples, and water-dispersable glucans further increased CCL2, IL-6 and IL-8 levels. The results suggest that Alum is more compatible with water-dispersable glucans, which may be explained by better interaction between the two ingredients. The combination of glucans extracted from SG strain with SE formulations led to an increase in the levels of the four cytokines/chemokines analysed. Overall, these findings indicate that glucans, when combined with Alum or SE, can potentiate the immunogenic effect of these adjuvants, highlighting the potential of glucans as adjuvants.

The COVID-19 pandemic highlighted the significance of vaccines and vaccine development, with several vaccine technologies rapidly developed to combat the spread of SARS-CoV-2 [38]. Aluminium salts have been commonly used as adjuvants in vaccines, but their limited ability to promote cellular mediated immunity is well-documented [39]. Squalene also has limitations concerning its sustainable production [8]. Therefore, the search for new adjuvant molecules, such as glucans, for

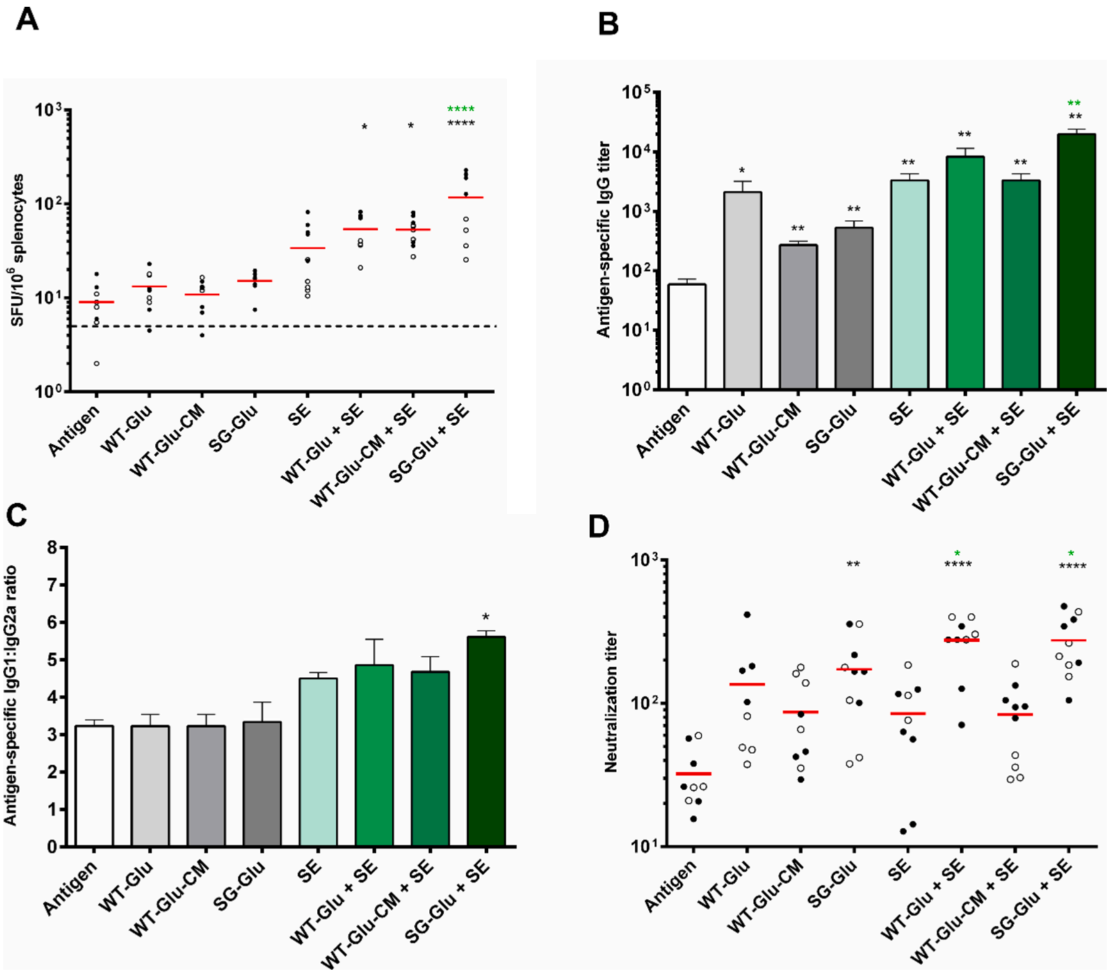


Fig. 7. Specific response against SARS-CoV-2 spike protein in mice immunized using SE and glucans as adjuvants. A – Levels of Antigen-specific IgG producing splenocytes in mice immunized with SE and glucans formulations. Black dots correspond to females and white dots to males. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by one-way ANOVA); B – Titer of spike protein S1 specific IgG present in the blood serum of mice immunized with glucans and SE vaccine formulations. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test); C – Antigen-specific IgG1:IgG2a ratio calculated using IgG1 and IgG2a titers. (Results are expressed as the average ± standard error of the mean of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ calculated by the Wilcoxon non-parametric test); D – Pseudovirus Spike protein neutralization assay for mice immunized with different formulations of glucans and SE. Black dots correspond to females and white dots to males. (The presented values correspond to each animal in its respective group and the group average. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by Wilcoxon non-parametric test; black asterisks indicate significant differences to antigen and green asterisks to SE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vaccine formulations that can overcome the limitations of existing adjuvants without compromising efficacy, would be an appealing outcome.

The combination of WT-Glu and WT-Glu-CM with Alum significantly increased the production of specific antibodies against the SARS-CoV-2 spike protein and increased the titre of antibodies capable of neutralizing the spike-expressing pseudovirus's binding to ACE-2 receptors in human cells, indicating an improved capacity to respond to SARS-CoV-2 infections. Similarly, formulations containing SG-Glu alone or combined with SE also increased antigen-specific antibody production and serum neutralizing activity against SARS-CoV-2 spike protein-expressing pseudovirus infection. However, water-dispersable glucans formulated with SE did not result in increased protection against the pseudovirus, possibly due to limited interaction of the oil-based squalene with aqueous dispersable glucans. Glucans formulated either with Alum or SE presented an increase in the antigen-specific IgG1:IgG2a ratio which may point to a more pronounced Th2 response. Although some literature [40,41] also point to the fact that, Alum, SE and glucans (generically) may favour Th2 response, more data regarding cellular immunity is needed to elicit the specific immune response triggered by the formulations used in this work.

The results also showed that the Alum plus WT-glucans formulation led to increased serum levels of IgG1 and IgG2a, with the latter associated with polysaccharide recognition [42]. In contrast, the SE and SG-Glu formulation led to increased IgM serum levels, known to be involved in primary immune response [42]. Additionally, formulations containing Alum plus WT-Glu or WT-Glu-CM, and containing SE plus SG-Glu, increased the levels of IgA, which is typically associated with mucosal protection [42]. As SARS-CoV-2 primarily targets the respiratory tract, an increase in IgA levels may indicate better protection at this site [43]. Overall, immunization using glucans as co-adjuvants increased the levels of general antibodies involved in immunological responses against infections. Further research should explore the relevance of increased antibody levels in the context of a SARS-CoV-2 infection.

This study demonstrates the potential of glucans as vaccine adjuvants, working synergistically with the approved adjuvants Alum and formulated squalene to stimulate the immune system and generate a stronger adaptive immune response. Taking into account the limitations pointed out for Alum and SE, glucans have proven to be a solution for both cases but for different reasons. Alum salts and glucans have different mechanisms of immune stimulation thus, the complementation of both mechanisms might explain the positive results observed in this study. Although SE is a broader immunostimulant adjuvant, its limited availability could be a drawback for its use in pandemic vaccines where demand can peak at a specific times or seasons. On the other hand, glucans present excellent levels of adjuvant capacity and are readily available in high quantities at lower prices.

It is important to note that this study utilized a monomeric fragment of the virus spike protein as the antigen, which has been shown to be less immunogenic compared to trimeric versions [44]. Additionally, the concentration of glucans used in the tested formulations was significantly lower than the concentrations successfully used in the *in vitro* whole blood studies and lower than the concentrations used by other research groups exploring the potential of glucans and other bioactive polysaccharides as vaccine adjuvants [19,45]. Furthermore, although the additive effect of glucans with Alum or SE observed *in vivo* is significant, it is not as strong as the effect observed in the *in vitro* studies. Fine tuning the glucan concentrations might be a critical step to improve the adjuvant effect of glucans both on their own and in combination with other adjuvants. Therefore, it is suggested that future research should investigate different antigen versions and concentrations of both the antigen and glucans to gain a better understanding of their role and potential as vaccine adjuvants. Moreover, it should be taken in account that the use of glucans as adjuvants should be further explored with other virus vaccines where a better immune response is needed. Also, the glucans used in this work were extracted from wild-type and engineered yeast with the later presenting a higher molecular-weight and vaccine adjuvant capacity. To better understand the molecular mechanisms behind glucans adjuvant capacity, this correlation between molecular-weight and immunological activity should be further explored.

5. Conclusions

In this study, we successfully purified glucans using an anionic detergent treatment without affecting their structure. The removal of the protein content from the glucans extracts is an important feature to assess its immunomodulatory properties as it might lead to cross-reactivity. Our results clearly demonstrate the potential of glucans as vaccine adjuvants. Glucans potentiate the effect of classical adjuvants according to their solubility status: synergic effects were observed in combinations of water-dispersable carboxymethylated glucans with aqueous alum salts and particulate glucans with oil-based squalene emulsion. These formulations significantly enhance the immune response against the presence of the antigen, in this case, the Spike S1 protein from SARS-CoV-2, with elevated levels of antigen-specific IgGs and neutralizing capacity in the pseudovirus assay.

Ethics and consent to participate

Biological sample collections (human blood used in *in vitro* assays) were approved by WCG IRB. All participants reviewed and signed informed consent forms.

All animal experiments were conducted in accordance with the guidelines and regulations of the competent Portuguese institutions, including the *Direção Geral da Alimentação e Veterinária* and *Orgão Responsável pelo Bem-estar dos Animais* (#1-2021).

Conflict of interest

JAS, MA, DTV, PS, CJP, MEP and JCF are inventors on a patent application covering the use of glucans as vaccine adjuvants (WO/2022/172087).

CRediT authorship contribution statement

João Azevedo-Silva: Writing – original draft, Investigation. **Manuela Amorim:** Writing – original draft, Investigation. **Diana Tavares-Valente:** Investigation. **Pedro Sousa:** Investigation. **Raodoh Mohamath:** Investigation. **Emily A. Voigt:** Investigation. **Jeffrey A. Guderian:** Investigation. **Robert Kinsey:** Investigation. **Sofia Viana:** Investigation. **Flávio Reis:** Investigation. **Manuela E. Pintado:** Funding acquisition. **Christopher J. Paddon:** Writing – review & editing, Conceptualization. **Christopher B. Fox:** Writing – review & editing, Conceptualization. **João C. Fernandes:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2024.114538.

Data availability

Data will be made available on request.

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