Adjuvants to increase immunogenicity of SARS-CoV-2 RBD and support maternal–fetal transference of antibodies in mice

Gabrielle Gimenes Lima, Amanda Izeli Portilho, Elizabeth De Gaspari

Abstract

Adjuvants are important components of vaccines, increasing immunogenicity and modulating the immune response. SARS-CoV-2 vaccines are still being developed in order to improve worldwide access to immunization. Specific populations should be addressed in these investigations, such as pregnant women—to protect both mothers and neonates. In this study, female adult mice were immunized with Receptor-binding domain (RBD) from SARS-CoV-2 adjuvanted by a mixture of DDA and Saponin and put to mating to verify the maternal transference of IgG. For comparison, other group received RBD adjuvanted by OMVs from Neisseria meningitidis and Alum. The adjuvants enhanced IgG production and neutralization. DDA/Sap contributed to increase IgG1, IgG2a, IgG2b, and IgG3 isotypes. Total IgG avidity was considered high, as well as IgG1, IgG2a, and IgG2b avidity. IgG antibodies were effectively transferred to the offspring, predominantly IgG2a, IgG2b, and IgG3. The passive transferred immunoglobulin maintained the neutralizing ability, although it lost avidity. ELISA data was confirmed in Dot-ELISA and immunoblotting assays. DDA and Saponin seem a promising adjuvant mixture to enhance the humoral response of SARS-CoV-2 antigens. Further studies considering the effects of maternal immunization in the protection of offspring are needed, regardless the platform used in COVID-19 vaccines.

Keywords: SARS-CoV-2, RBD, saponin, dioctadecyl dimethylammonium bromide, maternal–fetal transference, outer membrane vesicles

Introduction

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is the virus that causes COVID-19 (Coronavirus Disease 2019). SARS-CoV-2 has spread worldwide within a few months, causing a public health emergency (Mohan et al. 2021). The virus presents several immunogenic proteins—Spike (S), envelope (E), membrane (M), and nucleocapsid (N). The receptor-binding domain (RBD), is located in the Spike and mediates the initial binding to the angiotensin-converting enzyme 2 (ACE2), the host receptor (Dai and Gao 2021). The infection by SARS-CoV-2 is efficiently inhibited by blocking the interaction between RBD and the host receptor (Krammer 2020). In fact, most COVID-19 vaccines developed so far use the Spike as antigen (World Health Organization 2022).

Until high coverage rates of immunization are achieved, SARS-CoV-2 infections are likely to become endemic, so more cases of COVID-19 are expected in the future (Chong et al. 2021). COVID-19 vaccines are already licensed and in use. However, it is still relevant to investigate other vaccine platforms, considering the emergence of new variants, specific populations and favoring all countries’ access to vaccines (Forman et al. 2020). Adjuvants are added to vaccines to enhance and modulate the immune response (Shah et al. 2017). Other benefits from enhanced immunogenicity are reducing the number of doses and antigenic concentration required, improving the manufacturing (Coffman et al. 2010).

Saponin (Sap) is an anionic molecule capable of stimulating immune response (Sun et al. 2009). Diocadecyl dimethylammonium bromide (DDA) is a cationic adjuvant with a spherical lipid bilayer structure that favors interaction with APCs and slow antigen release (Hilgers and Snippe 1992, Lincopan et al. 2009). Both molecules were tested in adjuvants and presented promising results—DDA was used in the cationic formulation (CAF) 01 (Abraham et al. 2019) and Saponin in the Immunostimulatory complex (ISCOM) (Pandey and Dixit 2020). Our group has studied both DDA and Sap as Neisseria meningitidis adjuvants and found promising results, agreeing with the literature (de Almeida and De Gaspari 2018, Correa et al. 2022).

Maternal immunization not only protects mothers, but also fetuses and neonates through the transplacental transference of IgG. Vaccinating pregnant women has been a strategy for decades (Omer 2017). In that scenario, it is important to use adequate adjuvants to impact the maternal immune response, supporting the antibody transference and, consequentially, the fetus and neonatal protection (Gerds et al. 2016, Bok 2017).

Transference of antibodies through the placenta is described for different pathogens. Our laboratory evaluated the immune response of mothers and offspring immunized with outer membrane vesicles (OMV) of N. meningitidis, using DDA as an adjuvant (de Almeida and De Gaspari 2018). Regarding SARS-CoV-2, there are descriptions that transference of IgG after natural infection is
inefficient (Atyeo et al. 2021), while mothers immunized with the BNT162b2 vaccine presented a robust humoral response, which was effectively transferred to their infants (Beharier et al. 2021). However, there are only a few studies focused on this subject.

Here we evaluated, for the first time, a DDA/Sap mixture as adjuvant for SARS-CoV-2 RBD in mice. The humoral response of mothers and the passive transference of IgG antibodies to the offspring were studied.

**Materials and methods**

**Recombinant antigens**

Briefly, the RBD protein was produced in 293F cells, using the plasmid provided by Mount Sinai Hospital (New York, NY, USA) to Dr Carlos Prudencio, from Adolfo Lutz Institute (São Paulo, SP, Brazil). The obtaining of the protein is described in Stadlbauer et al. (2020).

**Adjuvants**

DDA (Sigma-Aldrich) and Sap (Sigma-Aldrich) were diluted in saline solution 0.01 mM and filtered using a 0.45-μm membrane. A different platform using *N. meningitidis* OMV/Aluminum hydroxide (AH) mixture as adjuvant was used for comparison, provided that it was used before and showed promising results (Gaspar and De Gaspari 2021, Gaspar et al. 2021). OMVs from *N. meningitidis* strain C:2a:P1.5 were obtained in our laboratory as described previously (Ito et al. 2009). AH (Rehydragel HPA, Reheis Chemicals) was prepared at a final concentration of 0.1 mM solution in saline and filtered on a 0.45-μm membrane.

**Hemolytic activity**

Since the natural extract of *Quillaja saponaria* can cause hemolysis, we conduct hemolytic activity testing to identify which dose of the adjuvants were adequate for experimental use, adapting the protocol of Pabreja et al. (2016). Briefly, a blood sample was collected through the opthalmic plexus of mice and transferred to a microtube, which was centrifuged at 2000 rpm for 5 minutes, then the supernatant was removed, and the red blood cells (RBC) were washed three times with 0.9% saline solution. A total of three suspensions were prepared with the RBCs at 4% v/v in saline. In a microtube, 150 μl of the RBC suspension plus 50 μl of the adjuvant Sap or DDA, at concentrations of 1, 500, 100, 50, 10, 5, and 1 μg/ml were added. Saline was used as a negative control and Triton x-100 (Fluka Chemika) as positive control. The mixtures were incubated for 30 minutes at 37°C. Finally, the contents were centrifuged at 2000 rpm for 5 minutes and the supernatant was transferred to a plate and read in a microplate reader (Labsystems Multiskan) at an optical density (OD) of 405 nm. The value of the readings was used to calculate the % of hemolysis using the following formula: % of hemolysis = OD_{sample}/OD_{positive control} × 100.

**Antigenic preparations**

Antigenic preparations were formulated from the mixture of adjuvants and antigen, diluted in 0.01 mM sterile saline. The mixtures were allowed to interact for 1 hour before administration. The final concentrations of antigen and adjuvant were: (1) the new antigenic preparation tested—0.5 μg RBD + 1 μg Sap + 1 μg DDA; (2) an antigenic preparation used previously for comparison—3 μg RBD + 10 μg OMVs of *N. meningitidis* C:2a:P1.5 + 0.1 mM AH; (3) an antigen control—3 μg RBD; and (4) an adjuvant control—1 μg Sap + 1 μg DDA. An extra group (5) was used as naive control and did not receive antigenic preparation.

**Materials and methods**

**Mice and immunization**

The Swiss, female, adult mice (*Mus musculus*) used in the study were obtained from the Breeding Animal Facility of the Adolfo Lutz Institute. The entire process of mice manipulation complies with the recommendations of the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA) and was approved by the ethics committees of the Adolfo Lutz Institute (CEUA/IAL number 02/2022). The immunization strategy was based on technical protocols established previously (de Almeida and De Gaspari 2018).

Briefly, mice received two intramuscular (IM) doses of the antigenic preparations described above, in a 21 days interval. Blood was collected before the immunization (pre-immune) and on days 21, 47, and 176 through puncture of the orbital plexus. Noteworthy, mice were followed from 2 to 8 months of age, so we could study the persistence of the immune response throughout their adult phase (Flurkey et al. 2007). To assess maternal transference of antibodies following RBD + DDA/Sap immunization, females of this group and its adjuvant control (DDA/Sap group) were put to mating after they received the booster dose. Blood was collected from the offspring 18 days after birth, through the axillary plexus. Adult mice were only anesthetized (ketamine 10 mg/kg and xylazine 10 mg/kg) and offspring mice were anesthetized and euthanized before blood collections. Figure 1 describes the immunization calendar.

**Electrophoretic characterization of RBD and OMVs**

A total of 2 μg of RBD was characterized using a polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), in a discontinuous buffer system, along with a molecular weight marker ranging from 14 to 97 kDa (Amersham Biosciences). The OMVs from the *N. meningitidis* strain were characterized as well, using a molecular weight marker ranging from 10 to 245 kDa (True Color, Synapse) (Laemmli 1970). After the electrophoresis, the gel was stained with Coomassie Blue (Pharmacia Biotech) and discolored with 7% glacial acetic acid solution until bands were visible.

**Enzyme-linked immunosorbent assay (ELISA)**

The 96-well polystyrene plates (High Binding, Costar) were adsorbed with 1 μg/ml of RBD diluted in 0.1 M carbonate bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. Blocking was performed with 5% skim milk (La Serenissima) for 2 hours at 37°C. Samples were diluted in 2.5% skim milk and incubated overnight at 4°C. For initial analysis, pooled sera were titrated from 1:50 to 1:6400 dilution. Afterwards, individual samples were tested at 1:100 dilution. To characterize IgG antibodies and their isotypes, it was incubated for 2 hours at 37°C: antismouse IgG γ-chain peroxidase-labeled (Kirkegaard & Perry Laboratories; 1:20000), IgG2a, IgG2b, or IgG3 peroxidase-labeled (Aviva Systems Biology; 1:10000); or anti-IgG1 biotinylated-labeled (Aviva Systems Biology; 1:5000). For IgG1, streptavidin-peroxidase (Zymed; 1:20000), IgG2a, IgG2b, or IgG3 peroxidase-labeled (Kirkegaard & Perry Laboratories; 1:10000). The OD was read at 450 nm in a microplate reader (Molecular Devices)
Figure 1. (A) Adult female mice received two IM doses of antigenic preparations and were bled on days 0 (pre-immune), 21, 47, and 176. (B) Immunized mice from RBA + DDA/Sap and DDA/Sap groups were put to mating after they received the booster dose. A total of 21 days later, the offspring was born and blood were collected 18 days after the neonates birth.

**Antibody avidity index (AI)**

The procedure followed ELISA steps as described previously, modified by the descriptions of Granoff et al. (1998). After serum incubation, 1.5 M potassium thiocyanate (KSCN) was incubated for 20 minutes at room temperature (RT, 20–25°C). Samples are tested, in parallel, in the presence and absence of KSCN. The AI is calculated as the ratio ODKSCN/ODraw sample and converted to percentage. The AI is classified as low if < 30%, intermediate between 30% and 49%, and high if ≥ 50% (Chackerian et al. 2001).

**Dot-ELISA**

We applied 0.5 and 0.25 μg of RBD on a 0.45-μm nitrocellulose membrane (BioRad Laboratories) for the evaluation of sera. RBD provided by Dr Florian Krammer and Dr Carlos Prudencio were tested in parallel, to compare the different batches. The membranes were blocked with 5% skim milk (La Serenissima) for 2 hours at RT. Afterwards, pooled sera of adult females and individual of offspring, diluted at 1:100, were incubated for 18 hours at 4°C. The antimouse IgG-chain γ peroxidase-labeled (Kirkegaard & Perry Laboratories; 1:10000) was incubated for 2 hours at RT. The reaction was developed with the substrate 4-chloro-naphthol. The enzymatic reaction was stopped with distilled water. Between each incubation step, the strips were washed 5x with PBS. Incubations were performed on an oscillating platform for homogenization of the reagents and washing of the membranes (Trzewikoswki de Lima et al. 2019).

**Neutralization**

The neutralization index of antibodies was detected by an inhibition ELISA-methodology using the commercial kit cPass SARS-CoV-2 Neutralization Antibody Detection kit (GenScript) and following the manufacturer’s instructions. Neutralization rates ≥ 20% were considered neutralizing (Gaspar and De Gaspari 2021).

**Statistical analysis**

Data were analyzed using the Kruskal–Wallis test followed by Dunn’s post-test for multiple comparisons, using GraphPad Prism v. 8 (Graph Pad Software). Values of P ≤ .05 were considered significant.

**Results and discussion**

**Hemolytic activity of the adjuvants**

Table 1 shows the results of the hemolysis test using different concentrations of the Sap and the DDA adjuvants. As shown in Table 1, the concentrations from 1 to 50 μg/ml of the adjuvants were hemotoxic, since the range of hemolysis remained between 98% and 100%. The concentration of 10 μg/ml presented low toxicity (less than 4% of hemolysis) and were used in the study—so final doses, of 100 μl each, would contain 1 μg of adjuvant (Fabreja et al. 2016).
Table 1. Hemolytic activity (%) of different concentrations of DDA/Sap adjuvants.

|       | 1 mg/ml | 500 μg/ml | 100 μg/ml | 50 μg/ml | 10 μg/ml | 5 μg/ml | 1 μg/ml |
|-------|---------|-----------|-----------|----------|----------|---------|---------|
| Sample 1 | 103.45/121.67 | 108.71/120.89 | 100.04/98.87 | 111.30/98.76 | 2.83/4.87 | 2.45/2.23 | 2.93/1.87 |
| Sample 2 | 143.54/97.28 | 109.78/105.56 | 98.70/84.32 | 97.56/87.98 | 2.54/3.78 | 2.42/1.85 | 2.99/0.98 |
| Sample | 168.33/80.32 | 98.17/89.11 | 78.12/98.98 | 96.40/87.67 | 3.00/5.54 | 2.22/1.33 | 2.69/1.02 |
| Mean | 138.44/99.76 | 105.53/101.58 | 92.28/94.05 | 99.08/91.47 | 2.79/3.73 | 2.36/1.80 | 2.87/1.29 |

The hemolytic activity of Sap and DDA are described. DDA results are given in bold numbers. DDA: dioctadecyl dimethylammonium bromide. Sap: saponin.

Figure 2. (A) SDS-PAGE of the RBD protein, used for immunization, in a 13% polyacrylamide gel electrophoresis. The band presented approximately 30 kDa, as the expected molecular weight (MW) of the antigen. (B) SDS-PAGE of the outer membrane vesicles of N. meningitidis C:2a:P1.5 strain, in 10% polyacrylamide gel electrophoresis.

Electrophoretic characterization of the antigen

Figure 2(A) shows the electrophoretic characterization of RBD protein in SDS-PAGE. As expected, only one band in the 30 kDa range was stained (Stadlbauer et al. 2020). Figure 2(B) shows the electrophoretic profile of the N. meningitidis strain used as adjuvant. Several bands were present.

Humoral response of adult females

Figure 3(A) shows the titration curve of IgG antibodies in pooled samples collected at the 47th day. Given that the 1:100 dilution is on the linear phase of the curve and allowed to distinguish between test and control groups, the following analysis used this dilution. The IgG levels of each group were compared different days after the immunization (Fig. 3B–D). As observed, on the 21st day, before the booster was administrated, the groups were not different. On day 47 (26 days after the booster), the groups RBD + DDA/Sap and RBD + OMV/AH presented statistically higher levels of IgG antibodies than pre-immune control. At the 176th day (155 days after the booster), the IgG levels decreased. Even though there is no statistical difference, the groups immunized with RBD + adjuvants presented slightly higher levels.

The IgG isotypes were assessed in individual samples collected on days 47 (Fig. 4) and 176 (Fig. 5), after the maturation of the immune response. The group RBD + DDA/Sap presented statistically higher levels of IgG1, IgG2a, and IgG2b than pre-immune sera. Although there was no statistical significance, IgG3 levels were higher as well. RBD + OMV/AH induced similar levels of IgG1, IgG2a, and IgG2b. The data was corroborated in the samples collected on day 176.

Figure 6 presents the results of Dot-ELISA. While pre-immune sera and control groups did not color the membrane, the RBD + DDA/Sap and RBD + OMV/AH groups colored the membrane in samples collected at three different time points (days 21, 47, and 176), corroborating ELISA data: adjuvants improved the humoral response.

Thus, the assay provides a comparison of two different batches of RBD antigens, (A) the one currently produced in Brazil and (B) the one provided by Dr Florian Krammer (Stadlbauer et al. 2020), used in our previous studies (Gaspar and De Gaspari 2021, Gaspar et al. 2021). On the left, it was applied 0.5 μg of the antigen in the nitrocellulose and on the right, 0.25 μg.

Figure 7 shows the results obtained by immunoblotting. Pooled sera of the RBD + DDA/Sap group were tested at 1:100 dilution, on days 21, 47, and 176. As observed, the antibodies recognized the denatured RBD after the booster dose (47 and 176 days).

Transference of antibodies to the offspring

Figure 8 shows the ELISA results of the offspring of females immunized with RBD + DDA/Sap and DDA/Sap. Pre-immune sera of the mothers were also used as a control. Figure 8(A) shows total IgG transference; the IgG1 (Fig. 8B), IgG2a (Fig. 8C), IgG2b (Fig. 8D), and IgG3 (Fig. 8E) were assessed as well. As expected, the immunized group presented a statistically higher anti-RBD IgG level than control. Regarding the isotypes, even though there is no statistical difference, we observed higher levels of IgG2a and IgG2b than control.

After ELISA, the results were confirmed in individual Dot-ELISA (Fig. 9). The RBD produced in Brazil (A) and in the USA (B) were tested at a 0.5 μg (left side) and a 0.25 μg (right side) concentration. Individual sera of the offspring corroborated ELISA results, provided that the offspring of immunized mothers recognized the antigen, and the offspring of adjuvant control did not.

Pooled sera of the offspring from RBD + DDA/Sap mothers also recognized the denatured RBD in Immunoblotting, as shown in Fig. 10, at (A) 1:100, (B) 1:200, and (C) 1:400 dilution.

Functionality of antibodies

Table 2 shows the mean neutralizing index (%NI) of the samples of adult mice collected at different points. The control groups (pre-immune, RBD, DDA/Sap and naive) were not considered positive for neutralization (indexes below 20%). As observed, the DDA/Sap was an effective adjuvant mixture to increase neutralization, while OMV + AH only presented an index considered neutralizing at day 47. The antibodies passively transferred to offspring from group RBD + DDA/Sap were neutralizing as well.

Table 3 describes the mean avidity index (%AI) of IgG antibodies and its isotypes, at different time points. Considering the
Figure 3. (A) Titration curve of pooled samples collected on day 47. Comparison of IgG levels in groups of mice (B) 21, (C) 47, and (D) 176 days after the beginning of immunization. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin. ∗: P < .05. ∗∗: P < .01. ns: non-significant.

Table 2. Mean neutralizing index of groups immunized with RBD and adjuvants at different time points.

| Group          | RBD + DDA/Sap | RBD + OMV/AH | RBD   | DDA/Sap | Offspring |
|----------------|---------------|--------------|-------|---------|-----------|
| Day            | 21            | 47           | 176   | 21      | 47        | 176       | 21      | 47    | 176       | 18       |
| NI (%)         | 36.73         | 70.92        | 20.02 | 16.61   | 23.49     | 16.81     | 17.09   | 15.27 | 3.83       | 16.75    | 15.88    | 12.61    | 38.08    |

AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin.

RBD + DDA/Sap group, IgG avidity was high at both time points, although it decreased from 98.56% to 64.40%. Analyzing the iso-types, on day 47, only IgG3 presented intermediate avidity—the others presented high avidity. On day 176, only IgG2b remained classified as high avidity, while the other isotypes were considered low. For RBD + OMV/AH, IgG avidity was considered high on day 47 and intermediate on day 176. IgG1 and IgG2a were considered high avidity in both time points, while IgG2b and IgG3 presented low avidity in day 47 but increased their indexes on day 176. The offspring presented intermediate IgG avidity; however, all isotypes presented low avidity when analyzed separately.

Observing Tables 2 and 3, we can verify that the peak of the AI agreed with the peak of the NI. Interestingly, the offspring presented neutralizing index even when the avidity was low.

Discussion

This report provides evidence that immunization with RBD before pregnancy results in the passive transference of antibodies...
Figure 4. Comparison of IgG isotypes—(A) IgG1, (B) IgG2a, (C) IgG2b, and (D) IgG3—between groups. The samples were collected 47 days after the beginning of immunization. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin. *: $P < .05$. **: $P < .01$. ns: non-significant.

Table 3. Mean AI of groups immunized with RBD and adjuvants at different time points.

| AI (%) - Groups | RBD + DDA/Sap | RBD + OMV/AH | Offspring |
|----------------|---------------|--------------|-----------|
| Days           | 47            | 176          | 47        | 176       | 18         |
| IgG            | 98.56         | 64.40        | 59.75     | 40.61     | 38         |
| IgG1           | 76.93         | 24.04        | 87.71     | 70.12     | 19.29      |
| IgG2a          | 60.66         | 22.63        | 69.21     | 58.50     | 7.04       |
| IgG2b          | 89.80         | 60.78        | 22.33     | 45.97     | 25.40      |
| IgG3           | 40.43         | 15.21        | 20.45     | 77.06     | 21.84      |

AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin.

to offspring. RBD was chosen to be used as the antigen because it is highly immunogenic and used in several SARS-CoV-2 vaccines (Chong et al. 2021). The addition of an adjuvant in the vaccines, besides boosting immunogenicity, would reduce the requirement of vaccine protein per dosage (Liang et al. 2020). Diverse compounds have been suggested as adjuvants for COVID-19 vaccines, including aluminium-based (Yadav et al. 2021), microbials products (Gaspar et al. 2021, Santana-Mederos et al. 2022), emulsions (Jangra et al. 2021), nanoparticles (Johnston et al. 2022), and others (Liang et al. 2020).

We proposed the combination of DDA and Sap as an adjuvant mix. Given the charged nature of Sap, an anionic compound, and
Figure 5. Comparison of IgG isotypes—(A) IgG1, (B) IgG2a, (C) IgG2b, and (D) IgG3—between groups. The samples were collected 176 days after the beginning of immunization. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin. *: $P < .05$. **: $P < .01$. ns: non-significant.

DDA, a cationic one, there are descriptions that these molecules can cause hemolysis (Vieira and Carmona-Ribeiro 2006, Pabreja et al. 2016). Our hemolysis test showed that the concentration of 10 μg/ml of both adjuvants were not hemotoxic, so we proceeded the immunization using 10 μg/ml of DDA and Sap (1 μg per 100 μl dose).

Through the separation of proteins by SDS-PAGE, it is possible to observe the RBD as a single band in the molecular range of 30 kDa, which was described previously (Stadlbauer et al. 2020). On the other hand, OMVs presented several bands, which could be related to different proteins, such as Neisseria Adhesin A (>100 kDa) (Comanducci et al. 2002), Chaperonin 60 (~ 60 kDa) (Phillips et al. 2013), Transferrin-binding protein (~ 80 kDa), Porin A (~ 46 kDa), and Opacity protein (~ 25 kDa) (Pollard and Frasch 2001). All these antigens confer good immunogenic properties to OMVs, even as an adjuvant option (Acevedo et al. 2014). In this study, we focused on the use of DDA/Sap as adjuvant; however, we immunized a group of mice with RBD + OMV/AH to compare the new antigenic preparation (RBD + DDA/Sap) with the one we described before (Gaspar et al. 2021).

The titration curve of pooled samples collected on day 47, after the booster dose (Fig. 3A), showed how adjuvanted groups (RBD + DDA/Sap and RBD + OMV/AH) presented higher IgG titer compared to the other groups. Enhanced immunogenicity was observed for SARS-CoV-2 antigens combined with adjuvant complexes, as diphtheria toxoid plus AS01 (composed of Monophosphoryl lipid A and Sap) (Scaria et al. 2022) and nanoemulsion and a RIG-I agonist (Jangra et al. 2021).

Following to individual analysis, we observed statistically higher IgG levels on day 47, after the second dose. It is well-described in the literature that booster doses improve the immune response. Regarding SARS-CoV-2, not only animal studies observed that (Guebre-Xabier et al. 2020, Jangra et al. 2021, Santana-Mederos et al. 2022, Scaria et al. 2022), but also clinical trials from vaccines in use (Folegatti et al. 2020, Polack et al. 2020, Zhang et al. 2021). Even though there were no statistical dif-
Figure 6. Dot-ELISA carried out with pooled sera collected at different time points, at a 1:100 dilution. A total of two batches of RBD antigen were used: (A) produced in Brazil, used in our immunization schedule and (B) produced in the USA, used in recently published manuscripts. The Dot-ELISA was performed using 0.5 μg (left) or 0.25 μg (right) of antigen to dot the nitrocellulose membrane. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin.

Figure 7. Immunoreactivity of the RBD+DDA/Sap pooled samples collected after (A) 21, (B) 47, and (C) 176 days after the first immunization. A total of 2 μg of RBD were transferred to the strips. MW: molecular weight.

ference, RBD + DDA/Sap and RBD + OMV/AH presented slightly higher levels on day 176. It is desirable that vaccines induce a long-lived immune response. Adjuvants contributed to maintain the antibodies titers against Bacillus anthracis (Kelly et al. 2021), influenza (Vujanic et al. 2012) and, in our studies, against N. meningitidis (Correa et al. 2022). The same was observed with humans (Budroni et al. 2021). Considering that anti-SARS-CoV-2 antibodies present a decay, which seems similar to other Coronavirus (Bauer 2021), it is important to study antigenic preparations to prolong the humoral response.

Dot-ELISA data (Fig. 6), performed using pooled samples, corroborated ELISA results, provided that sera collected on days 47 and 176 colored the membrane with higher intensity. Moreover, only the RBD + DDA/Sap and RBD + OMV/AH groups were positive, while sera of RBD, DDA/Sap and Naïve controls did not color the membrane. In addition, pooled sera from RBD + DDA/Sap collected on days 47 and 176 were able to recognize the RBD in a denatured presentation in Immunoblotting (Fig. 7), agreeing with the results presented so far.

The IgG isotypes can suggest the polarization of the immune response toward a Th1 or a Th2 pattern: while Th1 cytokines, such as IL-2 and IFN-γ contribute to IgG2a class switch, while Th2 cytokines, as IL-4, supports IgG1 (Mosmann and Coffman 1989). Here, we observed that RBD + DDA/Sap and RBD + OMV/AH presented higher levels of IgG1, IgG2a, and IgG2b, although only RBD + DDA/Sap was statistically different. Thus, this group was the only one to present IgG3 (Fig. 4). The data was confirmed in the last blood collection, on day 176 (Fig. 5). Both DDA and Sap are known to induce a Th1 pattern of response, which agrees with the high levels of IgG2a (Lincopan et al. 2009, Sun et al. 2009, Carneiro et al. 2015). However, Sap was described to induce both IgG1 and IgG2a previously (Huber et al. 2002, Cibulski et al. 2016) and, in N. meningitidis (Correa et al. 2022) and Zika virus (Cibulski et al. 2021) vaccines, it contributed to induce all IgG isotypes, including IgG3. Taken our results and literature observations together, we might point that DDA/Sap induced a mixed Th1/Th2 profile—future cytokine studies will help elucidate this hypothesis. However, we predict that a mixed profile might be beneficial to prevent COVID-19, since there are observations of highly polarized Th1 (Liu et al. 2020) and Th2 (Roncati et al. 2020) immune response.
Figure 8. (A) Total IgG and (B) IgG1, (C) IgG2a, (D) IgG2b, and (E) IgG3 isotypes passively transferred from immunized mother to offspring. The RBD + DDA/Sap offspring presented higher levels of IgG than DDA/Sap group. Even though there was no statistical difference considering the isotypes, it was observed a higher level of IgG2a and IgG2b in the offspring of immunized mothers. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. RBD: receptor-binding domain. Sap: saponin. ns: non-significant.

Figure 9. Dot-ELISA carried out with individual sera collected 18 days after the offspring birth, at a 1:100 dilution. A total of two batches of RBD antigen were used: (A) produced in Brazil, used in our immunization schedule and (B) produced in the USA, used in recently published manuscripts. The Dot-ELISA was performed using 0.5 μg (left) or 0.25 μg (right) of antigen to dot the nitrocellulose membrane. Each membrane represents one individual. AH: aluminium hydroxide. DDA: dioctadecyl dimethylammonium bromide. OMV: outer membrane vesicles. RBD: receptor-binding domain. Sap: saponin.
in severe COVID-19 patients, while a balanced humoral and cellular immunity, supported by a Th1/Th2 profile of cytokines, would relate to protection (Hasan et al. 2021, Havervall et al. 2022).

Our group tested DDA as an adjuvant for maternal–fetal transfer of antibodies before (de Almeida and De Gaspari 2018). The vaccination of pregnant women is required not only because they may present more severe COVID-19 (Alzamora et al. 2020), but also as a strategy to protect the newborns, that are particularly susceptible to infections, because their immune systems are still maturing. While neonates tend to present critical illness upon SARS-CoV-2 infection, seropositive newborns showed a reduced risk of developing respiratory morbidity (Kim et al. 2020, Helguera-Repetto et al. 2022).

We observed that anti-RBD IgG was effectively transferred from immunized mothers to the offspring (Fig. 8). Other adjuvants were studied to contribute to anti-SARS-CoV-2 IgG transfer, such as A503 and IVT-DI (Jangra et al. 2021, Dubé et al. 2022). When the isotypes were assessed, although we did not observe statistical difference, IgG2a and IgG2b were the predominant isotypes, followed by IgG3. The literature about subclasses transference in mice is scarce, however, a study found that human IgG1 is transferred more effectively through the placenta (Garty et al. 1994). Interestingly, this isotype is related to mouse IgG2a (Stewart et al. 2014, Temming et al. 2020).

Individual Dot-ELISA (Fig. 9) corroborates the results of ELISA, showing that only newborns from RBD + DDA/Sap group recognized the antigen. Thus, pooled samples presented reactivity until 1:400 dilution in Immunoblotting (Fig. 10), recognizing denatured RBD.

To verify the functionality of antibodies, we conducted an avidity-ELISA and a surrogate-neutralization test, based on an inhibition ELISA. Considering the adult females, the higher NI (Table 2) and AI (Table 3) were obtained in samples collected on day 47, at the peak of the immune response. These parameters were associated before, provided that a high affinity is required to impair the RBD-ACE-2 interaction (Khatri et al. 2020). We observed that RBD + DDA/Sap induced higher NI than RBD + OMV/AH. Improved neutralization was observed for Sap-based adjuvant and SARS-CoV-2 before (Hu et al. 2022, Scaria et al. 2022). Cationic liposomes, as DDA, and anionic adjuvants, as Sap, also contributed to functional responses against SARS-CoV (Bisht et al. 2005), Influenza (Lay et al. 2009), and Zika virus (Cibulski et al. 2021).

Concluding, the AI is related to the binding affinity between the antigen and the antibody and reflects the affinity maturation (Bauer 2021). While natural Coronaviruses infections do not elicit high avidity antibodies, vaccines seem to improve this parameter (Struck et al. 2021). In mice, adjuvants contributed to increased AI of SARS-CoV-2 (Jangra et al. 2021) and other microorganisms (Cibulski et al. 2021). Moreover, the combination of adjuvants supported AI better than one adjuvant alone before (Rudroni et al. 2021). AI was described as an important parameter for protection against different pathogens, especially viruses (Bauer 2021).

DDA/Sap not only induced total IgG of high AI, but separated isotypes maintained the classification—only IgG3 was considered intermediate. For OMV/AH, IgG2b and IgG3 showed low avidity whereas the total IgG presented high avidity. The literature describes how the IgG isotypes differ regarding their structure and function, mostly due to Fc characteristics (Stewart et al. 2014, Valenzuela and Schaub 2018). Meanwhile, we could not find other studies comparing the AI of each IgG isotype in an immunization context, considering the Fab region, which limits this discussion. We believe that it is important to address this aspect not only to improve our immunology understanding, but also to refine immunization approaches to elicit more adequate immunity. Nevertheless, a robust humoral response, presenting the more adequate isotypes and binding affinity is more likely to induce a protective response (Miura et al. 2019).

Contrary to adult mice, offspring presented positive neutralization but low avidity. We hypothesize that the high quantity of antibodies compensated for their impaired binding affinity (Bauer 2021), thus, the IgG isotype may have supported it. In humans, IgG1 was positively associated with neutralization of SARS-CoV-2 (Chen et al. 2022). Since mouse IgG2a is related to human IgG1, it might follow a similar pattern, however, more studies would be needed to address this question. Additional studies would also be required to investigate if placental transference compromised the IgG avidity, which was high in mothers and intermediate in the offspring—or low, if the isotypes are studied separately.

Furthermore, the characterization of maternal–neonatal transfer of IgG anti-SARS-CoV-2 antibodies and neonatal outcomes remains limited. More information on passive maternal immunity and neonatal morbidity is urgently needed to develop strategies for infant protection and vaccine campaigns.

Conclusion

The search for a new vaccine with longer durability and efficacy against SARS-CoV-2, which has presented a high degree of mutation, is a major challenge. The available vaccines are requiring booster doses to keep adequate titers of circulating antibodies—several studies described a decline in antibody levels months after vaccination. Therefore, new strategies to protect against the new variants of SARS-CoV-2 are needed. The lack of vaccines for children under 3 years of age is a greater concern and requires further studies. We know that when antibodies are transferred from the mother to the fetus, they last about a year. Beyond this point, finding out whether vaccines can be administered soon after birth is still a challenge for the scientific community.

Acknowledgments

The authors would like to thank Dr Florian Krammer and Dr Carlos Prudencio for providing the recombinant RBD; and NL Diagnostica (São Paulo, SP, Brazil) for providing the kit cPass SARS-CoV-2 Neutralization Antibody Detection kit (GenScript).
Conflict of interest statement. The authors declared no potential conflicts of interest regarding the publication of this article.

Funding
This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grant number 18/04202-0, E.D.G.); the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant number 131308/2021-1; G.G.L.), and the Coordination for the Improvement of Higher Education Personnel (CAPES; grant number 88887.661236/2022-00; A.I.P.).

Data availability
The manuscript data is available upon reasonable request to the corresponding author.

References
Abraham S, Juel HB, Bang P et al. Safety and immunogenicity of the chlamydia vaccine candidate CTHS22 adjuvanted with CAF01 liposomes or aluminium hydroxide: a first-in-human, randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Infect Dis 2019;19:1091–100.
Acevedo R, Fernández S, Zayas C et al. Bacterial outer membrane vesicles and vaccine applications. Front Immunol 2014;5:1–6.
Alzamora MC, Paredes T, Caceres D et al. Severe COVID-19 during pregnancy and possible vertical transmission. Am J Perinatol 2020;37:861–5.
Atheyo C, Pullen KM, Bordt EA et al. Compromised SARS-CoV-2-specific placental antibody transfer. Cell 2021;184:628–642.e10.
Bauer G. High avidity of vaccine-induced immunoglobulin g against SARS-CoV-2: potential relevance for protective humoral immunity. Explo Immunol 2021;2:133–56. doi: 10.37349/lei.2021.00025.
Beharier O, Plitman Mayo R, Raz T et al. Efficient maternal to neonatal transfer of antibodies against SARS-CoV-2 and BNT162b2 mRNA COVID-19 vaccine. J Clin Invest 2021;131:e150319.
Bisht H, Roberts A, Vogel L et al. Antibody avidity, persistence, and response to antigen recall: comparison of vaccine adjuvants. NPJ Vaccines 2021;6:78. DOI: 10.1038/s41541-021-00337-0.
Carneiro C, Correia A, Collins T et al. DODAB:monolein liposomes containing Candida albicans cell wall surface proteins: a novel adjuvant and delivery system. Eur J Pharm Biopharm 2015;89:190–200.
Chackravarthy B, Lowy DR, Schiller JT. Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. J Clin Invest 2001;108:415–23.
Chen W, Zhang L, Li J et al. The kinetics of IgG subclasses and contributions to neutralizing activity against SARS-CoV-2 wild-type strain and variants in healthy adults immunized with inactivated vaccine. Immunology 2022;167:1–12.
Chong WC, Chellappan DK, Shukla SD et al. An appraisal of the current scenario in vaccine research for COVID-19. Viruses 2021;13:1–18.
Cibulski S, Varela APM, Teixeira TF et al. Zika virus envelope domain III recombinant protein delivered with saponin-based nanoadjuvant from Quillaja saponifera enhances anti-zika immune responses, including neutralizing antibodies and splenocyte proliferation. Front Immunol 2021;12:632714. DOI: 10.3389/fimmu.2021.632714.
Cibulski SP, Mourglia-Ettlin G, Teixeira TF et al. Novel ISCOMs from Quillaja saponifera saponins induce mucosal and systemic antibody production, T-cell responses and improved antigen uptake. Vaccine 2016;34:1162–71.
Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity 2010;33:492–503.
Comanducci M, Bambini S, Brunelli B et al. NAD, a novel vaccine candidate of Neisseria meningitidis. J Exp Med 2002;195:1445–54.
Correa VA, Portilho AI, De Gaspari E. Immunological effects of dimethylidodecylammonium bromide and saponin as adjuvants for outer membrane vesicles from Neisseria meningitidis. Diagn 2008;10:46. DOI: 10.3390/disease10003046.
Dal L, Gao G. Viral targets for vaccines against COVID-19. Nat Rev Immunol 2021;21:73–82.
de Almeida AF, De Gaspari E. Dodecyl(dimethylammonium) bromide (DODAB-BF) as a new adjuvant for maternal-fetal immunization in mice against Neisseria meningitidis: evaluation of humoral response. Pathog Dis 2018;76:1–9.
Dubé C, Paris-Robidas S, Primakova I et al. Lack of effects on female fertility or pre- and postnatal development of offspring in rats after exposure to AS03-adjuvanted recombinant plant-derived virus-like particle vaccine candidate for COVID-19. Reprod Toxicol 2022;107:69–80.
Ferraz AS, Belo EFT, Coutinho LMCC et al. Storage and stability of IgG and IgM monoclonal antibodies dried on filter paper and utility in Neisseria meningitidis serotyping by Dot-blot ELISA. BMC Infect Dis 2008;8:1–8.
Flurkey K, Currer J, Harrison D. Mouse models in aging research. In: The Mouse in Biomedical Research. Amsterdam: Elsevier, 2007:637–72.
Folegatti PM, Ewer KJ, Aley PK et al. Safety and immunogenicity of the chadox1 nCoV-19 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial. Lancet 2020;396:467–78.
Forman R, Anderson M, Jit M et al. Ensuring access and affordability through COVID-19 vaccine research and development investments: a proposal for the options market for vaccines. Vaccine 2020;38:6075–7.
Garty BZ, Ludomirsky A, Donan YL et al. Placental transfer of immunoglobulin g subclasses. Clin Diagn Lab Immunol 1994;1:667–9.
Gaspar EB, De Gaspari E. Avidity assay to test functionality of anti-SARS-CoV-2 antibodies. Vaccine 2021;39:1473–5.
Gaspar EB, Prudencio CR, De Gaspari E. Experimental studies using OMV in a new platform of SARS-CoV-2 vaccines. Hum Vaccines Immunother 2021;17:2965–8.
Gerds T, van Drunen Littel-vanden Hurk S, Potter A. Protection of neonates and infants by maternal immunization. Expert Rev Vaccines 2016;15:1347–9.
Granoff DM, Maslanka SE, Carlone GM et al. A modified enzyme-linked immunosorbent assay for measurement of antibody responses to meningococcal C polysaccharide that correlate with bactericidal responses. Clin Diagn Lab Immunol 1998;5:479–85.
Guebre-Xabier M, Patel N, Tian JH et al. NVX-CoV2373 vaccine protects cynomolgus macaque upper and lower airways against SARS-CoV-2 challenge. Vaccine 2020;38:7892–6.
Hasan A, Al-Ozairi E, Al-Baqsumi Z et al. Cellular and humoral immune responses in covid-19 and immunotherapeutic approaches. *Immunotherapeutics* 2021;10:63–85.

Havervall S, Ng H, Jernborn Falk A et al. Robust humoral and cellular immune responses and low risk for reinfection at least 8 months following asymptomatic to mild COVID-19. *J Intern Med* 2022;291:72–80.

Helguera-Repetto AC, Villegas-Mota I, Arredondo-Pulido G et al. Cord blood SARS-CoV-2 IgG antibodies and their association with maternal immunity and neonatal outcomes. *Front Pediatr* 2022;10:1–8.

Hilgers LAT, Snippe H. DDA as an immunological adjuvant. *Res Immunol* 1992;143:494–503.

Hu Z, Chen JF, Xu JC et al. A two-dose optimum for recombinant S1 protein-based COVID-19 vaccination. *Virology* 2022;566:56–9.

Huber M, Baier W, Bessler WG et al. Modulation of the Th1/Th2 bias by lipopeptide and saponin adjuvants in orally immunized mice. *Immunobiology* 2002;205:61–73.

Ito AY, Neri S, Machado MSS et al. Homologous prime-boost strategy in neonate mice using *Neisseria lactamica*. *Vaccine* 2009;27:3422–8.

Jangra S, Landers JI, Rathnasinghe R et al. A recombinant vaccine for the induction of potent antiviral immune responses for a recombinant SARS-CoV-2 protein vaccine. *Front Immunol* 2021;12:1–17.

Johnston SC, Ricks KM, Lakhal-Naouar I et al. A SARS-CoV-2 spike ferritin nanoparticle vaccine is protective and promotes a strong immunological response in the cynomolgus macaque coronavirus disease 2019 (COVID-19) model. *Vaccines* 2022;10:717.

Kelly SM, Larsen KR, Darling R et al. Single-dose combination nanovaccine induces both rapid and durable humoral immunity and toxin neutralizing antibody responses against *Bacillus anthracis*. *Vaccine* 2021;39:3862–70.

Khatri I, Staal FJT, van Dongen JJM. Blocking of the high-affinity Kelly SM, Larsen KR, Darling R et al. Single-dose combination nanovaccine induces both rapid and durable humoral immunity and toxin neutralizing antibody responses against *Bacillus anthracis*. *Vaccine* 2021;39:3862–70.

Khatri I, Staal FJT, van Dongen JJM. Blocking of the high-affinity interaction-synapse between SARS-CoV-2 spike and human ACE2 proteins likely requires multiple high-affinity antibodies: an immune perspective. *Front Immunol* 2020;11:1–9.

Kim L, Whitaker M, O’Halloran A et al. Hospitalization rates and characteristics of children aged <18 years hospitalized with laboratory-confirmed COVID-19: COVID-NET, 14 states, March 1–July 25, 2020. *Morb Mortal Wkly Rep* 2020;69:1081–8.

Krammer F. SARS-CoV-2 vaccines in development. *Nature* 2020;586:516–27.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.

Lay M, Callejo B, Chang S et al. Cationic lipid/DNA complexes (JVR5-100) combined with influenza vaccine (Fluzone®) increases antibody response, cellular immunity, and antigenically drifted protection. *Vaccine* 2009;27:3811–20.

Liang Z, Zhu H, Wang X et al. Adjuvants for coronavirus vaccines. *Front Immunol* 2020;11:589833. DOI: 10.3389/fimmu.2020.589833.

Lincopan N, Espindola NM, Vaz AJ et al. Novel immunoadjuvants based on cationic lipid: preparation, characterization and activity in vivo. *Vaccine* 2009;27:5760–71.

Liu Y, Zhang C, Huang F et al. Elevated plasma levels of selective cytokines in COVID-19 patients reflect viral load and lung injury. *Natl Sci Rev* 2020;7:1003–11.

Miura K, Deng B, Wu Y et al. ELISA units, IgG subclass ratio and avidity determined functional activity of mouse anti-Pf5230 antibodies judged by a standard membrane-feeding assay with *Plasmodium falciparum*. *Vaccine* 2019;37:2073–8.

Mohan S, Anjum MR, Koidiasu A et al. SARS-CoV-2 infection: a global outbreak and its implication on public health. *Bull Natl Res Cent* 2021;45:139. DOI: 10.1186/s42269-021-00599-7.

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145–73.

Omer SB. Maternal immunization. *N Engl J Med* 2017;376:1256–67.

Pabreja S, Garg T, Rath G et al. Mucosal vaccination against tuberculosis using Ag85A-loaded immunostimulating complexes. *Artif cell s. Artif Cells Nanomed Biotechnol* 2016;44:532–9.

Pandey RS, DIXIT VK. Evaluation of ISCOM vaccines for mucosal immunization against hepatitis B. *J Drug Target* 2010;18:282–91.

Phillips R, Williams JN, Tan WM et al. Immunization with recombinant chaperonin60 (Chp60) outer membrane protein induces a bacterialid antibody response against *Neisseria meningitidis*. *Vaccine* 2013;31:2584–90.

Polack FP, Thomas SJ, Kitchin N et al. Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. *N Engl J Med* 2020;383:2635–51.

Pollard AJ, Frasch C. Development of natural immunity to *Neisseria meningitidis*. *Vaccine* 2001;19:1327–46.

Roncati L, Nasillo V, Lusenti B et al. Signals of Th2 immune response from COVID-19 patients requiring intensive care. *Ann Hematol* 2020;99:1419–20.

Santana-Mederos D, Perez-Nicrado R, Climenti Y et al. A COVID-19 vaccine candidate composed of the SARS-CoV-2 RBD dimer and *Neisseria meningitidis* outer membrane vesicles. *RSC Chem Biol* 2022;3:242–9.

Scaria PV, Rowe CG, Chen BB et al. Protein-protein conjugation enhances the immunogenicity of SARS-CoV-2 receptor-binding domain (RBD) vaccines. *Iscience* 2022;25:104739.

Shah RR, Hassett KJ, Brito LA. Overview of vaccine adjuvants: introduction, history, and current status. *Methods Mol Biol* 2017;1494:1–13.

Stadlbauer D, Aranfat F, Chromikova V et al. SARS-CoV-2 seroconversion in humans: a detailed protocol for a serological assay, antigen production, and test setup. *Curr Protoc Microbiol* 2020;57:1–15.

Stewart R, Hammond SA, Oberst M et al. The role of Fc gamma receptors in the activity of immunomodulatory antibodies for cancer. *J Immunother Cancer* 2014;2:1–10.

Struck F, Schreiner P, Staschek E et al. Vaccination versus infection with SARS-CoV-2: establishment of a high avidity IgG response versus incomplete avidity maturation. *J Med Virol* 2021;93:6765–77.

Sun H-X, Xie Y, Ye Y-P. Advances in saponin-based adjuvants. *Vaccine* 2009;27:1787–96.

Temming AR, Bentlage AEH, de Taeye SW et al. Cross-reactivity of mouse IgG subclasses to human Fc gamma receptors: antibody deglycosylation only eliminates IgG2b binding. *Mol Immunol* 2020;127:79–86.

Trzewikoswki de Lima G, Portilho AI, De Gaspari E. Cross-reactivity with outer membrane vesicles. *Ther Adv Vaccines Immunother* 2018;10:71–80.

Vujanic A, Snibson KJ, Wee JLK et al. Adjuvants for coronavirus vaccines. *Vaccine* 2020;38:71–90.

Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. *J Antimicrob Chemother* 2006;58:760–7.

Vujanic A, Snibson KJ, Wee JLK et al. Long-term antibody and immune memory response induced by pulmonary delivery of the influenza iscomatrix vaccine. *Clin Vaccine Immunol* 2012;19:79–83.

World Health Organization. COVID-19 vaccine tracker and landscape. 2022. https://www.who.int/publications/m/item/draft-la
Yadav PD, Ella R, Kumar S et al. Immunogenicity and protective efficacy of inactivated SARS-CoV-2 vaccine candidate, BBV152 in rhesus macaques. *Nat Commun* 2021,12 1–11.

Zhang Y, Zeng G, Pan H et al. Safety, tolerability, and immunogenicity of an inactivated SARS-CoV-2 vaccine in healthy adults aged 18–59 years: a randomised, double-blind, placebo-controlled, phase 1/2 clinical trial. *Lancet Infect Dis* 2021,21 181–92.