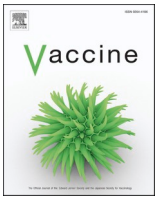
Vaccine 42 (2024) 125980

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Efficacy of a stable broadly protective subunit vaccine platform against SARS-CoV-2 variants of concern

Ravendra Garg [a,1], Qiang Liu [a,b,c,1], Jill Van Kessel [a], Akarin Asavajaru [a], Eva-Maria Uhlemann [a], Morgane Joessel [d], Glenn Hamonic [a], Zahed Khatooni [a], Andrea Kroeker [a], Jocelyne Lew [a], Erin Scruten [a], Paul Pennington [a], William Deck [a], Tracy Prysliak [a], Michaela Nickol [a], Falko Apel [d], Thomas Courant [d], Alyson A. Kelvin [a,e], Andrew Van Kessel [a], Nicolas Collin [d], Volker Gerdts [a,b], Wolfgang Köster [a,b], Darryl Falzarano [a,b], Trina Racine [a,c,*], Arinjay Banerjee [a,b,f,g,h,*]

[a] Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, SK S7N 5E3, Canada
[b] Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada
[c] School of Public Health, University of Saskatchewan, Saskatoon, SK S7N 2Z4, Canada
[d] Vaccine Formulation Institute (VFI), Plan-Les-Ouates, Switzerland
[e] Department of Biochemistry, Microbiology, and Immunology, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[f] Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada
[g] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[h] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

A B S T R A C T

The emergence and ongoing evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted the need for rapid vaccine development platforms that can be updated to counteract emerging variants of currently circulating and future emerging coronaviruses. Here we report the development of a "train model" subunit vaccine platform that contains a SARS-CoV-2 Wuhan S1 protein (the "engine") linked to a series of flexible receptor binding domains (RBDs; the "cars") derived from SARS-CoV-2 variants of concern (VOCs). We demonstrate that these linked subunit vaccines when combined with Sepivac SWE™, a squalene in water emulsion (SWE) adjuvant, are immunogenic in Syrian hamsters and subsequently provide protection from infection with SARS-CoV-2 VOCs Omicron (BA.1), Delta, and Beta. Importantly, the bivalent and trivalent vaccine candidates offered protection against some heterologous SARS-CoV-2 VOCs that were not included in the vaccine design, demonstrating the potential for broad protection against a range of different VOCs. Furthermore, these formulated vaccine candidates were stable at 2–8 °C for up to 13 months post-formulation, highlighting their utility in low-resource settings. Indeed, our vaccine platform will enable the development of safe and broadly protective vaccines against emerging betacoronaviruses that pose a significant health risk for humans and agricultural animals.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 to cause the coronavirus disease (COVID-19) pandemic [1,2]. To combat the rapid spread of this novel coronavirus, new vaccine candidates were quickly deployed, including mRNA-1273 and BNT162b2 messenger RNA (mRNA) vaccines that demonstrated 94.1% and 95% efficacy, respectively, in preventing severe COVID-19 [3,4]. Multiple vaccines have received approval globally or regionally and eleven vaccines are in Phase 4 clinical trials as of the time of writing this manuscript [3,5].

First-generation COVID-19 vaccines were developed using sequence information from the ancestral Wuhan strain of SARS-CoV-2, particularly the sequence of the surface spike (S) glycoprotein [3,4,6]. First-generation vaccines have been effective in preventing severe disease in vaccinated individuals, with one modelling study in 2022 estimating a global reduction of 63% total deaths and 41% reduction in excess COVID-19 mortality in COVAX advance market commitment countries [7]. As SARS-CoV-2 continues to infect and evolve in humans and non-human mammals, new variants of concern (VOCs) with immune escape potential and higher transmission rates have emerged [8,9]. We and others have demonstrated that sera from first-generation vaccinated

* Corresponding authors.
E-mail addresses: trina.racine@usask.ca (T. Racine), arinjay.banerjee@usask.ca (A. Banerjee).
[1] Equal contribution.

https://doi.org/10.1016/j.vaccine.2024.05.028
Received 9 April 2024; Received in revised form 1 May 2024; Accepted 14 May 2024
Available online 19 May 2024

individuals retain some ability to neutralize VOCs such as Beta, Delta, and Omicron, albeit to lower levels compared to neutralization of ancestral SARS-CoV-2 [10–12]. Due to the rise of immune evasive SARS-CoV-2 Omicron VOC and its subvariants, updated mRNA vaccines have been authorized for use [13]. Accumulating data demonstrate that neutralizing antibody levels in sera from individuals who received one or two monovalent booster shots or one bivalent booster shot remained lower against Omicron subvariants BA.1, BA.5, BA2.75.2, BQ.1.1, and XBB compared to ancestral Wuhan strain WA1/2020 [14].

Important for vaccine development, antibodies directed against the SARS-CoV-2 S protein, especially the receptor binding domain (RBD) within the S protein can neutralize the virus [15–17]. However, emerging VOCs have evolved antibody escape mutations within the RBD and S, making it harder for first-generation vaccine-induced antibodies to efficiently neutralize VOCs [12,14]. Indeed, there is a need to develop broadly protective next-generation SARS-CoV-2 vaccines that will provide better protection against emerging VOCs and further reduce virus shedding and transmission among the vaccinated population. These pan-SARS-CoV-2 VOC vaccine candidates will also inform the development of broadly protective pan-*Sarbecovirus* and pan-*Betacoronavirus* vaccine candidates [18], some of which have been published recently [19–21].

Here we report the development of a "train model" subunit vaccine platform consisting of SARS-CoV-2 Wuhan S1 protein as the train 'engine' and various VOC RBDs as the train 'cars', which can be placed after or both before and after the S1 subunit. We demonstrate that these subunit vaccine candidates confer broad protection against SARS-CoV-2 VOCs in the Syrian golden hamster model of COVID-19. The "train model" platform builds on our first-generation COVID-19 vaccines that are currently being assessed in clinical trials as either the primary regimen (COVAC-001 – NCT04702178 and COVAC-003 – NCT05209009) or as a booster vaccine (COVAC-004 – NCT05226702, and COVAC-005 – NCT05693272). We and others have demonstrated that first-generation authorized vaccines induce lower neutralizing antibodies against the highly divergent Omicron VOC [22,23]. Yet, first-generation vaccines offered protection against severe disease upon infection with Omicron VOC [24]. Thus, to increase the breadth of immunity induced by our S1 subunit vaccine candidates, we developed a new subunit vaccine platform where RBD sequences from VOCs are linked to a core SARS-CoV-2 Wuhan S1 sequence using a short amino acid glycine-serine flexible linker sequence (WuhanS1-linker-RBD or RBD-linker-WuhanS1-linker-RBD platforms).

We also report on the long-term stability and immunogenicity of our formulated vaccine candidates. The antigens are formulated with a squalene-in-water emulsion adjuvant originally developed by the Vaccine Formulation Institute (Switzerland) [25] and then manufactured and commercialized by Seppic (France) under the trademark Sepivac SWE^{TM}. The squalene-in-water family of adjuvants has a strong record of safety and efficacy and has great potential for use in vaccines during new outbreaks, especially due to its ease of manufacture, stability, and potential antigen dose-sparring properties [26]. SWE has been successfully used in preclinical and clinical studies with multiple vaccine candidates, [27–31]. To demonstrate the commercial and broad dissemination potential of our vaccine candidates, we performed long-term stability and immunogenicity testing. Indeed, our data suggest that a "train model" subunit vaccine platform based on S1-linker-RBD can be utilized to design stable and easily editable vaccine candidates to combat emerging SARS-CoV-2 VOCs. In the future, our platform can potentially be adapted to develop more broadly neutralizing vaccine candidates against emerging sarbecoviruses and other betacoronaviruses.

2. Materials and methods

2.1. Ethics statement

All work with infectious SARS-CoV-2 was performed in a containment level 3 laboratory at the Vaccine and Infectious Disease Organization according to approved protocols. The University of Saskatchewan's University Animal Care Committee (UACC) and Animal Research Ethics Board (AREB) approved the animal work as per guidelines of the Canadian Council of Animal Care's (CCAC) criteria. Experimental protocols for mice studies were reviewed and approved by the canton of Vaud Veterinary Authority as per guidelines of the Swiss Federal Law on the Protection of Animals regulations.

2.2. Cells and viruses

All virus isolates/variants were isolated and/or propagated in Vero76 cells (CRL-1587, ATCC) using previously established methods and authenticated by next-generation sequencing [23,32]. Virus stocks were propagated for 1–2 passages prior to use in animal challenge experiments. The ancestral SARS-CoV-2 Wuhan strain (GISAID Accession EPI_ISL_425177), Beta (GenBank Accession# OM131550.1), Delta (GenBank Accession#: OM131551.1), and Omicron BA.1 (GenBank Accession# OM131552.1) [23] VOCs were isolated from Canadian patients and cultured on Vero76 cells.

2.3. Structure prediction

The predicted three-dimensional conformations of three constructs were generated through AlphaFold2 (AlphaFold2 (Colab-Fold) (ChimeraX plugin for AlphaFold2/Colab-Fold with default criteria) and validated using SWISS-MODEL structural assessments [33,34]. PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) and ChimeraX were used to visualize them. Then, we conducted the energy minimization through ModRefiner, which employs physics- and knowledge-based force fields in two steps to relax the structures [35]. The Ramachandran plot for three constructs shows the residues are mapped in the allowed area for 93.8%, in VIDO4372, and over 95% for both VIDO4304 and VIDO4296 [36,37].

2.4. Expression and purification of VIDO4296, VIDO4304, and VIDO4372 chimeric proteins and vaccine formulation

VIDO4296 chimeric protein was expressed by stable transfection of an episomal plasmid in HEK 293 cells [38] whereas VIDO4304 and VIDO4372 chimeric proteins were expressed in ExpiCHO cells as per manufacturer's instruction (ThermoFisher Scientific). In brief, cells were transfected with 0.8 μg/mL of plasmid DNA and subsequently cultured for 3–8 days until protein production reached peak levels. The protein containing suspension culture was harvested by centrifugation at 4000 x g for 30 min at 4 °C. The clarified supernatant containing the expressed proteins was then brought to a final concentration of 300 mM NaCl and 0.05% Tween 80. A final filtration through a 0.2 μm filter was performed and the clarified supernatant was 8-10x concentrated via tangential flow filtration using a 30kDA cut-off membrane. The concentrated supernatant was loaded onto a column with Repligen NGL Covid-19 Spike protein AR 2.0 resin. Following the manufacturer's recommendations, column volume and residence times were adjusted based on the expected protein in the sample. The columns were washed with 10 CV of PBS pH 7.5 before elution in 8 CV of 100 mM sodium acetate pH 5.0 + 1 M arginine. After elution, the protein was immediately buffer exchanged into a PBS buffer containing 20 mM sodium phosphate and 277 mM sodium chloride. The pH of the buffer was in the range of 6.5–7.5 which was chosen at least 0.5 unit below the theoretical pI of the protein, as predicted by Expasy ProtParam tool (Swiss Institute of Bioinformatics). The identity of the glycosylated and deglycosylated (treatment with PNGase) chimeric proteins was confirmed by SDS-PAGE and immunoblotting. The concentration of the purified proteins was determined by BCA protein assay (Pierce®) to be 2.3 mg/L for VIDO4296, 6–8 mg/L for VIDO 4372, and 15 mg/L for VIDO4304. The purified proteins were separated on a 4–20% gradient Mini-PROTEAN

TGX gel (Biorad). For Western blot analysis, the proteins were transferred after SDS-PAGE onto 0.2 μm nitrocellulose using a Trans Turbo Transfer pack (BioRad) according to the manufacturer's recommendations. Following standard blocking and washing procedure, the membrane was probed with in-house generated rabbit polyclonal primary antibody CoV19-S1 (1:10,000). The immunoblot was visualized with a Li-Cor® NIR scanner using Li-COR® secondary anti-rabbit IRDye 800CW antibody (1:10,000). Sepivac SWE™ adjuvant (Seppic, France) was formulated with chimeric protein in a 1:1 ratio.

2.5. Animal vaccination and challenge

Six to eight-week-old male Syrian golden hamsters (Charles River Laboratories, Wilmington, Massachusetts, USA) were randomly assigned to four groups (n = 8/group) and vaccinated twice intramuscularly (IM) with either PBS or 25 μg VIDO4296, VIDO4304, or VIDO4372 chimeric antigen formulated in adjuvant just prior to administration. Three weeks after the second vaccination, animals were challenged intranasally (IN) with 10^5 $TCID_{50}$ in 100 μl of either the Beta, Delta, or Omicron SARS-CoV-2 VOC. Animal weight and clinical signs were monitored throughout the study. At 5 days post-challenge (dpc), half of the animals from each group (n = 4) were euthanized and nasal washes and lung samples were collected for virological and pathological analysis. At 10 dpc, the remaining animals were euthanized (n = 4) as above. Sera were collected before each immunization, before the challenge, and at euthanasia.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Ancestral SARS-CoV-2 S1-specific IgG was evaluated in serum samples by standard ELISA. Briefly, plates were coated with recombinant SARS-CoV-2 Wuhan S1 protein at a concentration of 1 μg/mL. Four-fold

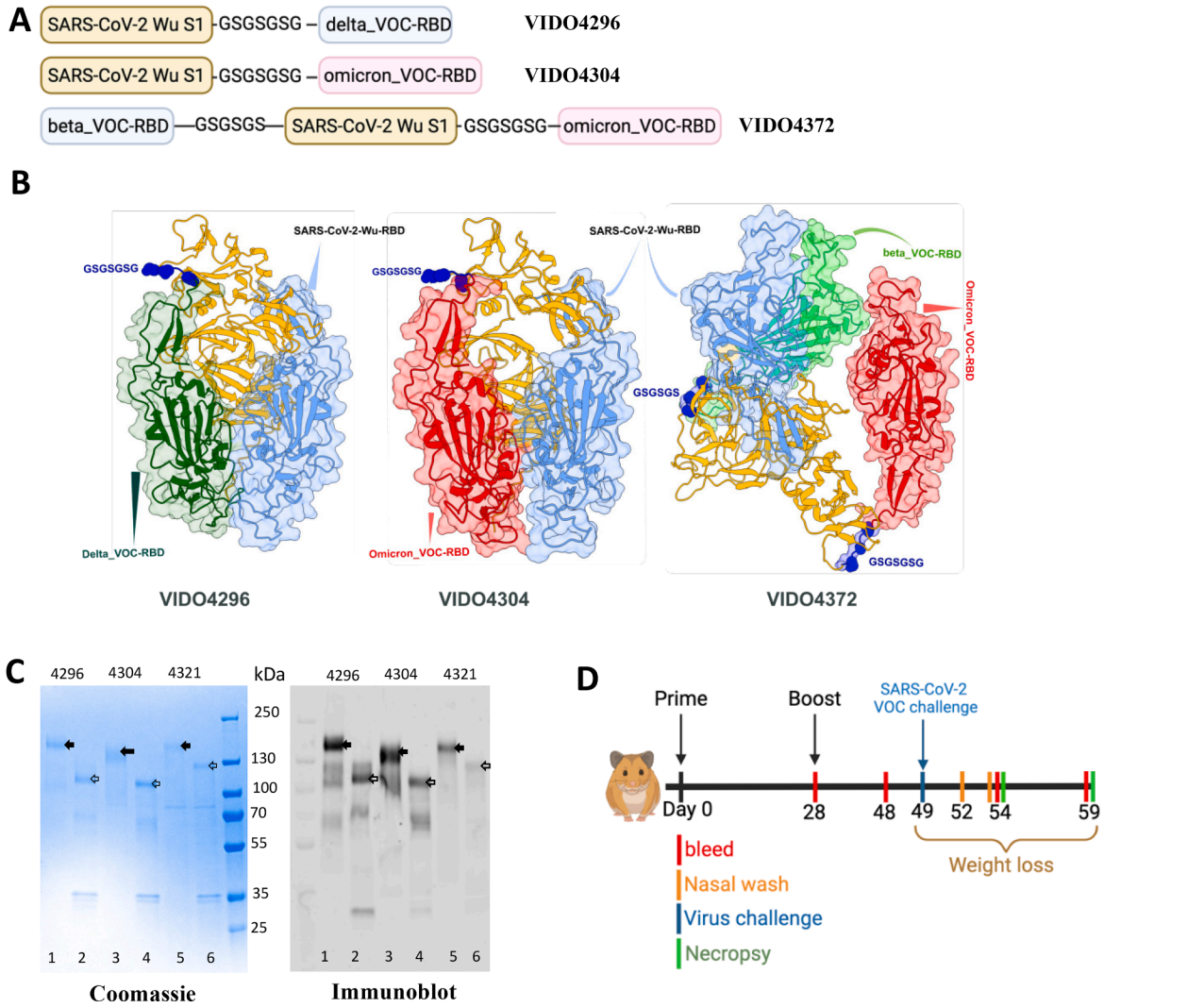


Fig. 1. Vaccine and study design. (A) Antigen designs. **(B)** Predicted 3D conformations of VIDO4296, VIDO4304, and VIDO4372. All components are colour-coded. SARS-CoV-2 Wuhan S1 RBD is shown in blue, Omicron VOC RBD in red, Beta VOC RBD in light green, Delta VOC RBD in spring green, N- and C-terminus of SARS-CoV-2 S1 is shown in yellow, and the glycine-serine linker sequences are highlighted as dark blue spheres. **(C)** VIDO4296, VIDO4304 and VIDO4372 glycosylated and deglycosylated proteins were analyzed by 4–20 % gradient SDS-PAGE and Western blotting. The molecular weight of the fully glycosylated proteins (solid arrows) versus deglycosylated proteins (open arrows) are shown. Glycosylated VIDO4296 (lane 1), deglycosylated VIDO4296 (lane 2), glycosylated VIDO4304 (lane 3), deglycosylated VIDO4304 (lane 4), and glycosylated VIDO4372 (lane 5) and deglycosylated VIDO4372 (lane 6). **(D)** Study design to assess the efficacy of vaccine candidates using the Syrian golden hamster model. Vaccine candidates and challenge viruses were altered between studies, but the overall study design and timeline remained the same. Hamsters were vaccinated twice intramuscularly with 25 μg of VIDO4296, VIDO4304, or VIDO4372 formulated 1:1 with Sepivac SWE™. Three weeks after the second vaccination, all animals were challenged with either Beta, Delta, or Omicron SARS-CoV-2 variants of concern (VOCs). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Schematics created using BioRender.com.

serially diluted serum samples were added to the coated plates. Antigen-specific antibodies were detected with Goat anti-Hamster IgG HRP at 1:7000 dilution (Thermo Fisher Scientific). Plates were developed with OPD peroxidase substrate (Thermo Fisher Scientific) and absorbance was read at 490 nm in a SPECTRAmax 340 PC Microplate Reader (Molecular Devices, CA, USA).

2.7. Virus neutralization assay

The hamster serum samples were tested for viral neutralization (VN) against the SARS-CoV-2 Beta, Delta and Omicron VOCs. Serum samples were heat-inactivated at 56 °C for 30 min and then serially diluted 1:2 in 96-well plates (Corning Incorporated, Corning, NY). Diluted samples were mixed with the virus at a 1:1 ratio and incubated for 1 h at 37 °C. The virus-serum sample mixtures were transferred to Vero76 cell monolayers and incubated at 37 °C. Endpoint neutralization titer was based on 100% inhibition of cytopathic effect (CPE) observed on day 5 after cell infection.

2.8. Infectious virus titration assay

Infectious virus titration assays were carried out on nasal washes and lung samples. Nasal washes were collected on days 3 and 5 post viral challenge (Fig. 1D). Nasal flushes were performed by inserting the teat cannula into the upper nares of the anesthetized hamster. Nasal washes were collected from the bottom nares. At necropsy, lung samples were collected, weighed, and homogenized in serum-free DMEM using a Qiagen TissueLyzer. After adding serially diluted samples to Vero76 cells for an hour, the samples were removed and replaced with fresh DMEM medium containing 2% FBS. Cells were examined for CPE five days after infection, and the 50% endpoint ($TCID_{50}$) was determined using the Spearman method [39].

2.9. Long-term stability and immunogenicity testing of formulated vaccine

The adjuvant Sepivac SWE™ is a squalene-based oil-in-water adjuvant, comprising squalene (3.9%, w/v), sorbitan trioleate (0.47%, w/v), and polyoxyethylene (80) sorbitan monooleate (0.47%, w/v) dispersed in 10 mM citrate buffer at pH 6.5. Sepivac SWE™ was developed by the Vaccine Formulation Institute (Switzerland) and made commercially available via an open-access model by Seppic (France). VIDO4296, VIDO4304, and VIDO4372 chimeric proteins were formulated with adjuvant in a 1:1 ratio and formulations were made isotonic by the addition of sodium chloride. The pH of the VIDO4296 + SWE and VIDO4372 + SWE formulations were adjusted to pH 6.5, while VIDO4304 + SWE was adjusted to pH 6.7.

For long-term stability and immunogenicity studies, formulations were vialed in ISO 2R type 1 glass vials (Schott) using bromobutyl 13 mm stoppers (West) and 13 mm flip-off aluminum seals (West) and stored at 2–8 °C for up to 13 months. Formulations were characterized by assessing visual appearance, pH (SevenCompact, Mettler Toledo, Switzerland), particle size, polydispersity index and zeta potential by dynamic/electrophoretic light scattering (Zetasizer Nano ZS, Malvern Panalytical, UK), osmolality (OsmoPro, Advanced Instruments, USA), squalene concentration by reverse-phase high-pressure liquid chromatography (1260 Infinity II, Agilent, USA), and antigen integrity by biolayer interferometry (BLI; Octet Red 96e, Sartorius, USA). *In vivo* immunogenicity studies were carried out at specified time points to confirm immunogenicity.

BLI assays were performed using the Octet Red96e instrument (Sartorius, USA). Protein A biosensors were dipped for 60 s into wells containing running buffer (PBS pH 7.2 with 0.1% w/v bovine serum albumin and 0.02% v/v tween 20) for baseline measurement. CR3022 recombinant Anti-SARS-CoV-2 Spike Glycoprotein S1 antibody (Abcam) was loaded at a concentration of 10 μg/mL onto biosensors for 300 s followed by a 40 s second baseline measurement in running buffer. VIDO4296 + SWE, VIDO4304 + SWE, and VIDO4372 + SWE formulations were diluted from 1:4 to 1:16 in the running buffer. The sensors were dipped into wells containing diluted formulations for 200 s to assess association. Curves were aligned at the association step and antigen concentration was determined using a 4P-unweighted dose–response analysis based on the initial slope of binding rates.

Six weeks old female BALB/c mice (Charles River Laboratories, France) were immunized IM on days 0 and 28 with extemporaneous formulations of either VIDO4296, VIDO4304, or VIDO4372 chimeric antigens adjuvanted with Sepivac SWE™, or preformulated long-term preparations stored at 2–8 °C. The control group of mice was immunized with PBS without any adjuvant. At day 42, mice were euthanized, and sera were collected for antibody response analysis.

SARS-CoV-2 S1-specific total IgG was evaluated in serum samples by standard ELISA. Briefly, plates were coated with soluble SARS-CoV-2 RBD at a concentration of 1.25 μg/mL. Four-fold serially diluted serum samples were added to the coated plates. Antigen-specific antibodies were detected with Goat anti-Mouse IgG(H + L) HRP (Southern Biotech). Plates were developed with OPD peroxidase substrate (ThermoFisher Scientific) and absorbance was read at 492 nm in a Synergy H1 Microplate Reader (Biotek, USA).

The mouse serum samples were tested for viral neutralization using the SARS-CoV-2 surrogate virus neutralization test (sVNT) kit (Genscript) against different VOCs. Diluted serum samples were mixed with HRP labeled SARS-CoV-2 RBD proteins (RBD-HRP) from Wuhan, Delta-, Omicron-, or Beta-VOC (Genscript), at a 1:1 ratio for 30 min at 37 °C. The RBD-HRP-serum sample mixtures were transferred to an ACE2-coated capture plate and incubated for 15 min at 37 °C. Plates were developed with TMB substrate (Genscript) and absorbance was read at 450 nm in a Synergy H1 Microplate Reader (Biotek).

2.10. Statistical analysis

Graphs and descriptive statistical analyses were performed on GraphPad Prism version 9. Statistical comparisons among different groups were conducted using either the nonparametric Mann-Whitney test or the Kruskal-Wallis test.

3. Results

3.1. Linked subunit proteins as a platform for multivalent SARS-CoV-2 vaccines

To express WuhanS1-linker-RBD or RBD-linker-WuhanS1-linker-RBD proteins, we cloned multiple coding sequences in frame and separated them by a glycine-serine linker sequence (Fig. 1A). We then predicted the 3D structures of the vaccine constructs, investigating how SARS-CoV-2 S1 and RBDs from different variants and GS linkers are folded together (Fig. 1B). We observed that the various antigen components bore a remarkable resemblance to experimental models already deposited in the Protein Data Bank (PDB). The structural alignments of these domains onto the crystallized RBD or N-terminus of the spike illustrate that both the RBDs (for SARS-CoV-2 Omicron, Delta, and Beta) and the SARS-CoV-2 S1 N-terminus that were linked through GS linkers matched well with the experimental models. Superimposition of the predicted SARS-CoV-2 Wuhan S1-RBD onto the crystal structure of SARS-CoV-2 S1 RBD (PDB: 6M0J) reveals a root-mean-square deviation (RMSD) of 0.69 Å [40] such that helices and beta-sheets closely aligned (supplementary Fig. 1) [41,42]. Overall, differences were observed in the flexible loops potentially due to the inherently disordered nature of these regions. Alignment of the predicted Beta VOC RBD with the reference crystallized Beta RBD (PDB: 7S5P) demonstrates a close conformational match, with an RMSD of 0.72 Å [43].

VIDO4296, VIDO4304 and VIDO4372 proteins were produced and purified from mammalian cell cultures as soluble secreted proteins. The main antibody-reactive band corresponds to the major band on the

Coomassie-stained gel for all proteins. The observed molecular weight of the glycosylated VIDO4296, VIDO4304 and VIDO4372 proteins were approximately 170 kD, 130 kD, and 150 kD, respectively, while the observed molecular weight of the deglycosylated forms were approximately 100.93, 101.02 and 128.15 kD, respectively (Fig. 1C-D). The higher molecular weight of the glycosylated form of VIDO4296 is a result of expression in HEK293 cells, which are known to more heavily glycosylate proteins compared to CHO cells [44]. After protein purification, vaccine antigen was formulated with Sepivac SWE™ adjuvant and were tested for protective efficacy against ancestral SARS-CoV-2 and VOCs using the hamster model of COVID-19 (Fig. 1D).

3.2. WuhanS1 – Delta RBD (VIDO4296) bivalent vaccine candidate induces strong protective immunity against homologous and heterologous SARS-CoV-2 challenge

To determine the protective efficacy of VIDO4296, hamsters were vaccinated IM twice with 25 μg of VIDO4296 formulated with Sepivac SWE™ (Fig. 1D). Three weeks after the second vaccine dose, all animals were challenged IN with 10^5 $TCID_{50}$ of SARS-CoV-2 Omicron, Delta, or Beta VOC. Samples were collected from animals as indicated (Fig. 1D). Vaccinated animals lost less weight compared to control animals infected with SARS-CoV-2 VOCs (Fig. 2A-C). VIDO4296 vaccinated animals quickly gained weight starting at day 6 post-infection with SARS-CoV-2 VOCs, unlike sham vaccinated animals that gained weight later (Fig. 2A-C). Sera from vaccinated animals contained high levels of neutralizing antibodies against SARS-CoV-2 Delta VOC (Fig. 2D-F), as well as anti-S1 (SARS-CoV-2 Wuhan S1) antibodies (Fig. 2G-I). Sera from sham vaccinated animals did not contain anti-S1 antibodies or neutralizing antibodies prior to challenge with SARS-CoV-2 VOCs (Fig. 2D-I). Five days post-challenge with SARS-CoV-2 VOCs, sera from vaccinated animals maintained robust levels of neutralizing antibodies (Fig. 2D-F). At ten days post-challenge with SARS-CoV-2 Omicron, Delta or Beta VOC, sera from sham vaccinated and VIDO4296 vaccinated animals contained comparable levels of neutralizing antibodies (Fig. 2D-F).

Limiting virus transmission in vaccinated individuals is a key priority for second-generation SARS-CoV-2 vaccines. Three days post-challenge, infectious virus was significantly lower in nasal washes collected from VIDO4296 vaccinated animals compared to PBS inoculated animals and remained lower at five days post-challenge for those animals challenged with either the Delta or Beta VOC (Fig. 2J-L). Furthermore, at five days post-challenge with SARS-CoV-2 Omicron, Delta, or Beta VOCs, no infectious virus could be recovered from the upper or lower respiratory tracts of the majority of VIDO4296 vaccinated animals. In contrast, sham-vaccinated animals had a high burden of infectious virus in sampled tissues. (Fig. 2M-O).

3.3. WuhanS1 – omicronRBD (VIDO4304) demonstrates broad protection and flexibility of the spike-RBD chimeric platform

To demonstrate the flexibility of the WuhanS1-linker-RBD platform, we replaced the deltaRBD in VIDO4296 with omicronRBD to generate a WuhanS1-omicronRBD (VIDO4304) subunit vaccine where WuhanS1 and omicronRBD are separated by a seven amino acid glycine-serine linker sequence (Fig. 1A). Next, we compared the efficacy of VIDO4304 in protecting against SARS-CoV-2 Omicron, Delta, and Beta VOC challenge in hamsters (Fig. 1D). Hamsters were vaccinated with VIDO4304 and challenged with either SARS-CoV-2 Omicron, Delta, or Beta VOC. Vaccinated animals lost less weight after virus infection compared to sham-vaccinated animals (Fig. 3A-C). Sera from VIDO4304 vaccinated animals contained neutralizing antibodies against SARS-CoV-2 Omicron and Delta VOCs at day 48 post-vaccination, prior to virus challenge, although neutralizing antibody titers against the Beta VOC were low (Fig. 3D-F). Sera from both PBS-inoculated and VIDO4304 vaccinated animals contained high levels of neutralizing antibodies against SARS-CoV-2 VOCs at days 5 and 10 post-infection (Fig. 3D-F). Sera from VIDO4304 vaccinated animals contained high levels of anti-S1 (SARS-CoV-2 Wuhan S1) antibody titers at days 28 and 48 post-vaccination (Fig. 3G-I). Sera from PBS-vaccinated animals did not contain detectable levels of anti-S1 antibodies (Fig. 3G-I).

Next, we assessed the impact of vaccination on virus replication and shedding. At three- and five-days post-challenge, infectious SARS-CoV-2 Omicron, Delta, and Beta VOC levels in nasal washes collected from VIDO4304 vaccinated animals were lower on average compared to sham vaccinated animals (Fig. 3J-L). Furthermore, at five days post-challenge with SARS-CoV-2 VOCs, infectious virus could not be recovered from either the upper or lower respiratory tracts of most VIDO4304 vaccinated animals (Fig. 3M-O). Detectable virus levels in tissues from sham vaccinated and SARS-CoV-2 Omicron VOC infected animals varied, which is consistent with other studies that have reported reduced virus replication in hamster lung tissues upon infection with SARS-CoV-2 Omicron VOC (Fig. 3M) [45].

3.4. BetaRBD-WuhanS1-omicronRBD (VIDO4372) trivalent vaccine candidate protects Syrian hamsters

To investigate if we could add additional RBDs to the S1-RBD vaccine platform, we generated an RBD-linker-WuhanS1-linker-RBD subunit vaccine candidate. VIDO4372 consisted of a SARS-CoV-2 Beta VOC RBD at the N terminus, followed by a six amino acid glycine-serine linker sequence and a core Wuhan S1. The core Wuhan S1 is followed by a seven amino acid glycine-serine linker sequence and a C terminus Omicron VOC RBD (Fig. 1A).

Hamsters were vaccinated with VIDO4372 and challenged IN with 10^5 $TCID_{50}$ of either SARS-CoV-2 Omicron, Delta, or Beta VOC. VIDO4372 vaccinated animals lost less body weight compared to sham vaccinated animals post-infection with SARS-CoV-2 Omicron and Beta VOCs (Fig. 4A & C). However, both vaccinated and PBS-inoculated animals lost weight upon challenge with the Delta VOC (Fig. 4B). Sera from four VIDO4372 vaccinated animals contained detectable levels of neutralizing antibodies at day 48 post-vaccination and before challenge against SARS-CoV-2 VOCs (Fig. 4D and E). Sera from both sham vaccinated and VIDO4372 vaccinated animals contained neutralizing antibodies against SARS-CoV-2 Omicron, Delta, and Beta VOCs at days 5 and 10 post-infection, albeit to different levels (Fig. 4D-F). Sera from VIDO4372 vaccinated animals contained high levels of anti-S1 (SARS-CoV-2 Wuhan S1) antibody titers at days 28 and 48 post-vaccination (Fig. 4G-I).

At three days post-challenge, infectious SARS-CoV-2 Omicron and Beta VOC levels in nasal washes collected from VIDO4372 vaccinated animals were lower than sham vaccinated animals or undetectable (Fig. 4J and L). Infectious virus levels in nasal washes collected from VIDO4372 vaccinated animals were no different between vaccinated and sham inoculated groups at three days post-challenge with SARS-CoV-2 Delta VOC, but virus levels were lower in vaccinated animals five days post-infection (Fig. 4K). Furthermore, at five days post-challenge with SARS-CoV-2 Omicron or Delta VOC, no infectious virus could be recovered from the upper and lower respiratory tracts of VIDO4372 vaccinated animals (Fig. 4M and 4 N). Virus levels in respiratory tissues were also significantly lower in VIDO4372 vaccinated animals at five days post-challenge with SARS-CoV-2 Delta or Beta VOCs (Fig. 4N and 4O). In summary, most VIDO4372 vaccinated animals had no detectable viral loads in their lower and upper respiratory tracts five days post-infection with SARS-CoV-2 Omicron, Delta, or Beta VOCs.

3.5. Long-term formulation of the bivalent or trivalent chimeric vaccine is stable and immunogenic

Typically, vaccines are formulated on the day of vaccination during pre-clinical testing, and thus, the long-term stability of these vaccines is not known until much later in the vaccine's development lifecycle. To demonstrate the long-term stability of our chimeric vaccines, and

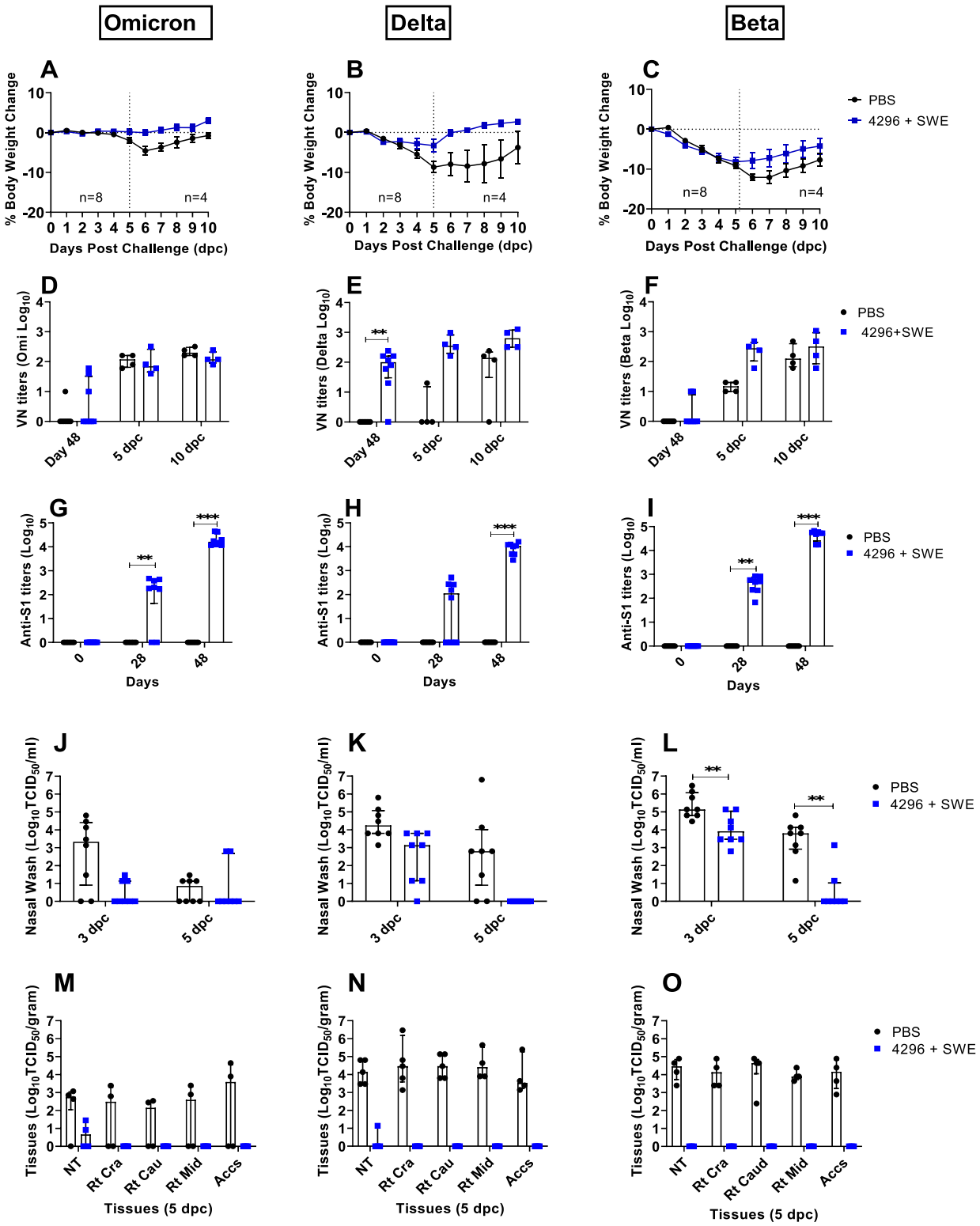


Fig. 2. Protective immunity of Wuhan S1 – delta RBD (VIDO4296) protein formulated with Sepivac SWE™ against either Omicron, Delta, or Beta VOCs challenge. Hamsters were vaccinated twice IM with 25 μg of VIDO4296 formulated with Sepivac SWE™. Three weeks after the second vaccination, all animals were challenged with either SARS-CoV-2 Omicron, Delta, or Beta VOC. Half the animals (n = 4) were euthanized on Day 5 post-challenge. **(A-C)** Percent body weight loss after challenge. Body weight was measured daily for 10 days post-challenge. **(D-F)** Serum-neutralizing antibody titers were measured before the virus challenge (day 48 post-first vaccination) and on euthanasia at days 5 and 10 post-challenge with the virus. **(G-I)** SARS-CoV-2 S1-specific serum IgG titers were assessed pre-vaccination and at days 28 and 48 after the first administration. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. **(J-L)** Viral load in nasal washes was measured on days 3 and 5 post-challenge with SARS-CoV-2 VOCs. **(M−O)** Viral load in lung tissue was measured at day 5 post-challenge with SARS-CoV-2 VOCs. Statistical analysis was performed using the non-parametric Mann-Whitney test. Bars or lines indicate median or median values with interquartile ranges. *P < 0.05; **P < 0.01; ***P < 0.001. NT: nasal turbinate, Rt Cra: right cranial lung lobe, Rt Cau: right caudal lung lobe, Rt Mid: right middle lung lobe, Accs: accessory lung lobe.

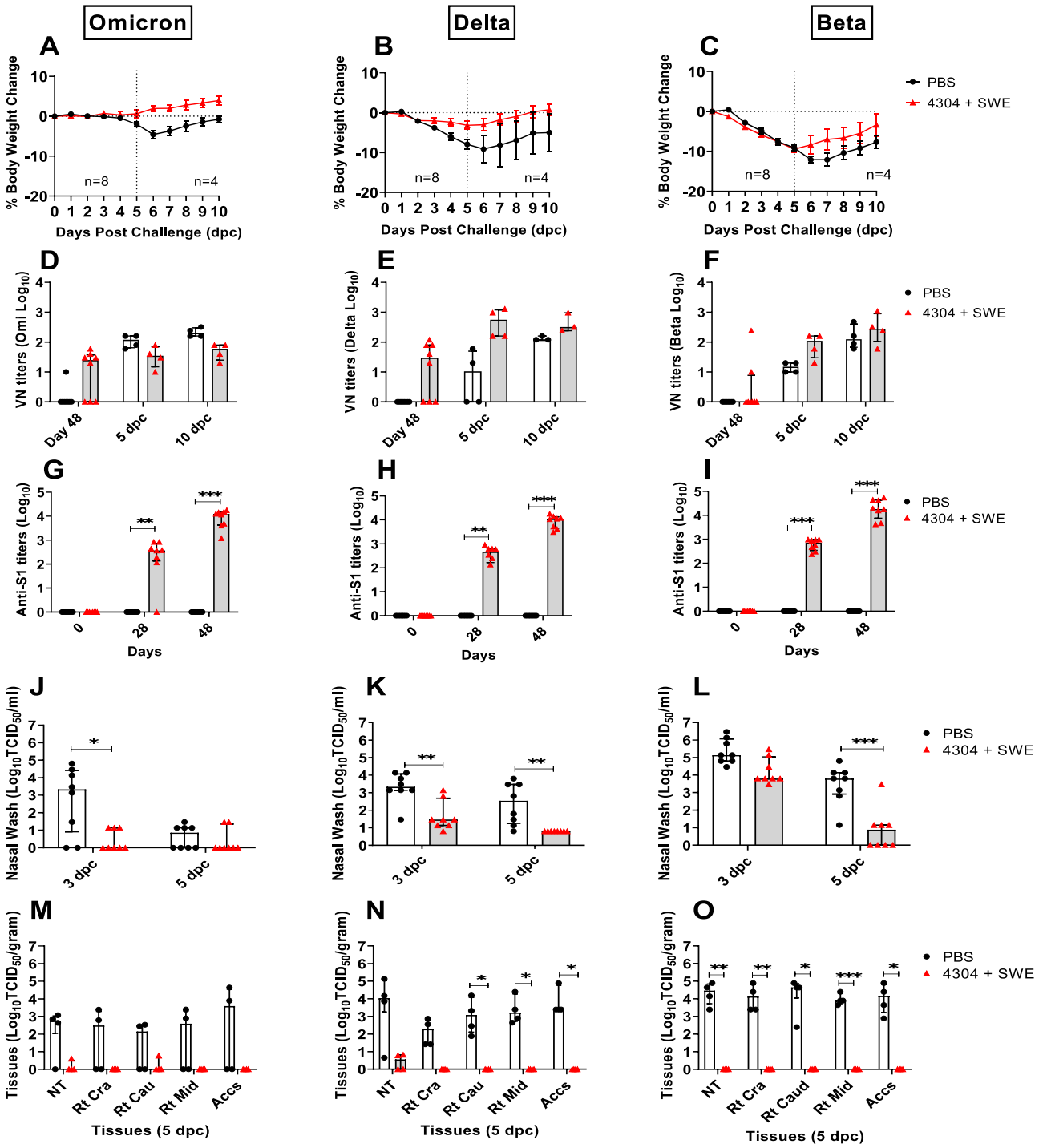


Fig. 3. Vaccination with Wuhan S1 – omicron RBD (VIDO4304) antigen formulated with Sepivac SWE™ offers protection against Omicron, Delta, and Beta VOCs. Hamsters were immunized and challenged as described in Fig. 1. **(A-C)** Percent body weight loss after virus infection in vaccinated and sham inoculated animals. Body weight was measured daily for 10 days post-challenge with SARS-CoV-2 VOCs. **(D-F)** Serum-neutralizing antibody titers were measured before the virus challenge (day 48 post-first vaccination) and on euthanasia at days 5 and 10 post-challenge with the virus. **(G-I)** SARS-CoV-2 S1-specific serum IgG titers were assessed pre-vaccination and at days 28 and 48 after the first administration. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. **(J-L)** Viral load in nasal washes was measured on days 3 and 5 post-challenge with SARS-CoV-2 VOCs. **(M—O)** Viral load in lung tissue was measured at day 5 post-challenge with SARS-CoV-2 VOCs. Statistical analysis was performed using the non-parametric Mann-Whitney test. Bars or lines indicate median or median values with interquartile ranges. *P < 0.05; **P < 0.01; ***P < 0.001. NT: nasal turbinate, Rt Cra: right cranial lung lobe, Rt Cau: right caudal lung lobe, Rt Mid: right middle lung lobe, Accs: accessory lung lobe.

therefore the potential commercial applicability of our vaccine candidates, we formulated the bivalent (VIDO4296 and VIDO4304) and trivalent (VIDO4372) subunit vaccines in a 1:1 vol ratio with Sepivac SWE™ and stored them at 2–8 °C for up to 13 months. Formulated Sepivac SWE™ was stable at 2–8 °C for at least 12 months as demonstrated by the absence of change in particle size, polydispersity index, pH, osmolality, and squalene concentration (Fig. 5A-E). While VIDO4296 adjuvanted with Sepivac SWE™ retained 100% antigen

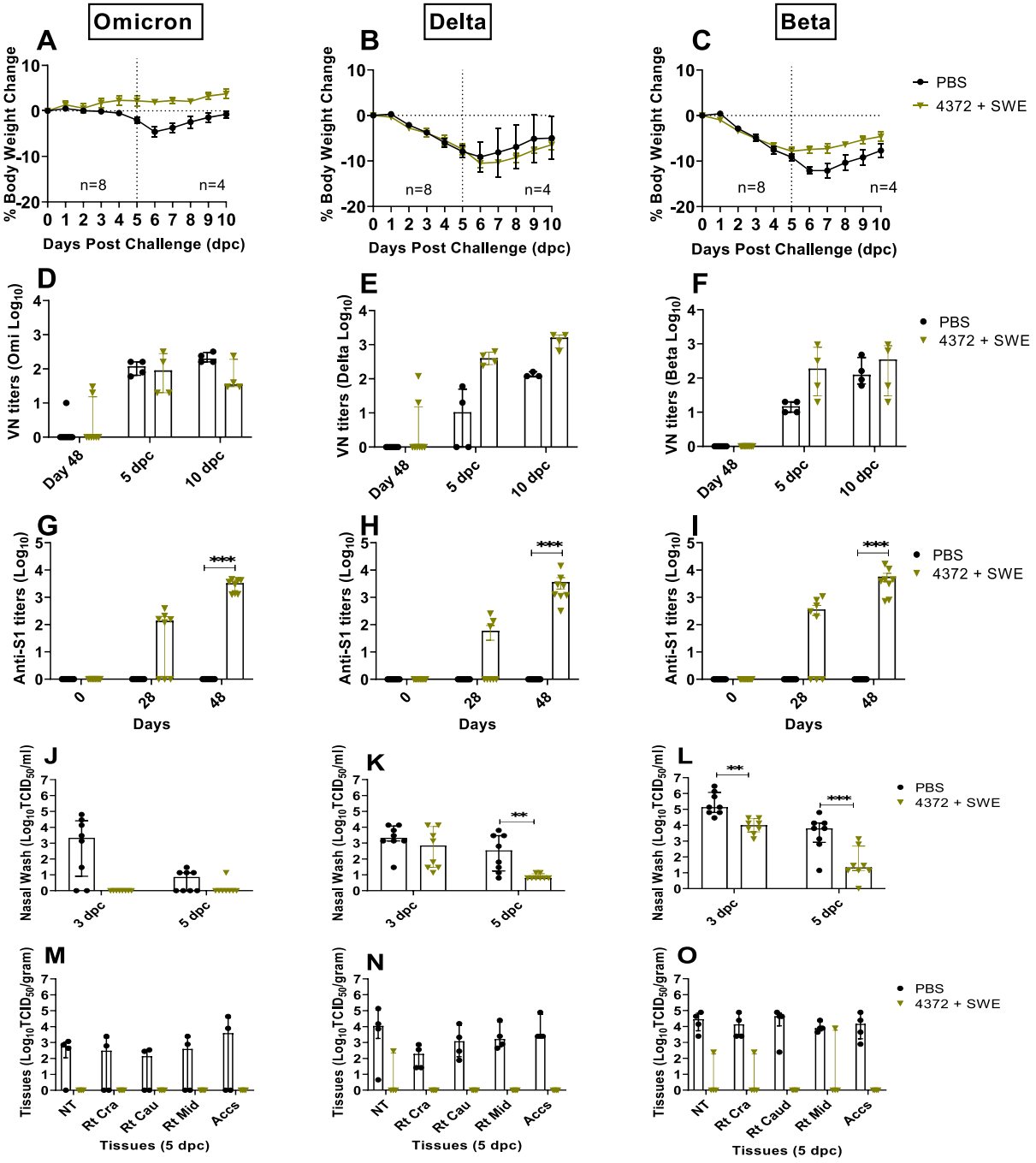


Fig. 4. Vaccination with betaRBD-WuhanS1-omicronRBD (VIDO4372) trivalent vaccine candidate antigen formulated with Sepivac SWE™ offers protective immunity against Omicron, Delta, and Beta VOCs. Hamsters were immunized and challenged as described in Fig. 1. **(A-C)** Percent body weight loss after virus infection in vaccinated and sham inoculated animals. Body weight was measured daily for 10 days post-challenge with SARS-CoV-2 VOCs. **(D-F)** Serum-neutralizing antibody titers were measured before the virus challenge (day 48 post-first vaccination) and on euthanasia at days 5 and 10 post-challenge with the virus. **(G-I)** SARS-CoV-2 S1-specific serum IgG titers were assessed pre-vaccination and at days 28 and 48 after the first administration. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. **(J-L)** Viral load in nasal washes was measured on days 3 and 5 post-challenge with SARS-CoV-2 VOCs. **(M−O)** Viral load in lung tissue was measured at day 5 post-challenge with SARS-CoV-2 VOCs. Statistical analysis was performed using the non-parametric Mann-Whitney test. Bars or lines indicate median or median values with interquartile ranges. *P < 0.05; **P < 0.01; ***P < 0.001. NT: nasal turbinate, Rt Cra: right cranial lung lobe, Rt Cau: right caudal lung lobe, Rt Mid: right middle lung lobe, Accs: accessory lung lobe.

integrity as assessed by biolayer interferometry (BLI) at 12 months post-formulation. VIDO4304 + SWE formulation and VIDO4372 + SWE retained 50% and 75% antigen integrity after 12 months, respectively (Fig. 5F).

To determine whether the vaccines retained their ability to induce a significant immune response after storage at 2–8 °C for several months, six-week-old female BALB/c mice were immunized IM at days 0 and 28 with preformulated vaccines that were stored for 6 or 12 months. As a control, freshly prepared formulations of either VIDO4296, VIDO4304, or VIDO4372 chimeric antigens adjuvanted with Sepivac SWE™ were used for immunization. Control groups of mice were immunized with PBS without any adjuvant. At day 42, mice were euthanized, and sera were collected for antibody response analysis. Serum samples were tested for neutralizing antibodies using the SARS-CoV-2 sVNT assay

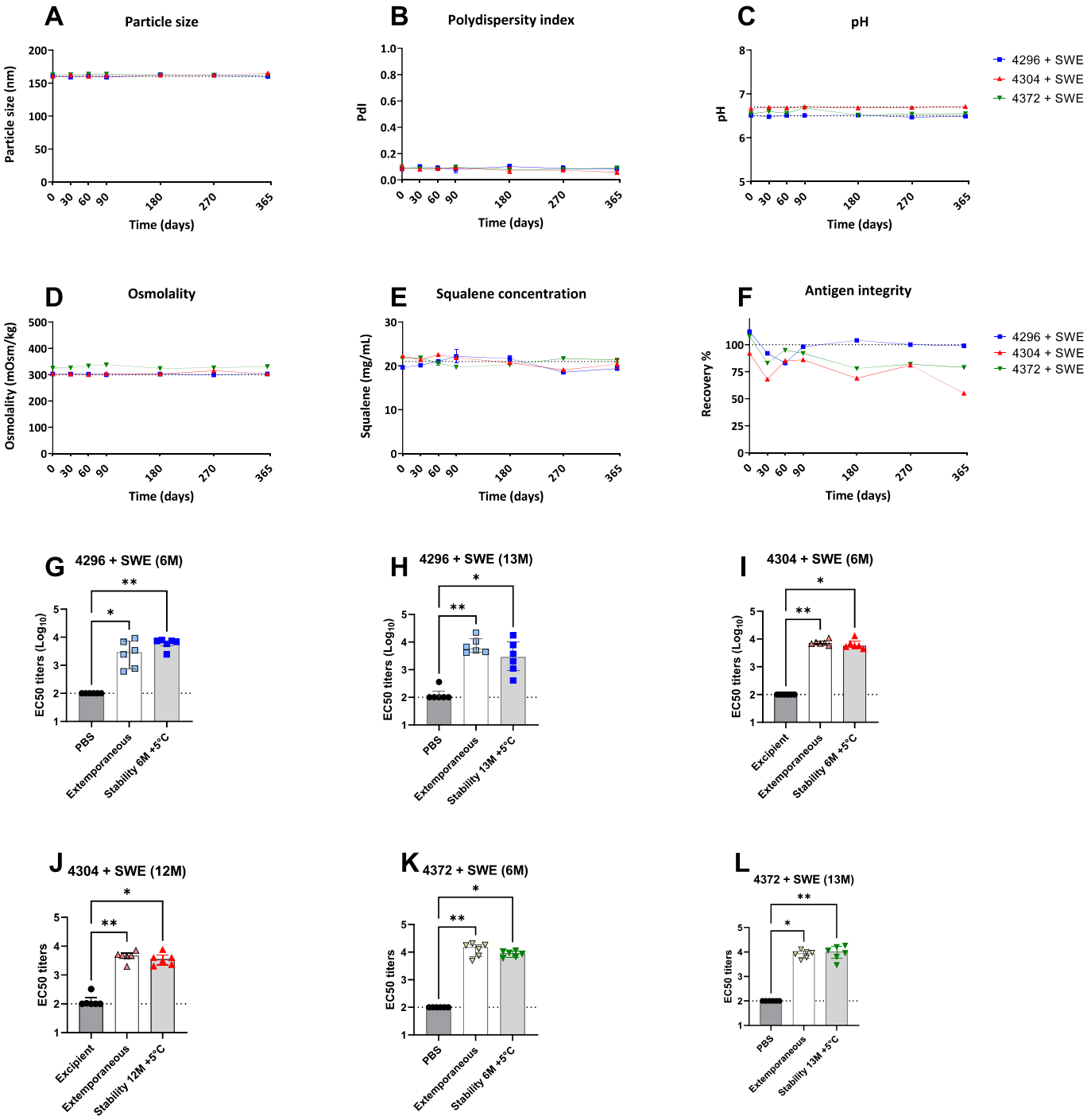


Fig. 5. Demonstration of long-term stability and immunogenicity of formulated vaccine. VIDO4296, VIDO4304, and VIDO4372 chimeric proteins were formulated with Sepivac SWE™ in a 1:1 ratio and formulations were made isotonic by the addition of sodium chloride. All quality control assays were performed in triplicate at specified time points for stability evaluation. Formulations were characterized by assessing **(A)** particle size, **(B)** polydispersity index, **(C)** pH, **(D)** osmolality **(E)** squalene concentration, and **(F)** antigen integrity. Mice were immunized intramuscularly at day 0 and day 28 with freshly prepared (extemporaneous) formulations of either VIDO4296, VIDO4304, or VIDO4372 chimeric antigens adjuvanted with Sepivac SWE™, or preformulated formulations stored at 2–8 °C as part of stability studies for 6–13 months (M). The control group of mice was immunized with PBS without any antigen or adjuvant. At day 42, mice were euthanized, and sera were collected. S1-specific serum IgG titers were assessed in animals vaccinated with VIDO4296 **(G and H)**, VIDO4304 **(I and J)**, or VIDO4372 **(K and L)**. Statistical analysis was performed using the non-parametric Kruskal-Wallis test. Bars or lines indicate median or median values with interquartile ranges. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

against different variants. VIDO4296 immunized animals had high neutralizing antibodies against SARS-CoV-2 Wuhan, Delta, and Beta VOCs when vaccinated with a formulated vaccine candidate that was stored at 2–8 °C for 6 months post formulation (Fig. 6A-D). The immunogenic response of VIDO4296 + SWE formulation was slightly lower, but not statistically different after 13 months of storage at 2–8 °C compared to a freshly prepared formulation (Fig. 6E-H). VIDO4304 vaccinated animals had high neutralizing antibodies against SARS-CoV-2 Wuhan, Delta, and Beta VOCs when vaccinated with a formulated vaccine that was stored at 2–8 °C for 6- or 12-months post formulation

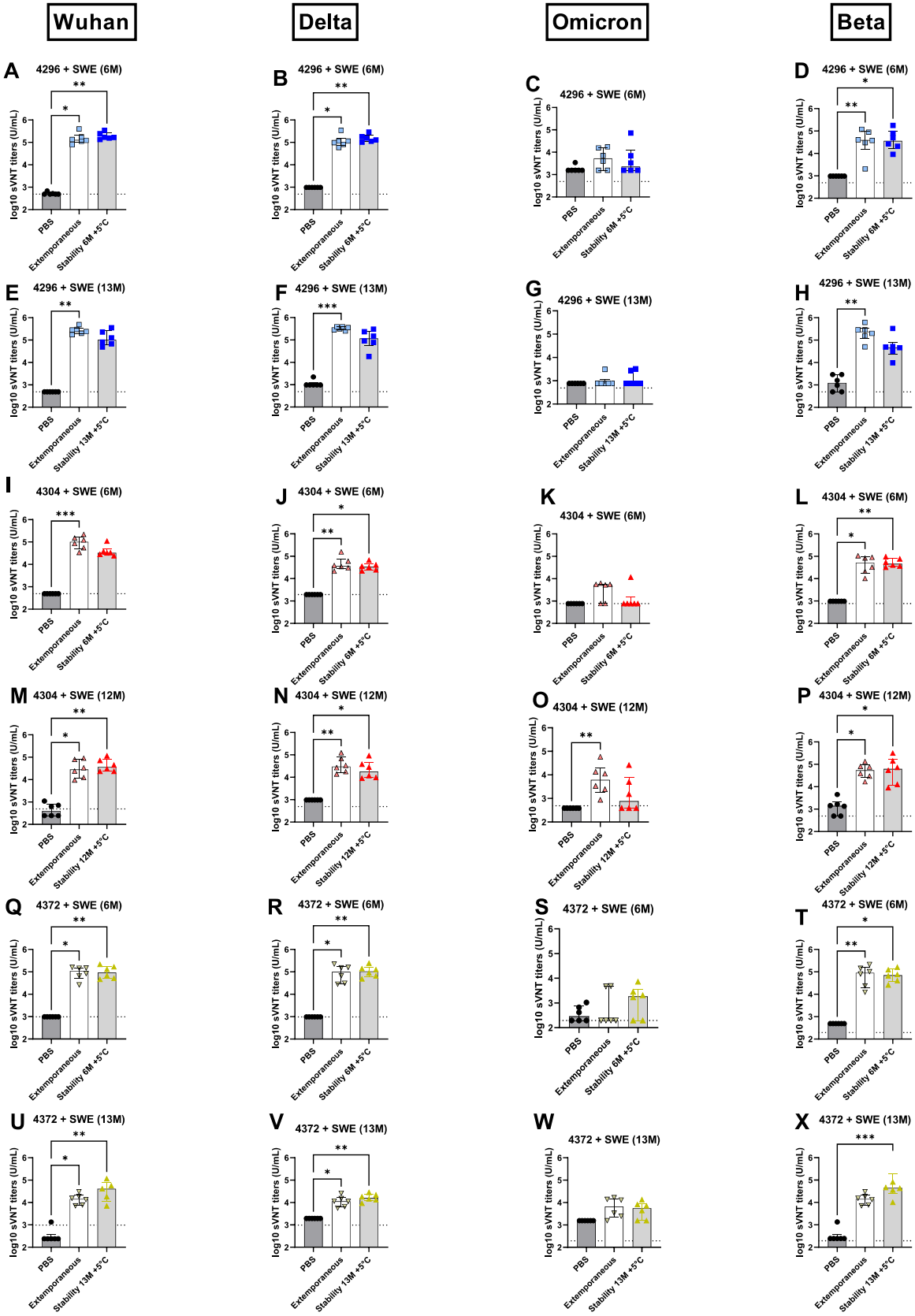


Fig. 6. Formulated vaccine is immunogenic for up to one year. Mice were immunized intramuscularly at day 0 and day 28 with extemporaneous formulations of either VIDO4296, VIDO4304, or VIDO4372 chimeric antigens adjuvanted with Sepivac SWE™, or preformulated formulations stored at 2–8 °C as part of stability studies for 6–13 months. The control group of mice was immunized with PBS without any adjuvant. At day 42, mice were euthanized, and sera were collected. Surrogate virus neutralizing titres (sVNT) of VIDO4296 (**A-H**), VIDO4304 (**I-P**), and VIDO4372 (**Q-X**) against SARS-CoV-2 Wuhan, Delta, Omicron, and Beta VOCs are shown. Bars or lines indicate median or median values with interquartile ranges. Statistical analysis was performed using the non-parametric Kruskal-Wallis test.

(Fig. 6I-P). VIDO4372 immunized animals had high levels of neutralizing antibodies against SARS-CoV-2 Wuhan, Delta, and Beta VOCs when vaccinated with a formulated vaccine that was stored at 2–8 °C for 6- or 13-months post formulation (Fig. 6Q-X).

4. Discussion

As SARS-CoV-2 continues to evolve, we and others have shown that the virus can escape humoral immunity that is generated upon both vaccination with first-generation vaccines and natural infection with a former SARS-CoV-2 variant [12,46,47]. In addition, mRNA vaccines can be expensive and require ultra-low temperatures for long-term stability, which makes their utility challenging in low-resource settings [48]. In contrast, inexpensive adjuvanted protein-based vaccines have a lot to offer to reduce the ongoing spread of SARS-CoV-2 and to prevent future coronavirus outbreaks and pandemics.

To develop a flexible subunit vaccine platform against multiple SARS-CoV-2 variants, we developed the "train model" subunit protein platform. By retaining the train engine and swapping out train cars, we demonstrate that our vaccine platform is versatile and adaptable, enabling broad protection against SARS-CoV-2 VOCs. The two-dose regimen of our vaccine candidates induced a robust antibody response, with both binding and neutralizing antibodies against most VOCs that we screened. Binding antibody levels remained elevated at 48 days post the first dose (20 days post second dose) and neutralizing antibody levels remained elevated at 10 days post challenge with SARS-CoV-2 VOCs. No adverse or severe events were noted in the vaccinated animals during the study.

Controlling virus replication and infectious virus shedding using vaccination has been challenging for SARS-CoV-2, although levels and duration of virus shedding and transmission can be reduced by vaccination [49,50]. In our study, all vaccine candidates reduced infectious virus load in nasal washes of SARS-CoV-2 VOC-challenged animals, demonstrating the potential of our broadly protective vaccines in reducing virus transmission. Importantly, viral titers in the respiratory tract of SARS-CoV-2 VOC-infected animals were significantly reduced with no detectable virus in most vaccinated animals at day 5 post-infection. Indeed, our vaccine candidates induced robust binding and neutralizing antibodies against both homologous and heterologous SARS-CoV-2 VOC challenge, suggesting a wide application potential of our vaccine platform to develop broadly protective vaccines against SARS-CoV-2 VOCs.

The presence of the "train engine", the Wuhan S1 antigen, in all our constructs enables a consistent upstream and downstream manufacturing process. Indeed, the vaccine antigen can be easily modified by switching out the "train cars" depending on the circulating virus without the need to modify the entire manufacturing process. This will increase the speed at which new vaccines are developed and brought into the clinic.

We also assessed the stability of our multivalent subunit vaccine candidates. All vaccine candidates were formulated and stored at 2–8 °C between 6–13 months. No change was observed in adjuvant particle size, polydispersity index, osmolality, pH, and squalene concentration in the formulated vaccines during the stability study. Furthermore, despite variabilities in antigen integrity, the formulated vaccines remained immunogenic during the storage period. Thus, our broadly protective subunit vaccines can be deployed at least 6–13 months post formulation, which will allow us to manufacture and stockpile these vaccines to potentially control the emergence of other immune evasive SARS-CoV-2 VOCs in the future. The ease with which subunit vaccines can be scaled up for GMP production, followed by formulation using accessible adjuvants promises to keep the cost of these vaccines low. Furthermore, compared to mRNA vaccines, our data suggest that our subunit vaccines do not require ultralow temperature freezers for storage, which should further reduce the cost of storing and distributing subunit vaccines. Indeed, temperature-stable and inexpensive subunit vaccines can be shipped globally to ensure better vaccine accessibility.

An added benefit to the "train model", beyond low cost of manufacture and storage, is the flexibility with which the "train cars" can be replaced should a new SARS-CoV-2 VOC arise, while maintaining a consistent antigen purification process that is based on the S1 Wuhan "train engine". The "platform" nature of our antigen design should enable rapid response to future VOCs and potentially other betacoronaviruses.

Hamsters are currently the most widely accepted small animal model for SARS-CoV-2. While vaccinated hamsters showed better clinical outcomes compared to unvaccinated animals upon SARS-CoV-2 infection in our study, we did not assess cell-mediated immunity in these animals. As we develop reagents for hamsters that are currently lacking, we will be able to perform in-depth assessments of all immune correlates of protection. Protective levels of neutralizing antibodies in vaccinated animals in our study, along with improving clinical signs demonstrate the translational potential of our vaccine candidates as broadly protective pan-variant SARS-CoV-2 vaccines. In the future, we will utilize this platform to develop broadly protective vaccines against betacoronaviruses and other zoonotic pathogens.

CRediT authorship contribution statement

Ravendra Garg: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Qiang Liu:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Jill Van Kessel:** Investigation. **Akarin Asavajaru:** Methodology, Investigation. **Eva-Maria Uhlemann:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Morgane Joessel:** Writing – review & editing, Investigation, Data curation. **Glenn Hamonic:** Resources, Project administration, Data curation. **Zahed Khatooni:** Writing – review & editing, Software, Investigation, Data curation. **Andrea Kroeker:** Investigation. **Jocelyne Lew:** Writing – review & editing, Investigation. **Erin Scruten:** Investigation. **Paul Pennington:** Investigation. **William Deck:** Investigation. **Tracy Prysliak:** Investigation. **Michaela Nickol:** Investigation. **Falko Apel:** Writing – review & editing, Investigation. **Thomas Courant:** Writing – review & editing, Investigation. **Alyson A. Kelvin:** Resources. **Andrew Van Kessel:** Resources. **Nicolas Collin:** Writing – review & editing, Supervision, Resources. **Volker Gerdts:** Supervision, Resources. **Wolfgang Köster:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **Darryl Falzarano:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Trina Racine:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition. **Arinjay Banerjee:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Arinjay Banerjee reports a relationship with Canadian Institutes of Health Research that includes: funding grants. Trina Racine reports a relationship with Coalition for Epidemic Preparedness Innovations that includes: funding grants. Darryl Falzarano reports a relationship with Canadian Institutes of Health Research that includes: funding grants. Arinjay Banerjee, Qiang Liu, Darryl Falzarano has patent fusion polypeptides, immunogenic compositions, methods and uses thereof pending to VIDO, University of Saskatchewan. The findings from this study have led to a patent application: fusion polypeptides, immunogenic compositions, methods and uses thereof. USPTO Patent application, United States of America. USPTO Patent application #63/452,586 (March 16, 2023). The authors declare no other conflict of interest.

Morgane Joessel, Falko Apel, Thomas Courant, and Nicolas Collin

from VFI used their trademarked Sepivac SWE™, a squalene in water emulsion (SWE) adjuvant in this study to formulate the vaccine candidates. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

Data availability

Data will be made available on request.

Acknowledgements

This study was funded by a grant from the Coalition for Epidemic Preparedness Innovations (CEPI). D.F. acknowledges funding from the Canadian Institutes of Health Research (PJT – 153319; VS1-175531). A. B. acknowledges funding from the Canadian Institutes of Health Research (CIHR)-Centre for Research on Pandemic Preparedness and Health Emergencies, Early Career Investigator Grant (FRN: PEE-183995). The authors would also like to thank the Vaccine Formulation Institute (VFI) for their collaboration and VIDO's veterinary staff for their tremendous efforts. VIDO receives operational funding from the Canada Foundation for Innovation through the Major Science Initiatives, from the Government of Saskatchewan through Innovation Saskatchewan, and the Ministry of Agriculture. Some figures were generated using BioRender.com.

Attestation

All authors attest they meet the ICMJE criteria for authorship.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.05.028.

References

[1] Wu C, Liu Y, Yang Y, Zhang P, Zhong W, Wang Y, et al. Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. Acta Pharm Sin B 2020;10:766–88.
[2] Yang J, Wang W, Chen Z, Lu S, Yang F, Bi Z, et al. A vaccine targeting the RBD of the S protein of SARS-CoV-2 induces protective immunity. Nature 2020;586:572–7.
[3] Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med 2021;384:403–16.
[4] Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. N Engl J Med 2020;383:2603–15.
[5] Jackson LA, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN, et al. An mRNA vaccine against SARS-CoV-2 - preliminary report. N Engl J Med 2020;383:1920–31.
[6] Falsey AR, Sobieszczyk ME, Hirsch I, Sproule S, Robb ML, Corey L, et al. Phase 3 safety and efficacy of AZD1222 (ChAdOx1 nCoV-19) Covid-19 vaccine. N Engl J Med 2021;385:2348–60.
[7] Watson OJ, Barnsley G, Toor J, Hogan AB, Winskill P, Ghani AC. Global impact of the first year of COVID-19 vaccination: a mathematical modelling study. Lancet Infect Dis 2022;22:1293–302.
[8] Sun C, Xie C, Bu GL, Zhong LY, Zeng MS. Molecular characteristics, immune evasion, and impact of SARS-CoV-2 variants. Signal Transduct Target Ther 2022;7:202.
[9] Singh J, Pandit P, McArthur AG, Banerjee A, Mossman K. Evolutionary trajectory of SARS-CoV-2 and emerging variants. Virol J 2021;18:166.
[10] Keegan LT, Truelove S, Lessler J. Analysis of vaccine effectiveness against COVID-19 and the emergence of delta and other variants of concern in Utah. J Am Med AssocNetw Open 2021;4:e2140906.
[11] Nasreen S, Chung H, He S, Brown KA, Gubbay JB, Buchan SA, et al. Effectiveness of COVID-19 vaccines against symptomatic SARS-CoV-2 infection and severe outcomes with variants of concern in Ontario. Nat Microbiol 2022;7:379–85.
[12] Banerjee A, Lew J, Kroeker A, Baid K, Aftanas P, Nirmalarajah K, et al. Immunogenicity of convalescent and vaccinated sera against clinical isolates of ancestral SARS-CoV-2, beta, delta, and omicron variants. Med (N Y) 2022;3(422–32):e3.
[13] WHO. COVID-19 vaccine tracker and landscape. In: WHO, editor. COVID-19 vaccine tracker and landscape: WHO; 2022.
[14] Davis-Gardner ME, Lai L, Wali B, Samaha H, Solis D, Lee M, et al. Neutralization against BA.2.75.2, BQ.1.1, and XBB from mRNA Bivalent booster. N Engl J Med 2022.
[15] Xiong H, Sun H, Wang S, Yuan L, Liu L, Zhu Y, et al. The neutralizing breadth of antibodies targeting diverse conserved epitopes between SARS-CoV and SARS-CoV-2. Proc Natl Acad Sci U S A 2022;119:e2204256119.
[16] Yuan M, Zhu X, He WT, Zhou P, Kaku CI, Capozzola T, et al. A broad and potent neutralization epitope in SARS-related coronaviruses. Proc Natl Acad Sci U S A 2022;119:e2205784119.
[17] Starr TN, Czudnochowski N, Liu Z, Zatta F, Park YJ, Addetia A, et al. SARS-CoV-2 RBD antibodies that maximize breadth and resistance to escape. Nature 2021;597:97–102.
[18] Morens DM, Taubenberger JK, Fauci AS. Universal coronavirus vaccines - an urgent need. N Engl J Med 2022;386:297–9.
[19] Wang Z, An J, Liu K, Yu P, Fang X, Li J, et al. A potent, broadly protective vaccine against SARS-CoV-2 variants of concern. npj Vaccines 2022;7:144.
[20] Halfmann PJ, Frey SJ, Loeffler K, Kuroda M, Maemura T, Armbrust T, et al. Multivalent S2-based vaccines provide broad protection against SARS-CoV-2 variants of concern and pangolin coronaviruses. EBioMedicine 2022;86:104341.
[21] Cohen AA, van Doremalen N, Greaney AJ, Andersen H, Sharma A, Starr TN, et al. Mosaic RBD nanoparticles protect against challenge by diverse sarbecoviruses in animal models. Science 2022;377:eabq0839.
[22] Lassaunière R, Polacek C, Frische A, Boding L, Sækmose SG, Rasmussen M, et al. Neutralizing antibodies against the SARS-CoV-2 omicron variant (BA.1) 1 to 18 weeks after the second and third doses of the BNT162b2 mRNA vaccine. J Am Med AssocNetw Open 2022;5:e2212073-e.
[23] Banerjee A, Lew J, Kroeker A, Baid K, Aftanas P, Nirmalarajah K, et al. Immunogenicity of convalescent and vaccinated sera against clinical isolates of ancestral SARS-CoV-2, beta, delta, and omicron variants. Med (N Y) 2022;3(42232.e3).
[24] Link-Gelles R, Levy ME, Natarajan K, Reese SE, Naleway AL, Grannis SJ, et al. Estimation of COVID-19 mRNA vaccine effectiveness and COVID-19 illness and severity by vaccination status during omicron BA.4 and BA.5 sublineage periods. J Am Med AssocNetw Open 2023;6:e232598-e.
[25] Ventura R, Brunner L, Heriyanto B, de Boer O, O'Hara M, Huynh C, et al. Technology transfer of an oil-in-water vaccine-adjuvant for strengthening pandemic influenza preparedness in Indonesia. Vaccine 2013;31:1641–5.
[26] O'Hagan DT, van der Most R, Lodaya RN, Coccia M, Lofano G. "World in motion" – emulsion adjuvants rising to meet the pandemic challenges. npj Vaccines 2021;6:158.
[27] Westdijk J, Koedam P, Barro M, Steil BP, Collin N, Vedvick TS, et al. Antigen sparing with adjuvanted inactivated polio vaccine based on Sabin strains. Vaccine 2013;31:1298–304.
[28] de Jonge J, van Dijken H, de Heij F, Spijkers S, Mouthaan J, de Jong R, et al. H7N9 influenza split vaccine with SWE oil-in-water adjuvant greatly enhances cross-reactive humoral immunity and protection against severe pneumonia in ferrets. NPJ Vaccines 2020;5:38.
[29] Xu S, Duan H, An Y, Jin X, Duan M, Dubois PM, et al. Effect of adjuvanting RBD-dimer-based subunit COVID-19 vaccines with Sepivac SWE™. Vaccine 2023;41:2793–803.
[30] Khaleeq S, Sengupta N, Kumar S, Patel UR, Rajmani RS, Reddy P, et al. Neutralizing efficacy of encapsulin nanoparticles against SARS-CoV2 variants of concern. Viruses 2023;15:346.
[31] Wong TY, Russ BP, Lee KS, Miller OA, Kang J, Cooper M, et al. RBD-VLP vaccines adjuvanted with alum or SWE protect K18-hACE2 mice against SARS-CoV-2 VOC challenge. mSphere 2022;7:e0024322.
[32] Francis ME, Goncin U, Kroeker A, Swan C, Ralph R, Lu Y, et al. SARS-CoV-2 infection in the Syrian hamster model causes inflammation as well as type I interferon dysregulation in both respiratory and non-respiratory tissues including the heart and kidney. PLoS Pathog 2021;17:e1009705.
[33] Tunyasuvunakool K, Adler J, Wu Z, Green T, Zielinski M, Zidek A, et al. Highly accurate protein structure prediction for the human proteome. Nature 2021;596:590–6.
[34] Meng EC, Goddard TD, Pettersen EF, Couch GS, Pearson ZJ, Morris JH, et al. UCSF ChimeraX: tools for structure building and analysis. ProteinSci 2023;32:e4792.
[35] Xu D, Zhang Y. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. Biophys J 2011;101:2525–34.
[36] Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 2018;46:W296–303.
[37] Guex N, Peitsch MC, Schwede T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis 2009;30(Suppl 1) (S162-73).
[38] Garg R, Latimer L, Gerdts V, Potter A, van Drunen Littel-van den Hurk S. Vaccination with the RSV fusion protein formulated with a combination adjuvant induces long-lasting protective immunity. J Gen Virol. 2014;95:1043-54.
[39] Spearman C. The method of right and wrong cases (Constant Stimuli) without Gauss's formulae. Br J Psychol 1908;2:227.
[40] Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature 2020;581:215–20.
[41] Ma J, Su D, Sun Y, Huang X, Liang Y, Fang L, et al. Cryo-EM structure of S-Trimer, a subunit vaccine candidate for COVID-19. J Virol 2021;95.
[42] Ma J, Su D, Sun Y, Huang X, Liang Y, Fang L, et al. Cryo-EM structure of S-Trimer, a subunit vaccine candidate for COVID-19. LID - .00194-21 [pii] LID - 10.1128/JVI.00194-21 [doi] LID - e00194-21. J Virol. 2021.

[43] Reincke SM, Yuan M, Kornau HC, Corman VM, van Hoof S, Sánchez-Sendin E, et al. SARS-CoV-2 Beta variant infection elicits potent lineage-specific and cross-reactive antibodies. Science 2022;375:782–7.
[44] Croset A, Delafosse L, Gaudry JP, Arod C, Glez L, Losberger C, et al. Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells. J Biotechnol 2012;161:336–48.
[45] Yuan S, Ye Z-W, Liang R, Tang K, Zhang AJ, Lu G, et al. Pathogenicity, transmissibility, and fitness of SARS-CoV-2 Omicron in Syrian hamsters. Science 2022;377:428–33.
[46] Willett BJ, Grove J, MacLean OA, Wilkie C, De Lorenzo G, Furnon W, et al. SARS-CoV-2 Omicron is an immune escape variant with an altered cell entry pathway. Nat Microbiol 2022;7:1161–79.
[47] Uraki R, Ito M, Furusawa Y, Yamayoshi S, Iwatsuki-Horimoto K, Adachi E, et al. Humoral immune evasion of the omicron subvariants BQ.1.1 and XBB. Lancet Infect Dis 2023;23:30–2.
[48] Uddin MN, Roni MA. Challenges of storage and stability of mRNA-based COVID-19 vaccines. Vaccines (Basel) 2021:9.
[49] Garcia-Knight M, Anglin K, Tassetto M, Lu S, Zhang A, Goldberg SA, et al. Infectious viral shedding of SARS-CoV-2 Delta following vaccination: a longitudinal cohort study. PLoS Pathog 2022;18:e1010802.
[50] Jung J, Kim JY, Park H, Park S, Lim JS, Lim SY, et al. Transmission and infectious SARS-CoV-2 shedding kinetics in vaccinated and unvaccinated individuals. J Am Med AssocNetw Open 2022;5:e2213606.