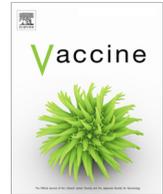




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Covax-19/Spikogen[®] vaccine based on recombinant spike protein extracellular domain with Advax-CpG55.2 adjuvant provides single dose protection against SARS-CoV-2 infection in hamsters



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ABSTRACT

COVID-19 presents an ongoing global health crisis. Protein-based COVID-19 vaccines that are well-tolerated, safe, highly-protective and convenient to manufacture remain of major interest. We therefore sought to compare the immunogenicity and protective efficacy of a number of recombinant SARS-CoV-2 spike protein candidates expressed in insect cells. By comparison to a full length (FL) spike protein detergent-extracted nanoparticle antigen, the soluble secreted spike protein extracellular domain (ECD) generated higher protein yields per liter of culture and when formulated with either Alum-CpG55.2 or Advax-CpG55.2 combination adjuvants elicited robust antigen-specific humoral and cellular immunity in mice. In hamsters, the spike ECD when formulated with either adjuvant induced high serum neutralizing antibody titers even after a single dose. When challenged with the homologous SARS-CoV-2 virus, hamsters immunized with the adjuvanted spike ECD exhibited reduced viral load in day 1–3 oropharyngeal swabs and day 3 nasal turbinate tissue and had no recoverable infectious virus in day 3 lung tissue. The reduction in lung viral load correlated with less weight loss and lower lung pathology scores. The formulations of spike ECD with Alum-CpG55.2 or Advax-CpG55.2 were protective even after just a single dose, although the 2-dose regimen performed better overall and required only half the total amount of antigen. Pre-challenge serum neutralizing antibody levels showed a strong correlation with lung protection, with a weaker correlation seen with nasal or oropharyngeal protection. This suggests that serum neutralizing antibody levels may correlate more closely with systemic, rather than mucosal, protection. The spike protein ECD with Advax-CpG55.2 formulation (Covax-19[®] vaccine) was selected for human clinical development.

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1. Introduction

Almost two years from the initial outbreak, the COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS CoV 2) remains a global public health crisis. As at 1 April

Abbreviations: CBA, cytokine bead array; ECD, extracellular domain; FBS, foetal Bovine Serum; FCS, furin cleavage site; FL, full length; HRP, horse radish peroxidase; IC50, inhibitory concentration 50%; MEM, minimum Essential Media; PFU, plaque forming unit; rSp, recombinant spike protein; RT, room temperature; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLR9, toll-like receptor-9; Tni, tricholusia ni.

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2022, SARS-CoV-2 had infected over 470 million people globally, with many countries now entering a fourth or even fifth disease wave [1]. Vaccines are a key tool to control the spread and impact of SARS-CoV-2, with various candidates having received emergency use authorization [2]. However, these COVID-19 vaccines are not without technical limitations with new technologies, such as mRNA-based platforms, being highly temperature sensitive [3] with strict cold storage requirements [4]. Developing regions, e.g. Africa, continue to have limited access to COVID-19 vaccines [5,6] and lack the required extreme cold storage infrastructure required for mRNA vaccines. In addition, the adenovirus vector and mRNA vaccines have been associated with adverse reactions including anaphylaxis [7], central venous thrombosis [8] and

myocarditis [9,10]. This highlights the need for a diversity of vaccine platforms to tackle this still-evolving global crisis.

Recombinant protein subunit vaccines have been highly successful against many viral and non-viral diseases [11]. A potential limitation of protein-based vaccines is their low immunogenicity, necessitating an adjuvant to enhance their immunogenicity [12]. Traditional aluminum hydroxide (Alum) and oil emulsion adjuvants remain prominent in COVID-19 vaccines in development [13]. Advax-CpG55.2 is a proprietary adjuvant formulation which combines Advax, a non-reactogenic adjuvant based on delta inulin, with a human toll-like receptor 9 (TLR-9) agonist oligonucleotide (CpG55.2) that was developed using artificial intelligence [14,15]. Advax-CpG55.2 adjuvant has been tested in many preclinical studies [16–19] and also in human clinical trials, including in vaccines against Influenza [NCT03945825; NCT03038776] and Hepatitis B [NCT01951677], where it enhanced vaccine immunogenicity while maintaining a positive safety profile. Advax-CpG adjuvant was previously shown to enhance coronavirus vaccine protection in models of severe acute respiratory syndrome (SARS) [20] and Middle East respiratory syndrome (MERS) [21].

Previously, we reported on a vaccine based on SARS-CoV-2 spike protein ECD formulated with Advax-CpG55.2 adjuvant which provided protection in ferrets [22]. In the current study, we describe the results from our screening of a range of vaccine formulations, comparing; 1. ECD versus full length (FL) spike protein both manufactured in insect cells, 2. Advax-CpG55.2 versus alum-CpG55.2 adjuvant, and 3. a single versus 2-dose regimen. These vaccine formulations were first evaluated in mice for immunogenicity, with protection then confirmed in hamsters. The hamster model has a number of advantages as SARS-CoV-2 replicates more efficiently in the lungs of hamsters and they display clinical signs of disease, i.e. weight loss and lung pathology, that can be quantified to assess vaccine efficacy and potency [23,24].

2. Methods

2.1. Vaccines and adjuvants

The FL vaccine antigen corresponded to the full-length of the SARS-CoV-2 spike of the original Wuhan-Hu-1 strain (accession number: NC 045512), whilst the ECD construct corresponded to aa 14–1213 of the Wuhan-Hu-1 spike protein sequence with the furin cleavage site deleted (see Fig. 1A). The constructs were produced using a Bac-to-Bac baculovirus expression system as previously described [22]. The size and purity of the recombinant FL and ECD spike proteins were confirmed by SDS-PAGE gels. Endotoxin was measured using a PyroGene Endotoxin Detection System (Cat. No. 50-658U, LONZA, Walkersville, MD, USA) and residual DNA content in final vaccine product was measured using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, P7589) following the manufacturer's instructions. Advax, Advax-CpG55.2, and Alum-CpG55.2 adjuvants were from Vaxine Pty Ltd (Adelaide, Australia).

2.2. Mouse immunisation protocol

The murine studies were carried out at Flinders University, Australia as approved by the Animal Welfare Committee of Flinders University and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013). Female, BALB/c and C57BL/6 (BL6) mice (6–10 weeks old) were supplied by the central animal facility of Flinders University. Mice were immunised intramuscularly (i.m.) in the thigh muscle at weeks 0 and 2 with 0.5, 1, 2.5, 5 or 10 µg recombinant spike protein (rSp) ECD or FL formulated with either Advax-CpG55.2

(1 mg/10 µg) or Alum-CpG55.2 (50 µg/10 µg) comprising Alhydrogel (Croda, Denmark) formulated with CpG55.2. Blood samples were collected by cheek vein bleeding 2 weeks after each immunisation. Serum was separated by centrifugation and stored at –20°C prior to use. Animals were sacrificed at week 4, and spleens were collected and used immediately for cell-based assays (CBA and ELISPOT).

2.3. Antigen-specific ELISA for murine studies

Spike-specific antibodies were determined by ELISA. Briefly, 1 µg/ml rSp [corresponding to SARS-CoV-2 (Wuhan) reference sequence Q13 to P1209] or 0.5 µg/ml spike receptor-binding domain (RBD) in PBS were used to coat 96-well ELISA plates (100 µL/well). After blocking, 100 µL of diluted serum samples were added followed by biotinylated anti-mouse IgG (Sigma-Aldrich) with horseradish peroxidase (HRP)-conjugated Streptavidin (BD Biosciences) for 1 h. After washing, 100 µL of TMB substrate (KPL, SeraCare, Gaithersburg, MD, USA) was added and incubated for 10 min before the reaction was stopped with 100 µL 1 M phosphoric acid (Sigma-Aldrich). The optical density was measured at 450 nm (OD₄₅₀ nm) using a VersaMax plate reader and analysed using SoftMax Pro Software. Average OD₄₅₀ nm values obtained from negative control wells were subtracted.

2.4. SARS-CoV-2 spike pseudotyped neutralisation assay for murine sera

A replication-deficient SARS-CoV-2 Spike pseudotyped lentivirus-based neutralisation assay was developed to measure neutralising activity of murine immune sera. Single-cell sorted HEK-293T stable cell line expressing human ACE2 on the plasma membrane was maintained in DMEM-10 medium [25]. Expression cassette of human-codon optimized SARS-CoV-2 spike with C-terminal 18aa truncation was cloned into pCAGGS vector. Spike-pseudotyped lentiviral particles were produced by co-transfecting HEK-293T cells with firefly luciferase encoding 3rd generation lentiviral vector pCDH-EF1-Luc-IRES-Puro, packaging plasmid psPAX2 and spike expressing plasmid pCAGGS-Spike using Lipofectamine 2000 according to the product manual and recombinant virus particles were harvested at 72 h post transfection. Neutralisation activity of immune sera was measured with a single round transduction of 293T-hACE2 cells with Spike-pseudotyped lentiviral particles. Briefly, prior to infecting cells, immune sera was serially diluted and incubated with pseudotyped virus particle for 1 h at 37 °C. Then 100 µL of virus-serum was added into 50 µL of 293 T-hACE2 cells freshly plated at 125,000 cells per well in a 96-well white tissue culture plate. The cells were then cultured at 37 °C for 72 h, followed by removing the culture medium and removing 30 µL of phenol red-free DMEM medium. Then 30 µL of ONE-Glo EX (Promega) reagent was added into each well and incubated at RT with shaking at 400 rpm on a thermoblock before luciferase activity reading on BMG FluoStar plate reader. Neutralization was calculated by reduction in % Luciferase units relative to pseudotyped virus alone group without any serum treatment. Neutralization antibody titers were then calculated using Sigmoidal 4PL robust fit regression method in GraphPad Prism Ver. 9.

2.5. Murine T-cell response

BALB/c mice were sacrificed 2 weeks after the last immunization, and individual spleens were collected aseptically. Single-cell suspensions in sterile 3% FBS in PBS were prepared using a 70 µm easy strainer (Greiner Bio-One) with a 5 mL syringe plunger. Isolated spleen cells were pelleted and incubated in red blood cell (RBC) lysis buffer for 10 min. For Cytometric Bead Array (CBA)

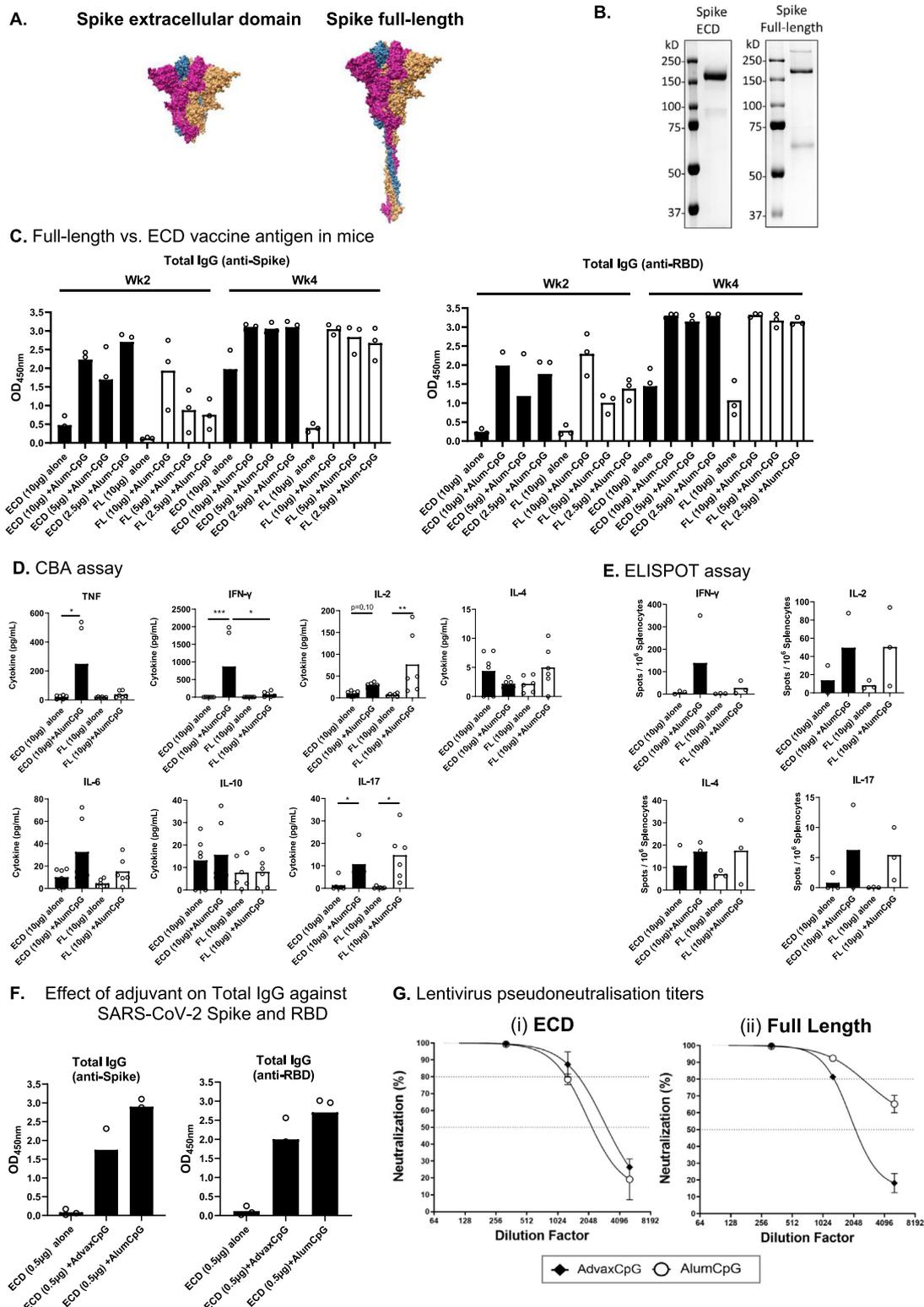


Fig. 1. Spike protein vaccine immunogenicity in mice. (A) 3D models of SARS-CoV-2 extracellular domain (ECD) and full length (FL) spike protein. (B) Representative SDS-PAGE gels showing purified ECD and FL spike proteins. BALB/c mice were immunised i.m. twice at 2-week intervals with 0.5–10 µg rSp ECD or FL with alum-CpG55.2 or Advax-CpG55.2 adjuvant. (C) ELISA results for rSp- and RBD-binding IgG (mean + SD). (D) CBA cytokine levels and (E) ELISPOT results for rSp-stimulated splenocytes. (F) ELISA results for serum rSp- and RBD-binding IgG (O.D. mean + SD) in BL6 mice immunized with 0.5 µg ECD alone or with Advax-CpG or alum-CpG adjuvant 2 weeks after second immunization. (G) SARS-CoV-2 spike pseudoneutralisation titers for sera from BL6 mice immunized twice with 1 µg ECD or FL vaccine antigen with Advax-CpG or alum-CpG adjuvant. Statistical analysis was performed using Kruskal-Wallis test with Dunn correction for multiple comparisons between groups (*; $p < 0.05$, **; $p < 0.01$, *** and $p < 0.001$).

assay, splenocytes were cultured at 5×10^5 cells/well in 96-well plates with 3 µg/ml of rSp antigen at 37 °C and 5% CO₂. Two days later, the supernatants were harvested and cytokine concentrations determined by mouse Th1/Th2/Th17 CBA kit (BD) and analysed by FCAP array Software (BD). In addition to CBA assay, enzyme-linked immune absorbent spot (ELISPOT) assay was performed using mouse Interleukin-2 (IL-2), Interleukin-4 (IL-4) or Interferon gamma (IFN-γ) ELISPOT set (BD PharMingen) or Interleukin-17 (IL-17) antibodies (BioLegend) according to the manufacturer's instruction. Briefly, single-cell suspensions were prepared from spleens of mice and plated in Millipore MultiScreen-HA 96-well filter plates (Millipore) pre-coated with anti-mouse IL-2, IL-4, IL-17 or IFN-γ antibodies overnight at 4 °C and blocked by RPMI-1640 containing 10% FBS. Cells were incubated for 48 h in the presence or absence of rSp protein at 37 °C and 5% CO₂. Wells were washed and incubated with biotinylated labelled anti-mouse IL-2, IL-4, IL-17 or IFN-γ antibody at room temperature (RT). After washing, wells were incubated with HRP-conjugated Streptavidin (BD Biosciences) for 1 h at RT. Wells were extensively washed again and developed with 3-amino-9-ethyl-carbazole (AEC) substrate set (BD Biosciences). After drying, spots were counted on an ImmunoSpot ELISPOT reader (CTL ImmunoSpot Reader, software version 5.1.36).

2.6. Hamster immunization protocol

All hamsters were held at Colorado State University in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited animal facilities. Animal testing and research received ethical approval by the Institutional Animal Care and Use Committee (IACUC) (animal protocol #1559). Golden Syrian hamsters (*Mesocricetus auratus*) at 6 weeks of age were acquired from Charles River Laboratories (Wilmington, MA). Hamsters were maintained in a Biosafety Level-2 (BSL-2) animal facility at the Regional Biocontainment Lab at Colorado State University during the vaccination period. The hamsters were group-housed and fed a commercial diet with access to water *ad libitum*. Each hamster was ear notched for animal identification. Hamsters (n = 4/group) were vaccinated at day 0 and boosted at day 21 with SARS-CoV-2 extracellular domain (ECD) or FL spike protein at either 2.5 or 10 µg formulated with either Advax-CpG55.2 (2–0.02 mg), Advax (2 mg), Alum-CpG (0.2–0.02 mg) adjuvant or alone. Two groups received a single dose of 10 µg of ECD or FL spike protein formulated with Advax-CpG55.2. Control groups either received saline (n = 8) or Advax-CpG55.2 adjuvant alone (n = 4) injections. At day 35, sera were collected to determine the antibody response prior to virus challenge.

2.7. Hamster challenge model

All hamsters were transferred to a Biosafety Level-3 animal facility at the Regional Biocontainment Lab at Colorado State University prior to live virus challenge. At day 35 following prime immunization, animals were challenged with $1e^4$ PFU of SARS-CoV-2 (isolate USAWA1/2020) acquired originally through BEI Resources (product NR-52281) and passaged twice in Vero E6 cells as previously described [26]. In brief, the hamsters were first lightly anesthetized with a 10:1 mixture of ketamine hydrochloride and xylazine hydrochloride. Each hamster was administered virus via pipette into the nares (50 µL/nare) for a total volume of 100 µL per hamster. Virus back-titration was performed on Vero cells immediately following inoculation. Hamsters were observed until fully recovered from anesthesia. All hamsters were maintained for three days then humanely euthanized and necropsied. Oropharyngeal swabs were also taken on days 1–3 after challenge to evaluate viral shedding. Swabs were placed in BA-1 medium

(Tris-buffered MEM containing 1% BSA) supplemented with antibiotics then stored at –80 °C until further analysis. Tissues were collected for virus quantification and histopathology. For virus quantitation, approximately 100 mg of the right cranial lung lobe and nasal turbinates from each hamster were homogenized in 9 volumes of BA-1 media with antibiotics then frozen to –80 °C for later analysis. The tissue homogenates were briefly centrifuged and virus titers in the clarified fluid was determined by plaque assay. Viral titers of tissue homogenates are expressed as pfu/100 mg (log₁₀). For histopathology, portions of the left and right medial lung lobes were fixed in 10% neutral buffered formalin for seven days then paraffin embedded, sectioned and stained with hematoxylin and eosin using routine methods for histological examination.

2.8. Virus titration

Plaque assays were used to quantify infectious virus in oropharyngeal swabs and tissue homogenates. Briefly, 10-fold serial dilutions were prepared in BA-1 media supplemented with antibiotics. Confluent Vero E6 cell monolayers were grown in 6-well tissue culture plates. The growth media was removed from the cell monolayers and each well was inoculated with 0.1 mL of the appropriate diluted sample. The plates were rocked every 10–15 min for 45 min and then overlaid with 0.5% agarose in MEM without phenol red and incubated for 1 day at 37 °C, 5% CO₂. A second overlay with neutral red dye was added at 24–30 h and plaques were counted at 48–72 h post-plating. Viral titers are reported as the pfu per swab or per 100 mg of tissue. Samples were considered negative for infectious virus if viral titers were below the limit of detection (LOD). For oropharyngeal swabs the LOD was 10 pfu/swab. For tissues the LOD was 10 pfu/100 mg.

2.9. Plaque reduction neutralization test (PRNT)

Neutralizing antibody levels were determined by plaque reduction neutralization test (PRNT). Briefly, sera were first heat-inactivated for 30 min at 56 °C in a waterbath, then a series of two-fold dilutions in BA-1 media prepared in a 96-well plate starting at a 1:5 dilution. An equal volume of SARS-CoV-2 virus (isolate USA-WA1/2020) was added to the serum dilutions and the sample-virus mixture was gently mixed. The plates were incubated for 1 h at 37 °C. Following incubation, serum-virus mixtures were plated onto Vero E6 plates as described for virus plaque assays. Antibody titers were recorded as the reciprocal of the highest dilution in which > 90% of virus was neutralized. All hamsters were tested for the presence of antibodies against SARS-CoV-2 prior to vaccination.

2.10. Histopathology

Histopathology was blindly interpreted by a veterinary pathologist (HBO). H&E-stained lung tissue sections were examined and microphotographed using a Nikon Eclipse 50i microscope equipped with a Nikon DS-Fi1 microscope and NIS-Elements F4.60.00 software. The H&E-stained slides were assessed for morphological evidence of inflammatory-mediated pathology in lung and trachea and reduction or absence of pathological features used as an indicator of vaccine-associated protection. Each hamster was assigned a score of 0–5 based on absent, mild, moderate, or severe manifestation, respectively, for each manifestation of pulmonary pathology including overall lesion extent, bronchitis, alveolitis, pneumocyte hyperplasia and vasculitis and then the sum of all scores for each hamster calculated.

2.11. Statistical analysis

GraphPad Prism 8.3.1 for Windows was used for drawing graphs and statistical analysis (GraphPad Software, San Diego, CA, USA). Neutralization titers, antibodies, T cell based assays and tissue severity scores were evaluated using Kruskal-Wallis test with Dunn correction for multiple comparisons between groups. The limit of detection for viral plaque titres was 10 PFU, so titers lower than this were given a value of 5 PFU (half the limit of detection) for the purposes of statistical analysis. PRNT90 are presented as geometric mean titres, the minimum dilution tested for neutralizing antibody was 1:10, and a titer of 5 was used for a negative result. T-test was used for analysis of daily weight change. Statistical analyses of the correlation of PRNT90, total weight loss, viral load titers and tissue severity scores was performed by linear regression, PRNT90 and viral titers were log transformed for the purposes of the analysis. For all comparisons, $p < 0.05$ was considered to represent a significant difference. In figures * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and ****, $p < 0.0001$.

3. Results

3.1. Adjuvanted spike protein vaccine provides robust cellular and humoral immunity

In the current study we evaluated two vaccine antigens constructs, a full length (FL) spike protein versus the extracellular domain (ECD), both with the furin cleavage site deleted (see Fig. 1A). The final proteins had a purity of > 90% as confirmed by SDS-PAGE (Fig. 1B) and were sterile with negligible endotoxin and residual host-cell DNA content. The secreted ECD antigen gave significantly higher protein yields of 15–20 mg per liter of insect cell culture compared to the FL protein which had yields of only < 4 mg per litre (data not shown).

To initially compare the cellular and humoral immunogenicity of the FL versus ECD protein, mice were immunized twice 2 weeks apart with either ECD or FL antigen (0.5–10 μg) alone or with control adjuvant, Alum-CpG55.2. At week 2, the ECD (10 μg) construct showed a trend towards increased anti-spike total IgG compared to the equivalent dose of FL protein (10 μg) (Fig. 1C). ECD or FL alone (induced similar levels of anti-RBD total IgG at both week 2 and 4. The addition of the Alum-CpG55.2 adjuvant had an antigen-dose sparing effect but didn't change the overall pattern of response.

Cytokine production was measured using a CBA assay in culture supernatants of rSp-stimulated splenocytes obtained from immunised mice (Fig. 1D). IFN- γ release was significantly higher in the adjuvanted ECD group compared to the adjuvanted FL group. TNF α and IL-6 trended higher in the adjuvanted ECD compared to the FL group, but this difference did not reach statistical significance. Other cytokines, such as IL-2, IL-4, IL-10, IL-17, were comparable between the ECD and FL spike immunized mice. Cytokine ELISPOT results on rSp-stimulated splenocytes showed a trend to a higher frequency of IFN- γ secreting T cells in the splenocytes from the adjuvanted ECD group compared to the adjuvanted FL group, although this was not statistically significant (Fig. 1E). The frequencies of IL-2, IL-4 and IL-17 secreting T cells were comparable between ECD and FL proteins.

Next we compared the effect of two different combination adjuvants on immune responses to ECD or FL spike protein. Advax-CpG55.2 and alum-CpG55.2 adjuvants induced comparable levels of spike-binding and RBD-binding IgG, that were several fold (1.5–3) higher than with either antigen injected alone without adjuvant (Fig. 1F). The neutralizing activity of antibodies induced by the different adjuvanted formulations was assessed using a pseudoneutralization assay (Fig. 1G). Formulations of the ECD

protein showed a higher IC₅₀ when adjuvanted with Advax-CpG55.2 as compared to alum-CpG55.2, whereas the FL protein did better with the alum-CpG adjuvant.

3.2. Advax-CpG and Alum-CpG adjuvants induce robust neutralizing antibody against SARS-CoV-2 in hamsters

Golden Syrian hamsters were immunized intramuscularly either with a single vaccine dose, or with two doses 3 weeks apart. Sera was obtained 2 weeks after the final vaccination for measurement of SARS-CoV-2 neutralizing antibodies against the ancestral strain using a plaque reduction neutralization test with a 90% cut-off (PRNT90) (Fig. 2B). As expected, none of the saline nor adjuvant-alone control injected hamsters developed neutralizing antibodies. For the spike ECD 2-dose groups, only those animals which received spike protein formulated with Advax-CpG or alum-CpG adjuvants achieved neutralizing titers > 1:640 in all animals in the group. In the single-dose Advax-CpG adjuvanted vaccine groups, the spike ECD protein induced higher neutralization titers (GMT = 640) than the spike FL protein (GMT = 113). A similar pattern was seen in the 2-dose groups, with spike ECD + Advax-CpG inducing >5-fold higher neutralization titers (GMT = 761) than spike FL + Advax-CpG (GMT = 135).

3.3. Advax-CpG and Alum-CpG adjuvant protect against weight loss

At day 35, all hamsters were challenged intranasally with $1e^4$ PFU of SARS-CoV-2 (isolate USAWA1/2020). The control groups (adjuvant alone and saline) lost an average of 6.4–6.8% body weight during the first two days post-challenge and with further weight loss at termination on day 3, while the spike immunized animals only lost 4.1–6.4% weight up to Day 2 then began to recover weight by day 3 (Fig. 2C). Cumulative weight loss throughout the 3 days was lower for all immunized groups compared to either control group, but with no significant differences between the various vaccine groups (Fig. 2D). There was a strong negative correlation ($R = -0.8063$, $p < 0.0001$) between the serum PRNT90 titer pre-challenge (day 35) and cumulative weight loss after challenge (Supplementary Fig. 1i). This suggests that the level of serum neutralizing antibodies prior to virus exposure may play an important role in determining systemic disease severity.

3.4. Spike protein vaccine reduces peak oropharyngeal swab viral loads

To assess vaccine effects on viral replication and shedding over time, oropharyngeal swabs were taken daily from hamsters on days 1–3 after challenge and viral loads measured using a viral plaque assay (Fig. 2Ei–iv). Viral loads in swabs from control groups reached a peak on day 2 before beginning to decrease on day 3. By contrast, the viral loads in all the actively immunized groups fell progressively from day 1 to day 3. Only the Spike ECD + Alum-CpG55.2 group had significantly lower throat swab viral load titers on day 1 (GMT = 7; $p < 0.01$) and day 2 (GMT = 8.4; $p < 0.01$) compared to the saline control group (GMT = 383) (Fig. 2Eii). There was a large reduction in the throat swab viral load titers in the Spike ECD + Advax-CpG55.2 group on day 2 (GMT = 31; $p = 0.1$) compared to the saline control group (GMT = 383) (Fig. 2Eiii–iv). By day 3, several immunized animals had no detectable throat swab virus, including 100% in FL + Advax-CpG55.2 group, 75% in 1-dose ECD + Advax-CpG55.2 and 2-dose ECD + Alum-CpG55.2 and 2-dose ECD + Advax and 50% in ECD + Advax-CpG55.2, ECD alone and 1-dose FL + Advax-CpG55.2 groups.

For cumulative throat swab virus load over days 1–3, the 2-dose ECD + Alum-CpG55.2 had the lowest overall viral load (viral titer = 28) compared to the saline control group (viral titer = 7310)

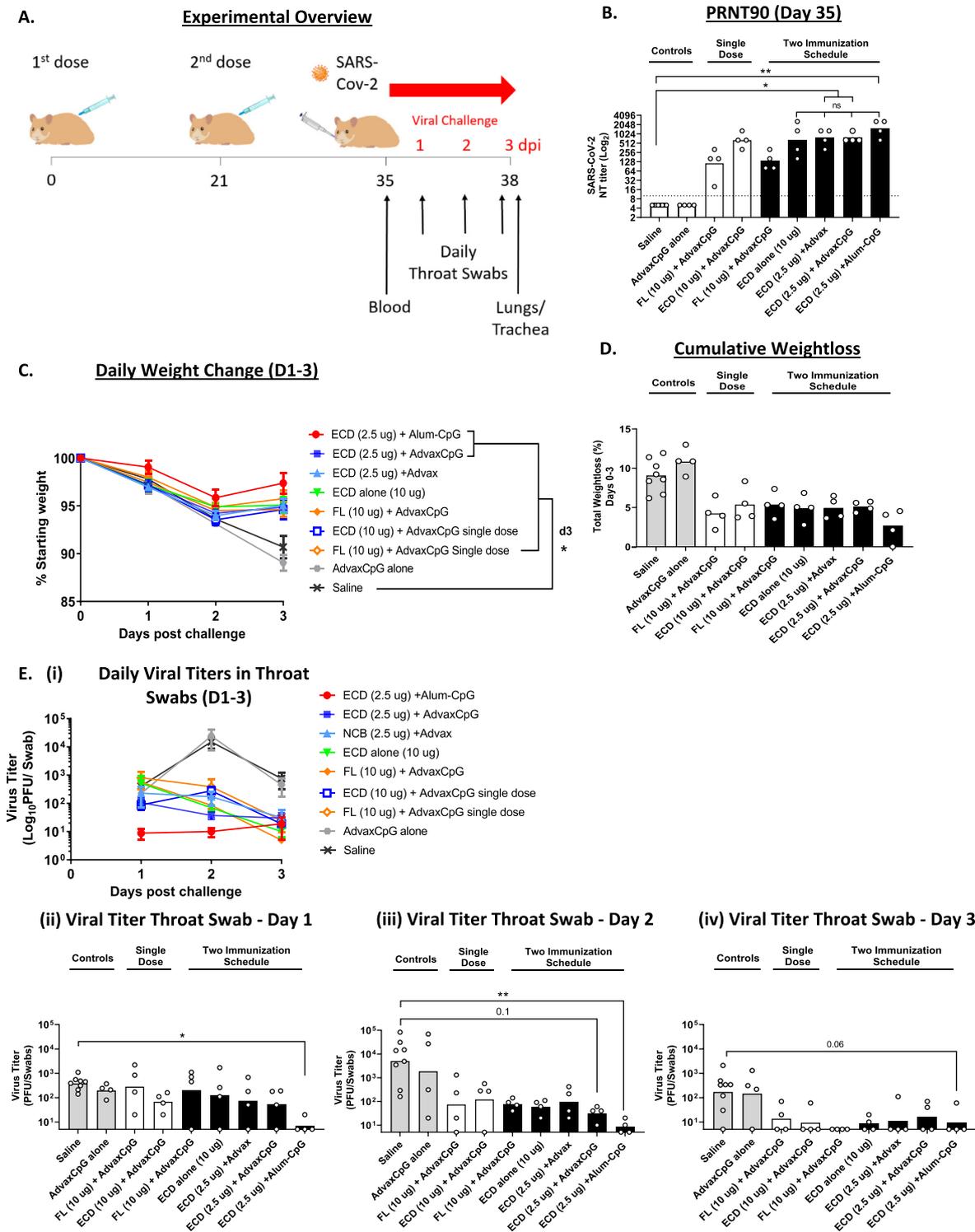


Fig. 2. Adjuvanted SARS-CoV-2 ECD protein induces strong neutralizing antibodies in hamsters and reduces clinical disease. (A) Hamsters (n = 4) were vaccinated at day 0 and boosted at day 21 with SARS-CoV-2 extracellular domain (ECD) or Full length (FL) spike protein at either 2.5 or 10 µg alone or formulated with Advax-CpG55.2 (2–0.02 mg), Advax (2 mg), Alum-CpG55/2 (0.2–0.02 mg). A 1-dose group was administered 10 µg of ECD or FL spike protein formulated with Advax-CpG55.2. Control hamsters received either saline (n = 8) or Advax-CpG55.2 alone (n = 4). Blood was collected on day 35 and then the animals were challenged nasally with 1e⁴ PFU of SARS-CoV-2 (WA-01 strain) and monitored for weight change. Oropharyngeal swabs were collected each day and on day 3 post-infection animals were sacrificed and nasal turbinates and cranial lobe of lungs were collected to determine SARS-CoV-2 viral loads. (B) Neutralization activity of sera at day 35 was determined by a PRNT90 assay. Results are presented as GMT. (C) Daily weight change normalized as percentage of starting weight. (D) Cumulative weight loss and (E) daily viral titers in throat swabs for 3 days. Statistical analysis of daily weight change D1-3 was performed using Multiple T-test and PRNT90, cumulative weight loss and viral titers was performed using Kruskal-Wallis test with Dunn correction for multiple comparisons (*; p < 0.05 and **; p < 0.01).

followed by 2-dose ECD + Advax-CpG55.2 (viral titer = 159, p < 0.05). A reduction in viral load was also observed in the 2-dose ECD + Advax (viral titer = 377) and the 2-dose ECD alone

(viral titer = 40) groups compared to saline control group, however these differences were not statistically significant (Fig. 3A). There was a negative correlation between PRNT90 antibodies

pre-challenge and throat swab viral load, with the highest negative correlation between PRNT90 and throat swab viral load on day 3 ($R = -0.6528, p < 0.0001$) then day 2 ($R = -0.6526, p < 0.0001$) and lastly day 1 ($R = -0.4777, p = 0.0018$) (Supplementary Fig. 1ii-iv).

3.5. Effect of spike protein vaccination on virus load in hamster nasal turbinates and lungs

To evaluate viral load in lungs and nasal turbinate tissues, hamsters were euthanized on day 3 and portions of right cranial lung lobe and nasal turbinate were collected for virus quantification using a plaque assay. Both control groups had extremely high day 3 viral loads in the range of $10^6 - 10^8$ PFU/100 mg tissue the nasal turbinates (Fig. 3B). By contrast, both the 2-dose ECD + Advax-CpG55.2 and 2-dose ECD + Alum-CpG55.2 groups showed significantly lower viral load in the nasal turbinates on day 3 with viral titers of 4701 and 28, respectively ($p < 0.05$).

Differences were particularly striking in day 3 lung viral loads where the control groups continued to have very high viral loads of $10^5 - 10^6$ PFU/100 mg tissue. By contrast, a number of immunized groups, including 1-dose ECD + Advax-CpG55.2 and 1-dose FL + Advax-CpG55.2 groups plus the 2-dose

ECD + Advax-CpG55.2 and 2-dose ECD + Alum-CpG55.2 groups showed absence of recoverable lung virus in all animals (Fig. 3C). Similarly, only 1 of 4 (25%) of the animals in the 2-dose ECD + Advax and 2-dose ECD groups had detectable lung virus. There was a stronger negative correlation between pre-challenge serum neutralizing antibody titers and lung viral load ($R = -0.8763, p < 0.0001$) than for cumulative throat swabs ($R = -0.7323, P < 0.0001$) or nasal turbinate ($R = -0.7659, P < 0.0001$) viral load (Supplementary Fig. 1v-vii) which suggests that serum neutralizing antibodies may play a larger role in controlling viral loads in the lower versus upper respiratory tract.

To examine how viral load in different tissues related to disease severity, cumulative weight loss was plotted against cumulative throat swab viral load, and day 3 lung and nasal turbinate viral load (Supplementary Fig. 2). Cumulative weight loss correlated highest with day 3 lung viral load ($R = 0.7483, p < 0.0001$) followed by day 3 nasal turbinate viral load ($R = 0.6293, P < 0.0001$) and least with cumulative day 1–3 throat swab viral load ($R = 0.5584, P = 0.0002$). Altogether, these results suggest that lung viral load determines COVID-19 disease severity in hamsters with serum neutralizing antibodies, playing an important role in controlling lung viral replication and disease severity.

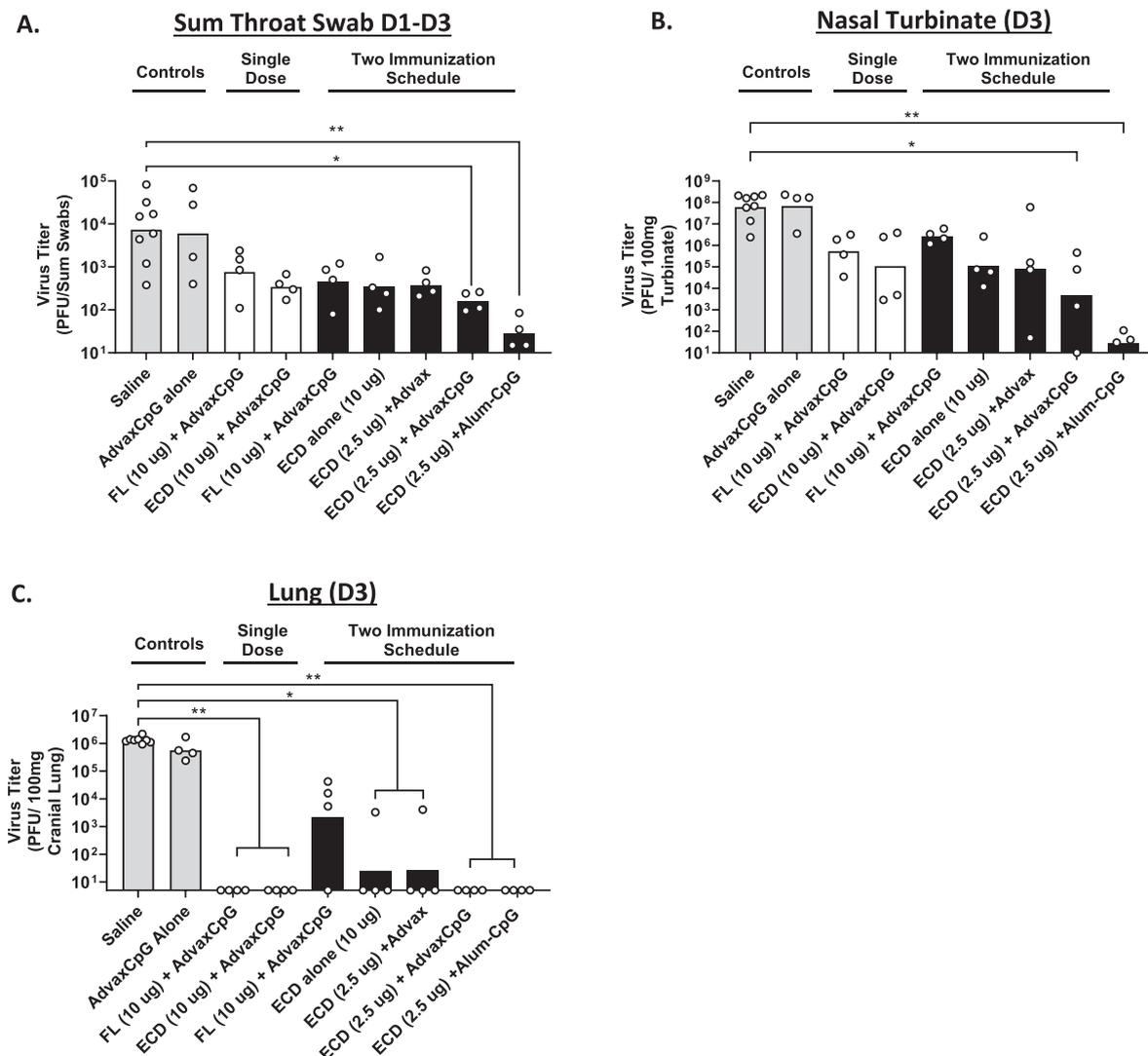


Fig. 3. Adjuvanted spike protein immunization reduces SARS-CoV-2 viral loads in throat swabs, nasal turbinates and lungs of hamsters. Virus loads in (A) cumulative days 1–3 throat swabs (PFU/swab), (B) Day 3 nasal turbinate and (C) Day 3 lung (PFU per 100 mg tissue). Results presented as GMT. Statistical analysis of viral titers was performed using Kruskal-Wallis test with Dunn correction for multiple comparisons (*; $p < 0.05$ and **; $p < 0.01$).

3.6. Single-dose Advax-CpG-adjuvanted spike protein vaccine protects lungs against virus replication and severe pathology

To evaluate whether spike protein vaccines could protect against lung pathology, portions of the lung were collected from all animals at Day 3 for H&E staining. Fig. 3 shows representative lung section images from a normal uninfected control lung (Fig. 4Ai), an infected saline control animal (total histopathology score 25) (Fig. 4Aii), and protected animals from the ECD + Advax-CpG55.2 and ECD + Alum-CpG55.2 groups (total histopathology score < 10) (Fig. 4Aiii-iv) at 20x and 100x magnification.

Tissue scoring showed lower total lung scores for the immunised versus the control groups (Fig. 4B), with the 2-dose ECD + Alum-CpG55.2 group having the lowest overall lung pathology score (1.75, $p < 0.01$) followed by 2-dose ECD + Advax (6.75, $p = 0.09$), ECD + Advax-CpG55.2 (10.25) and ECD alone (10.5). Some immunized animals had a total lung score of 0, indicating their lungs were free of any signs of disease, including 75% of animals in the ECD + Alum-CpG55.2 group and 50% of animals in the ECD + Advax-CpG55.2 group (Fig. 4B). The 1-dose ECD + Advax-CpG55.2 and 2-dose ECD + Advax-CpG55.2 groups had a similar mean total lung score of 9 and 10.25, respectively, consistent with

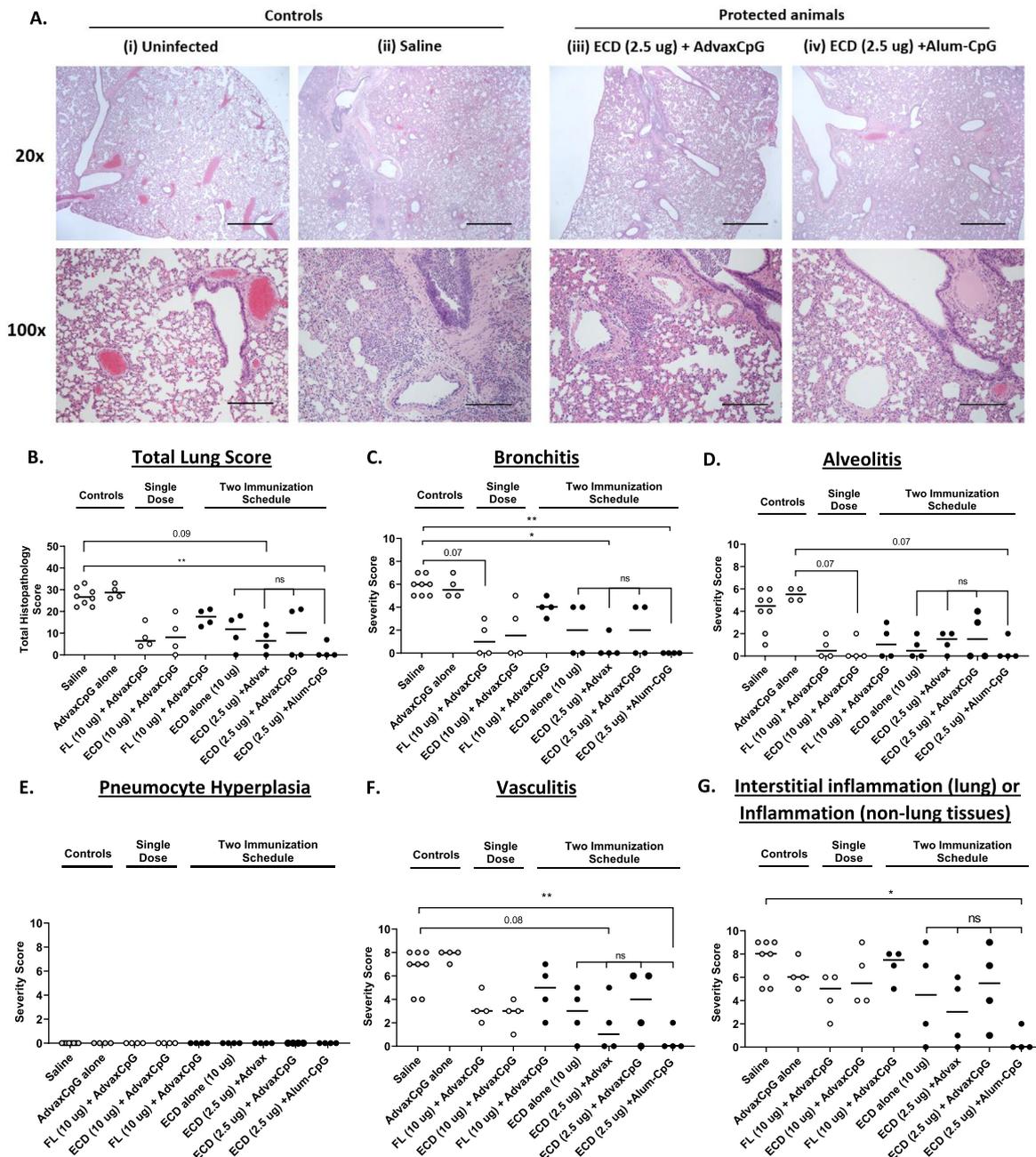


Fig. 4. Adjuvanted spike ECD reduces day 3 post challenge lung histopathology. Animals were sacrificed at 3 days post-infection and lungs were collected and processed for histopathology analysis. (A) Representative Hematoxylin & Eosin (H&E) stained sections (i-iv) of control uninfected and protected animals (groups Alum-CpG55.2 and Advax-CpG55.2 at 20x and 100x magnification (scale bar 1500 μm and 300 μm, respectively). Lung (right medial), Lung (left) and Trachea were scored by a trained pathologist. Quantification of (B) total lung score, (C) Bronchitis, (D) Alveolitis, (E) Pneumocyte hyperplasia, (F) Vasculitis and (G) Interstitial inflammation (lung) or Inflammation (non-lung tissues). Results presented as mean. Statistical analysis of viral titers was performed using Kruskal-Wallis test with Dunn correction for multiple comparisons (*; $p < 0.05$ and **; $p < 0.01$).

absence of detectable day 3 lung virus in both these groups. Conversely, the 2-dose FL + Advax-CpG55.2 group, which had the highest day 3 lung virus load of the immunized groups, also had the highest mean lung severity scores of the immunized groups with a mean lung severity score of 17.25. Lung scores of challenged control animals were all in the range of 20–30.

Total lung pathology scores were highly correlated with cumulative day 1–3 weight loss ($R = 0.8204$, $P < 0.0001$) (Supplementary Fig. 3). Pre-challenge serum PRNT90 titers were negatively correlated with total lung severity scores ($R = -0.7894$, $p < 0.0001$) (Supplementary Fig. 1viii). The day 3 lung viral load demonstrated a high negative correlation with total lung score ($R = 0.7937$, $p < 0.0001$), followed by day 3 nasal turbinate viral load ($R = 0.7616$, $p < 0.0001$) and cumulative day 1–3 throat swab viral load ($R = 0.5976$, $p < 0.0001$) (Supplementary Fig. 4i–vi).

4. Discussion

The COVID-19 pandemic remains highly active with 0.5–3 million new cases per day, globally [1]. Control measures such as contact tracing, quarantine and general lockdowns have had variable success [27]. Recombinant protein-based vaccines have a strong safety record and make a useful addition to the inactivated whole virus, adenovirus vector and mRNA Covid-19 vaccines. During the initial design of Covax-19/Spikogen[®] vaccine we sought to screen a number of different vaccine formulations, including different protein constructs, adjuvants and doses, as described here, in order to select the most favourable.

Antigen yield is a critical parameter in pandemic vaccine selection and the ECD protein produced much higher protein yields than the FL construct per liter of insect cell culture. In murine immunogenicity studies, when both FL and ECD proteins were formulated with a control alum-CpG adjuvant, the ECD protein induced slightly higher rSp-binding IgG against after the first dose and also induced higher T cell IFN γ recall responses than the FL protein. Spike ECD when formulated with Advax-CpG55.2 adjuvant induced levels of rSp- and RBD-binding IgG and neutralizing antibody not significantly different to those induced by alum-CpG adjuvant. Interestingly, the FL protein with alum-CpG55.2 adjuvant yielded slightly higher neutralizing antibody levels, suggesting some adjuvant effects might be antigen specific. Overall, based on the immunogenicity and protein yield data the spike ECD protein formulated with Advax-CpG adjuvant was chosen as the lead Covid-19 vaccine candidate to go forward with.

The down-selected vaccine candidate, named Covax-19[®]/Spikogen[®], was next tested for its ability to prevent viral replication/shedding and disease pathology in the well-established hamster model [26,28]. The candidate ECD vaccine was compared against the FL protein and the Advax-CpG adjuvant was compared to the alum-CpG adjuvant formulation. Both single and 2-dose regimens of adjuvanted spike protein provided robust protection of hamsters against lung infection and pathology, with a high correlation between serum neutralizing antibody levels, pre-challenge, and lung protection. post-challenge. Other Covid-19 studies have similarly shown a high correlation between serum spike antibody levels and protection against systemic disease [29–31].

Novavax's Nuvaxovid recombinant Covid-19 vaccine is based on FL spike protein nanoparticles [32], and thereby bears the greatest similarity to the FL protein used in our study. Covax-19/Spikogen vaccine is distinct to Nuvaxovid as whereas Nuvaxovid is an insoluble FL protein expressed in SF9 insect cells that forms nanoparticles with lipid and detergent components, Covax-19/Spikogen vaccine is based on a secreted soluble spike ECD expressed in T.ni insect cells. Our spike ECD antigen has the furin cleavage site (FCS) removed to inhibit transition from pre-fusion to post-fusion confor-

mation [33] with a stabilized pre-fusion state hypothesized to lead to better neutralizing antibody production [34,35]. In Nuvaxovid, the FCS residues are still present but mutated. Nuvaxovid uses a saponin adjuvant, whereas Covax-19 uses Advax-CpG55.2, a new combination adjuvant formulation containing CpG55.2 oligonucleotide TLR9 agonist developed using artificial intelligence. Various earlier forms of Advax-CpG adjuvant have been used in human clinical trials of vaccines against influenza [NCT03945825; NCT03038776] and hepatitis B [NCT01951677] where it was found to be safe and effective at boosting vaccine immunogenicity. Covax-19/Spikogen[®] vaccine received an emergency use authorization in Iran in early October 2021, making it the first recombinant spike protein vaccine in the world to be authorized, with Nuvaxovid vaccine receiving its first approval several weeks later.

Interestingly, in addition to the large protein yield differences, we saw some immunogenicity differences between the spike ECD and FL proteins. In both mice and hamsters, the spike ECD protein induced slightly higher spike antibody titers than the FL protein, and in the murine immunogenicity studies it induced higher T cell IFN- γ recall responses. All animals that received either single or two-dose spike ECD had neutralization titers of 1:160 or greater, whilst in the FL groups 75% of animals had titers equal to or below 1:160. This titer of 1:160 may be significant as it is a key selection threshold for convalescent plasma to be considered therapeutic for COVID-19 patients [36]. Serum neutralization titers were highest in the 2-dose ECD with Advax-CpG55.2 or alum-CpG55.2 groups which achieved titers of $> 1:640$ in all animals. Reassuringly, no adverse effects of the Advax-CpG adjuvanted formulations were observed in either the mice or hamsters.

In the hamster study there was a strong correlation between high serum neutralizing antibody titers, pre-challenge, and reduced weight loss and lung severity scores, post-challenge. The 2-dose ECD groups with Advax-CpG55.2 or Alum-CpG55.2 had the highest percentage of animals with no lung pathology, at 50% and 75%, respectively. Eosinophilic lung immunopathology which was a feature of alum-adjuvanted SARS CoV vaccines [20] was not observed in any group in this SARS-CoV-2 study. This suggests that either SARS-CoV-2 is not associated with eosinophilic lung immunopathology or that any Th2 bias in our study imparted by the alum adjuvant may have been countered by its co-formulation with CpG55.2, which drives a strong Th1 signal. There was also no evidence of vaccine-enhanced disease in any group as the lung scores in all immunised groups were consistently lower than the lung scores of the control animals which were all > 20 .

One aim of this study was to evaluate whether any of the vaccine formulations could confer single-dose protection. A vaccine candidate that can provide robust protection after a single dose would be highly desirable in a pandemic as it would speed the global vaccine rollout. One dose of ECD with Advax-CpG55.2 conferred protection comparable to that provided by the 2-dose regimen, with similar attenuation of weight loss (5.3% vs. 5.1%), lack of lung virus on day 3 and reduced lung severity scores. However, the single-dose vaccine did use twice the total amount of antigen. Two vaccine doses were required for maximum protection in our earlier ferret challenge study [22], so single-dose protection seen in the hamsters may not translate to larger animals such as monkeys and humans.

The viral load in lungs and nasal turbinates of the challenged hamsters strongly correlated with weight-loss and lung pathology scores. Serum neutralizing antibody levels prior to challenge strongly correlated with reduction in viral load in lung and nasal turbinate, consistent with other studies suggesting that serum neutralizing antibody plays an important role in systemic control of coronavirus infection [37]. Interestingly, throat swab viral loads were a poor predictor of disease severity and showed only a low correlation with weight loss or lung scores. Serum neutralizing

antibody levels, pre-challenge, only showed a modest negative correlation with throat swab viral loads. This suggests that serum antibody levels may be less effective against the mucosal component of the infection. Instead the role of serum neutralizing antibodies may be to help restrict the SARS-CoV-2 virus to the upper respiratory tract, thereby preventing severe lower respiratory tract infections.

An ability of a vaccine to inhibit viral replication in the nasal mucosa might not only help protect the lower respiratory tract against infection, but could also assist in blocking spread of infection and community transmission. All ECD and FL protein immunized groups in the hamster study showed a trend towards reduced nasal turbinate and throat swab viral loads compared to the saline control group, although only in the 2-dose ECD with Advax-CpG55.2 or alum-CpG55.2 adjuvant groups were they significantly lower than the control group. Future transmission studies are planned to examine whether our vaccine can prevent virus transmission from a vaccinated animal to a naïve recipient. How a parenterally-administered vaccine might help control mucosal virus replication is not known but could, for example, involve trafficking of memory T or B cells to the mucosal compartments. In a previous mouse immunogenicity study, the addition of Advax-CpG55.2 adjuvant to SARS-CoV-2 spike protein produced a balanced Th1/Th2 response, increased the breadth of serum neutralizing antibody to cover the alpha variant of concern, and induced a strong cellular response characterized by polyfunctional T cells and robust *in vivo* cytotoxic T lymphocyte activity against spike-labelled target cells [22]. Furthermore, in the ferret challenge model, susceptibility to lung infection was dependent on the number of vaccine doses, with only the 2-dose vaccine schedule able to prevent lung infection with SARS-CoV-2. Hence, multiple features are likely to contribute to mucosal protection. Human studies all suggest the importance of two or more doses of Covid-19 vaccine for maximal protection, with only modest and short-lasting protection seen with after single vaccine doses [38,39].

Potential limitations of this study were the small hamster group size and the short duration of the virus challenge period, i.e., 3 days prior to termination. However, there was very high consistency of responses within each group and significant differences were apparent in most major parameters, including virus load and clinical signs, between the vaccinated and the control groups. The hamster results were highly consistent with the murine immunogenicity results. The termination of hamsters at day 3 post-challenge was based on findings in previous studies that hamsters show peak SARS-CoV-2 viral titers by day 2 and even unimmunized control animals subsequently rapidly clear the virus by day 4–6, making later time points not useful for assessing vaccine effects [40]. Another limitation was that similar to other COVID-19 studies [41–44], our hamsters were challenged just 2 weeks after the second immunization [45]. Emerging data suggests that immunity induced by adenoviral and mRNA vaccines might rapidly wane over months, leading to loss of protection [46]. We recognize it will be important to undertake future studies to evaluate the long-term durability of Covax-19[®] vaccine protection. While only a homologous SARS-CoV-2 virus was used in this study to perform the infection challenges in this study, future studies will also need to examine the ability of Covax-19 vaccine to provide durable protection against variant strains.

In summary, we show in mice and hamsters that Covax-19 vaccine, based on recombinant spike ECD formulated with Advax-CpG55.2 adjuvant, induces high serum neutralization titers, and T cell IFN γ responses, and provides robust protection against SARS-CoV-2 infection. The candidate vaccine provided single-dose protection against the homologous virus and was well-tolerated. This data supports the use of spike protein ECD with Advax-CpG55.2 adjuvant as a promising Covid-19 vaccine candidate.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: LL, JB, and NP are affiliated with Vaxine Pty Ltd which holds the rights to COVAX-19[®]/Spikogen[®] vaccine and Advax[™] and CpG55.2[™] adjuvants.

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Author Contributions

NP and RB conceptualized the study. LL, YH, RB carried out the experimental work. LL, YH, RB, HBO, JB and NP analyzed data. JB and NP wrote the manuscript. All authors have read and approved the manuscript.

Appendix A. Supplementary material

Supplementary Figure 1: Pre-challenge serum neutralization titers correlate with disease protection and viral load. Correlation between PRINT90 at day 35 with (i) total weight loss day 1–3, viral load (ii–iv) individual throat swabs/(v) accumulative throat swabs/(vi) nasal turbinate/(vii) lung, and (viii) total lung severity score. PRINT90 titers are presented as log₂ and values below threshold are presented as half the lowest dilution (1:10) tested. Viral titers are presented as log₁₀ (n=40, groups indicated in legend). **Supplementary Figure 2: Total weight loss correlates with viral load.** Correlation between total weight loss over days 1–3 versus viral titers of (i) cranial lung, (ii) nasal turbinate and (iii) accumulative throat swabs day 1–3 (n=40, groups indicated in legend). Viral titers are presented as log₁₀. **Supplementary Figure 3: Weight loss correlates with severity of lung pathology.** Correlation between total weight loss over days 1–3 versus lung viral titers (n=40, groups indicated in legend). Viral titers are presented as log₁₀. **Supplementary Figure 4: Lung and nasal turbinate viral load correlates strongly with lung pathology.** Correlation between total lung severity score versus viral load of (i) cranial lung, (ii) nasal turbinate and (iii) accumulative and (iv–vi) individual daily throat swabs day 1–3 (n=40, groups indicated in legend). Viral titers are presented as log₁₀.

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