



Recombinant protein subunit SARS-CoV-2 vaccines formulated with CoVaccine HT™ adjuvant induce broad, Th1 biased, humoral and cellular immune responses in mice

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ABSTRACT

The speed at which several COVID-19 vaccines went from conception to receiving FDA and EMA approval for emergency use is an achievement unrivaled in the history of vaccine development. Mass vaccination efforts using the highly effective vaccines are currently underway to generate sufficient herd immunity and reduce transmission of the SARS-CoV-2 virus. Despite the most advanced vaccine technology, global recipient coverage, especially in resource-poor areas remains a challenge as genetic drift in naïve population pockets threatens overall vaccine efficacy. In this study, we described the production of insect-cell expressed SARS-CoV-2 spike protein ectodomain constructs and examined their immunogenicity in mice. We demonstrated that, when formulated with CoVaccine HT™ adjuvant, an oil-in-water nanoemulsion compatible with lyophilization, our vaccine candidates elicit a broad-spectrum IgG response, high neutralizing antibody (NtAb) titers against SARS-CoV-2 prototype and variants of concern, specifically B.1.351 (Beta) and P.1. (Gamma), and an antigen-specific IFN- γ secreting response in outbred mice. Of note, different ectodomain constructs yielded variations in NtAb titers against the prototype strain and some VOC. Dose response experiments indicated that NtAb titers increased with antigen dose, but not adjuvant dose, and may be higher with a lower adjuvant dose. Our findings lay the immunological foundation for the development of a dry-thermostabilized vaccine that is deployable without refrigeration.

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Introduction

The emergence and rapid spread of a new infectious respiratory disease, Coronavirus Disease 2019 (COVID-19) has caused an unprecedented public health emergency worldwide since emerging in December 2019. A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is closely related to SARS-CoV, was identified as the etiologic agent of the new respiratory disease. As of July 2021, there are over 185 million

confirmed cases with over 4 million deaths worldwide and over 33 million cases and 606,000 fatalities reported in the U.S. [1], accentuating the continued need for safe and effective, inexpensive and easily deployable vaccines that would rapidly establish global herd immunity. Facilitated by the broad nature of the outbreak, significant SARS-CoV-2 variants have been identified, some of which appear to be more resistant to first-generation vaccines [2]. More than 200 COVID-19 vaccine candidates using various technology platforms are in development [3]. Three vaccines including two of the mRNA platforms, Pfizer/BioNTech BNT162b2 and Moderna mRNA-1273, and the third, a viral-vector platform, Janssen Ad26.COV2.S, with reported interim efficacy rates of 95%, 94.1% and 85% respectively, have been approved by the U.S. Food and Drug Administration (FDA) for emergency use as of July 2021 [4–6].

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These vaccines are now being administered in the U.S. and have also been approved for use in other jurisdictions. As no single vaccine can be produced rapidly in sufficient quantities to satisfy the global demand, diversification using different vaccine platforms would enable worldwide vaccine coverage as well as address the needs of the most vulnerable populations, particularly elderly and immunocompromised individuals or those with other comorbidities. With mutations emerging even in the absence of strong selective pressure placed on SARS-CoV-2, variants of concern (VOC) will continue to arise under inequitable vaccine availability [2,7]. Thus, continued research into adaptable and more easily distributed vaccines, compatible with rapid deployment and significant cost efficiencies, must continue unabated.

The recombinant subunit vaccine platform offers a safety advantage over virally vectored vaccines and a distribution advantage relative to many other vaccine platforms. Purified recombinant protein antigens can be engineered to achieve optimal immunogenicity and protective efficacy. Furthermore, a thermostabilized subunit vaccine can be deployed in the field, eliminating stringent cold-chain requirements. Formulation of the vaccine immunogen with a potent adjuvant enhances and focuses immunogenicity while lowering the antigen dose requirement, thereby enabling vaccination of more people with a product carrying significantly more clinical and regulatory precedence compared to nucleic acid-based and viral-vectored approaches.

CoVaccine HT™, is an oil-in-water nanoemulsion adjuvant with an excellent safety profile [8]. In combination with properly selected antigens, CoVaccine HT™ can achieve potent immunogenicity and protective efficacy in rodents and non-human primates (NHPs) [9–12]. In previous work, we have successfully demonstrated the use of the recombinant protein subunit *Drosophila* S2 expression system in combination with CoVaccine HT™ to produce vaccines to combat global health threats such as Zika virus (ZIKV) and Ebola virus (EBOV). Immunization with recombinant ZIKV E protein induced potent neutralizing titers in mice [10] and non-human primates [9] and protection against viremia after viral challenge. Similarly, immunization with recombinant subunit formulations consisting of the EBOV glycoprotein and matrix proteins VP40 and VP24 was able to induce potent antibody titers and protection in both mouse [11] and guinea pig models of EBOV disease [12]. In healthy adults, CoVaccine HT™ was shown to be safe and well-tolerated with no risk of severe adverse effects in phase 1 clinical trials (NCT01015703) [8].

The spike (S) glycoprotein, comprised of a receptor binding subunit (S1) and a membrane-fusing subunit (S2) [13] is the main surface protein and present as homotrimers on the viral envelope of SARS-CoV-2. Based on previous preclinical studies of vaccines against the highly pathogenic SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) [14–16] as well as recent studies of patients with SARS-CoV-2 infections [17–20], the S protein appears to be the antigenic target of both neutralizing antibody and T cell responses. The majority of current COVID-19 vaccines under preclinical and clinical development use full-length S proteins as antigen targets with further modifications such as removal of the polybasic sites [21–23], introduction of proline mutations [21,24,25], or addition of trimerization domains to preserve the native-like trimeric prefusion structure of S proteins. These antigens have been shown to mimic the native S protein presented on viral particles and preserve neutralization-sensitive epitopes [16,26]. In a prior study, we evaluated the utility of CoVaccine HT™ adjuvant to induce properly balanced immunity against SARS-CoV-2, when formulated with a commercially available SARS-CoV-2 spike S1 protein [27]. In the current study we produced a native-like trimeric S protein ectodomain with and without stabilizing mutations using the *Drosophila* S2 cell expression system and assessed the immunogenicity of these S

ectodomain trimers formulated with CoVaccine HT™ in mice. The scope of this work demonstrates that CoVaccine HT™ is an effective adjuvant that promotes rapid induction of balanced humoral and cellular immune responses and in combination with spike proteins warrants further development as an effective medical intervention against coronavirus disease.

Materials and methods

Ethics statement

All animal work was conducted in accordance with the Animal Welfare Act and the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Hawaii at Manoa (UHM) and carried out in the UHM American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited Laboratory Animal Facility.

Recombinant protein expression and purification

Plasmids were generated to express the native-like, trimeric, transmembrane (TM)-deleted spike (S) glycoprotein (SdTM) from SARS-CoV-2 strain Wuhan-Hu-1 (Genbank Accession number NC_045512). The SdTM sequence was designed to encode the SARS-CoV-2 S protein sequence spanning Gln14 to Ser1147. The SdTM gene was produced by de novo synthesis (ATUM, Newark, CA). The gene was also codon-optimized for expression in *Drosophila* S2 cells, with an altered furin cleavage site (RRAR changed to GSAR) between S1 and S2 domains to prevent cleavage, and contains a trimerization domain of T4 bacteriophage fibrin (foldon) at the C-terminus. Two additional proline substitutions (K986P and V987P) between the heptad repeat 1 and central helix regions and the removal of the S2' protease cleavage site were introduced by site-directed mutagenesis to generate the stabilized prefusion structure of S protein (SdTM2P). The SARS-CoV-2 receptor binding domain with a foldon trimerization domain at the C-terminus (RBD-F) was also prepared. The RBD-F sequence was designed to encode the SARS-CoV-2 S protein sequence spanning Phe318 to Gly594. The proprietary expression vector pHH202 (Hawaii Biotech Inc., Honolulu, HI) containing S gene variants was transfected into *Drosophila* S2 cells using the Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Stably transformed cell lines were created by selection with culture medium containing hygromycin B at 300 µg/mL. To verify selection, transformants were induced with culture medium containing 200 µM CuSO₄. Expression was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

Recombinant S proteins were purified from filtered cell culture supernatants by affinity chromatography (AC) using NHS-activated Sepharose (Cytiva, Marlborough, MA) coupled with 2 mg of a his-tagged human angiotensin I converting enzyme 2 (hACE2), which was also produced using the *Drosophila* S2 cell expression system and purified by Ni-affinity chromatography in our laboratory. Purified recombinant S proteins were concentrated using Amicon filtration devices (EMD Millipore, Billerica, MA), buffer-exchanged into PBS and analyzed by SDS-PAGE and Western blotting. Antigens were quantified by UV absorbance at 280 nm and stored at –80 °C.

A conventional immunoaffinity chromatography (IAC) method was also established to purify SdTM2P proteins. For this, the monoclonal antibody (mAb) CR3022 (provided by Mapp Biopharmaceutical, San Diego, CA), was coupled to NHS-activated Sepharose

at a concentration of 10 mg/mL and used for IAC in tandem with a HiPrep 26/10 desalting column (Cytiva, Marlborough, MA) equilibrated with PBS allowing quick buffer exchange of the eluted protein from low pH buffer into PBS.

Mouse experiments

Groups ($n = 7$ or 15 per group) of 7 to 10-week-old Swiss Webster mice of both sexes (bred from original breeding stocks obtained from Taconic Biosciences, Germantown, NY) were immunized intramuscularly (i.m.) at days 0 and 21 with $5 \mu\text{g}$ of SARS-CoV-2 spike proteins SdTM or SdTM2P (purified by hACE2 affinity chromatography) alone or in combination with 1 mg of CoVaccine HT™ adjuvant (Protherics Medicines Development Ltd, a BTG company, London, United Kingdom). The negative control group received equivalent doses of adjuvant only. Vaccines were prepared by mixing the liquid adjuvant with antigen diluted in 1x PBS to the desired concentration immediately prior to administration to mice. To determine the optimal antigen and adjuvant dosing, mice were also immunized with either 5, 2.5, or 1.25 μg of SdTM2P (purified by mAb CR3022 IAC) and 1 mg of CoVaccine HT™ or 5 μg of SdTM2P formulated with 0.3 mg CoVaccine HT™. Serum samples were collected on days 7, 14, 28, and 35. Three to five mice from each group were euthanized on the seventh day after the first and second vaccinations and splenectomies were performed for preparation of splenocytes. The remaining four to five mice from each group were euthanized 14 days after the second vaccination and terminal bleeds collected by cardiac puncture.

Analysis of antibodies by multiplex microsphere immunoassay (MIA)

The IgG antibody in mouse sera was measured by a multiplex microsphere-based immunoassay as described previously [10,27,28]. Briefly, internally dyed, magnetic MagPlex® microspheres (Luminex Corporation, Austin, TX) were coupled to purified receptor binding domain (RBD-F), spike protein (SdTM2P) or bovine serum albumin (BSA) as control [10,27]. A mixture of RBD, spike SdTM2P, and BSA-coupled beads (approximately 1,250 beads each) was incubated with diluted sera in black-sided 96-well plates for 3 h at 37 °C with gentle agitation in the dark. Following two washes with MIA buffer (1% BSA and 0.02 % Tween 20 in 1x PBS), 50 μl of 1 $\mu\text{g}/\text{mL}$ red phycoerythrin (R-PE)-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch, Inc., West Grove, PA) were added and incubated at 37 °C for another 1 h. After washing twice with the MIA buffer, the beads were resuspended in MAGPIX® drive fluid and analyzed on a MAGPIX® Instrument (Luminex Corporation, Austin, TX).

The median fluorescence intensity (MFI) readouts of the experimental samples were converted to antibody concentrations using purified antibody standards prepared from pooled mouse anti-serum to SdTM or SdTM2P as described below. For this, IgG was purified from mouse antisera by protein A affinity chromatography and then subjected to immunoaffinity chromatography (IAC) using SdTM2P-coupled NHS-Sepharose (Cytiva, Marlborough, MA) to select only S-reactive IgG. The concentration of purified S-specific IgG was quantified by measuring the UV absorbance of the solution at 280 nm. The purified anti-spike IgG was diluted to concentrations in the range of 4.88–5000 ng/mL and analyzed in the MIA assay. The resulting MFI values were analyzed using a sigmoidal dose-response, variable slope model (GraphPad Prism, San Diego, CA), with antibody concentrations transformed to log₁₀ values. The resulting curves yielded r^2 values >0.99 with a well-defined top and bottom and the linear range of the curve was determined. The experimental samples were analyzed side-by-side with the antibody standards at different dilutions (1:50, 1:200, 1:1000, 1:40,000, 1:80,000 or 1:160,000) to obtain MFI values that fall

within the linear range of the standard curve. The experimental sample IgG concentrations were interpolated from the standard curves using the same computer program. Finally, the interpolated values were multiplied by the dilution factors and plotted as antibody concentrations (ng/mL).

The IgG subclass profile in serum samples was analyzed using IgG subclass-specific secondary antibodies (Southern Biotech, Birmingham, AL), and the ratios of IgG2a/IgG1 and IgG2b/IgG1 were calculated using the MFI readouts at the serum dilution (1:2000) that is within the linear range of the antibody binding standard curve.

Recombinant vesicular stomatitis virus (rVSV) neutralization assay

Replication-competent rVSV expressing SARS-CoV-2 S protein (Wuhan-Hu-1) with a 21 amino acid deletion in the cytoplasmic tail (rVSV-SARS-CoV-2-S) was generated as described previously [29] and the virus stocks were amplified in Vero E6 cells. For the plaque reduction neutralization test (PRNT) using rVSV-SARS-CoV-2-S, pooled or individual mouse serum samples were heat-inactivated at 56 °C for 30 min. Eight 3-fold serial dilutions of serum samples starting at final 1:10 dilution were prepared and incubated with 100 plaque-forming units (PFU) of rVSV-SARS-CoV-2-S at 37 °C for 1 h. Antibody-virus complexes were added to Vero E6 cell monolayers in 6-well plates and incubated at 37 °C for another hour followed by addition of overlay media mixed with 1% agarose. Three days later, cells were fixed and stained with a solution containing 1% formaldehyde, 1% methanol, and 0.05% crystal violet overnight for plaque enumeration. The neutralization titers (PRNT₅₀) were defined as the highest serum dilution that resulted in 50% reduction in the number of plaques.

Wild-type SARS-CoV-2 virus neutralization assay

PRNT was also performed in a biosafety level 3 facility at BIOQUAL, Inc. (Rockville, MD) using 24-well plates. Mouse sera pooled from individual mice within each group, were diluted to 1:10, and a 1:3 serial dilution series was performed 11 times. Diluted samples were then incubated with 30 plaque-forming units of wild-type SARS-CoV-2 USA-WA1/2020 (BEI NR-52281), B.1.351 (Beta) (BEI NR-55282), or P.1. (Gamma) (BEI NR-54982) variants, in an equal volume of culture medium (DMEM with 10% FBS and gentamicin) for 1 h at 37 °C. The serum-virus mixtures were added to a monolayer of confluent Vero E6 cells and incubated for 1 h at 37 °C in 5% CO₂. Each well was then overlaid with 1 ml of culture medium containing 0.5% methylcellulose and incubated for 3 days at 37 °C in 5% CO₂. The plates were then fixed with methanol at –20 °C for 30 min and stained with 0.2% crystal violet for 30 min at room temperature. Neutralization titers (PRNT₅₀) were defined as the highest final serum dilution that resulted in 50% reduction in the number of plaques.

Splenocyte preparation and FluoroSpot assays

Three or five mouse spleens from each group were harvested seven days after the first and second vaccinations, and single cell suspensions were prepared using a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). The cells were passed through a cell strainer, resuspended in freezing medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO) after lysis of red blood cells, and cryopreserved in liquid nitrogen. The FluoroSpot assay was performed using the mouse IFN- γ /IL-4 FluoroSpot^{PLUS} kit according to the manufacturer's instructions (Mabtech, Inc., Cincinnati, OH). Briefly, splenocytes were rested in a 37 °C, 5% CO₂ incubator for 3 h after rapidly thawing in a 37 °C water bath followed by slow dilution with culture medium to allow removal of cell debris. A

total of 2.5×10^5 cells per well in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) were added in a 96 well PVDF membrane plate pre-coated with capture monoclonal antibodies. The cells were stimulated for 24 h with a peptide pool consisting of 17-mer peptides with 10 amino acids overlapping, covering the spike protein of SARS-CoV-2 (BEI Resources NR-52402) at 10 µg/mL or medium containing an equal concentration of DMSO (0.05%) as negative control. Fifty thousand cells were incubated with a cell activation cocktail (BioLegend, San Diego, CA) at 1:500 dilution containing phorbol-12-myristate 13-acetate (PMA, 0.081 µM) and Ionomycin (1.3386 µM) as positive control. Each stimulation condition was set up in triplicate. Plates were developed using specific monoclonal detection antibodies and fluorophore-conjugated secondary reagents. Finally, plates were treated with a Fluorescence enhancer (Mabtech) to optimize detection and then air-dried. The spots were enumerated using the CTL ImmunoSpot[®] S6 Universal Analyzer (Cellular Technology Limited, CTL, Shaker Heights, OH), and the number of antigen-specific cytokine secreting spot forming cells (SFCs) per million cells for each stimulation condition was calculated by subtracting the number of spots detected in the medium only wells.

Statistical analysis

Statistical analysis was performed using a one-sample *t* test and Wilcoxon test or Mann-Whitney *t* test to compare the neutralization titers, and IFN-γ secreting response between adjuvanted and unadjuvanted groups. The difference in the neutralizing antibody titers and the numbers of IFN-γ secreting cells between groups receiving different dosages of vaccines, or IgG subclass antibody profile, anti-S and anti-RBD IgG concentrations between unadjuvanted and adjuvanted groups were determined by one-way, or two-way, ANOVA with Tukey's multiple comparison test. The correlation between the measured neutralization titers using rVSV-SARS-CoV-2 and authentic SARS-CoV-2 was analyzed by Pearson correlation analysis.

Results

Drosophila S2 cell-expressed recombinant S proteins adjuvanted with CoVaccine HT[™] induce robust IgG antibody responses

Using the *Drosophila* S2 cell expression system, we generated trimeric SARS-CoV-2 S protein devoid of transmembrane domain (SdTM) as well as a stabilized prefusion structure of S protein (SdTM2P). To explore different methods for purification of S protein, we employed two methods of purification using hACE2 protein, a known receptor for SARS-CoV-2, for AC, and the SARS-CoV RBD specific mAb CR3022 for IAC. This mAb, originally constructed from an immune svFc phage display library of lymphocytes from a convalescent SARS-CoV patient, was selected due to its cross-reactivity with both SARS-CoV and SARS-CoV 2 receptor binding sites, which contain the majority of neutralizing epitopes [30,31]. The immunogenicity of recombinant S proteins was evaluated with and without CoVaccine HT[™] adjuvant in outbred Swiss Webster mice. Animals were immunized with one or two doses at a 3-week interval by intramuscular injection with 5 µg of SARS-CoV-2 SdTM or SdTM2P (purified by hACE2 AC) alone or formulated with 1 mg CoVaccine HT[™] (Fig. 1A). We developed a quantitative IgG assay in which purified anti-S polyclonal antibody was used as a standard and therefore the antigen-specific IgG levels are expressed as concentration (ng/mL) (Fig. 1B). Analysis of IgG antibody in sera obtained on days 7, 14, 28, and 35 indicated that the co-formulation of CoVaccine HT[™] with SdTM or SdTM2P

significantly increased anti-S IgG antibody levels over those induced by proteins alone (Fig. 1C). In addition, two doses of CoVaccine HT[™] adjuvanted SdTM or SdTM2P elicited high levels of IgG antibodies to RBD, which is the target of neutralizing antibodies (Fig. 1D). To further optimize vaccine formulations, we evaluated the IgG antibody responses in mice that received a lower dosage (2.5 or 1.25 µg) of SdTM2P proteins or that received a reduced amount (0.3 mg) of adjuvant. The results demonstrated that vaccination with reduced amounts of antigen or adjuvant did not decrease the levels of antigen-specific IgG antibodies (Fig. 1E).

Recombinant S proteins adjuvanted with CoVaccine HT[™] elicit potent neutralizing antibody responses

We next examined whether our vaccine candidates induced neutralizing antibody responses. Measurement of SARS-CoV-2 neutralizing antibodies requires biosafety level 3 (BSL3) laboratory facilities, and therefore, alternative assays have been developed, such as one utilizing replication-competent rVSV-ΔG expressing SARS-CoV-2 S glycoprotein (rVSV-SARS-CoV-2-S) [32,33]. We first compared the neutralizing antibody titers of pooled mouse sera using both SARS-CoV-2 and rVSV-SARS-CoV-2-S (Table 1) and found that the titers are significantly correlated between these two assays (Fig. 2C). To further evaluate the neutralizing antibodies of individual animals, we employed only the PRNT using rVSV-SARS-CoV-2-S. The PRNT₅₀ titers of sera obtained from mice vaccinated with SdTM or SdTM2P in combination with CoVaccine HT[™] were significantly higher than those from animals receiving protein alone (Fig. 2A), suggesting that the CoVaccine HT[™] adjuvant enhances induction of neutralizing antibody responses. Furthermore, a trend of dose-dependent decreases of neutralization titers was observed when animals were given lower dosages of SdTM2P proteins (Fig. 2B). Of note, the serum PRNT₅₀ titers of mice receiving a lower amount (0.3 mg) of CoVaccine HT[™] were comparable or even higher than those of mice given 1 mg of adjuvant indicating that the optimal vaccine formulation may be achieved using lower dosages of adjuvant.

Similar trends were seen with the pooled sera in PRNT using the SARS-CoV-2 wt, lineage B.1.351 Beta, and P.1 Gamma VOC (Table 1). Although two doses of 5 µg SdTM or SdTM2P formulated with CoVaccine HT[™] yielded PRNT₅₀ titers approximately 3–9 fold-lower than the Lineage A, USA-WA1/2020 strain, both adjuvanted spike antigens were able to generate a humoral response capable of cross-variant neutralization. Interestingly, 2.5 and 1.25 µg of the SdTM2P antigen sustained the same neutralization level across different VOC, suggesting lower concentrations of antigen may generate a minimal threshold of neutralizing antibodies targeting conserved epitopes. Furthermore it appears that the B.1.351 Beta variant was more evasive than the P.1 Gamma variant, an observation consistent with clinical studies using convalescent sera and sera from vaccinated individuals [34].

Recombinant S proteins in combination with CoVaccine HT[™] induce a balanced IgG subtype antibody response

Vaccine-associated enhanced respiratory disease (VARED) has been reported in infants and young children immunized with inactivated whole virus vaccine against respiratory syncytial virus (RSV) and measles virus [35–37], and associated with Th2-biased immune responses [38]. A similar pulmonary immunopathology was also observed in animals with SARS-CoV vaccines [39–41]. Thus, we evaluated the balance of Th1 and Th2 by comparing the levels of S-specific IgG2a/b and IgG1, which are indicative of Th1 and Th2 responses, respectively. Both SdTM and SdTM2P adjuvanted with CoVaccine HT[™] elicited high anti-S IgG2a/2b and

Table 1

Virus neutralizing antibody titers against SARS-CoV-2 lineage A USA-WA-1/2020, B.1.351, and P.1 variants, and rVSV-SARS-CoV-2-S (Wuhan-Hu-1) in pooled antisera from mice immunized with two doses of S proteins formulated with CoVaccine HT™ adjuvant.

Immunogen ^a	Adjuvant ^a	PRNT ₅₀ ^b SARS-CoV-2 USA-WA1/2020	PRNT ₅₀ ^b rVSV-SARS-CoV-2-S Wuhan-Hu-1	PRNT ₅₀ ^b SARS-CoV-2 B.1.351	PRNT ₅₀ ^b SARS-CoV-2 P.1
5 µg SdTM	1 mg CoVaccine HT™	540	30	60	180
5 µg SdTM2P	1 mg CoVaccine HT™	1620	810	180	180
5 µg SdTM	None	<20	<10	<20	<20
5 µg SdTM2P	None	<20	<10	<20	<20
None	1 mg CoVaccine HT™	<20	<10	NT ^c	NT ^c
5 µg SdTM2P	1 mg CoVaccine HT™	NA ^d	270	180	540
2.5 µg SdTM2P	1 mg CoVaccine HT™	180	90	60	180
1.25 µg SdTM2P	1 mg CoVaccine HT™	60	30	60	60
5 µg SdTM2P	0.3 mg CoVaccine HT™	4860	2430	540	1620

^a Two vaccinations with protein with or without adjuvant given intramuscularly.

^b Pooled antiserum (n = 3 or 5 per group) collected 14 days post-dose 2. Titer equals the highest serum dilution yielding 50% reduction in plaque counts.

^c NT: Not tested.

^d NA: Assay results not available.

IgG1 subclass antibodies whereas protein alone induced high IgG1 with lower IgG2a and IgG2b (Fig. 3A). To further assess the effect of CoVaccine HT™ adjuvant on IgG subclass antibody profiles, the ratios of IgG2a versus IgG1 as well as IgG2b versus IgG1 were calculated. The results indicate that CoVaccine HT™ enhanced the induction of Th1 responses as evidenced by the significantly higher ratios of IgG2a/IgG1 and IgG2b/IgG1 obtained from groups given either SdTM or SdTM2P with adjuvant compared to those without adjuvant (Fig. 3B, 3C).

CoVaccine HT™ adjuvanted SARS-CoV-2 recombinant S protein vaccine elicits IFN-γ T cell response

To assess vaccine-induced T cell responses, we analyzed the number of IFN-γ secreting cells after *ex vivo* stimulation of splenocytes with SARS-CoV-2 spike peptides by a FluoroSpot assay. Stimulation of cells prepared from mice receiving 2 doses of SdTM or SdTM2P with CoVaccine HT™ resulted in robust production of IFN-γ secreting cells (Fig. 4A). Interestingly, one dose of CoVaccine HT™-adjuvanted S proteins also elicited a rapid IFN-γ secreting response (Fig. 4A). Additionally, a slight decrease in the numbers of IFN-γ secreting cells was observed when mice received a lower amount (2.5 or 1.25 µg) of adjuvanted SdTM2P proteins or a lower amount (0.3 mg) of CoVaccine HT™ (Fig. 4B). However, these differences were not statistically significant. In addition, spike peptide stimulation of immune splenocytes was also analyzed for production of IL-4, but no significant IL-4 production was detected at any timepoint with less than 10 spot forming cells (SFCs) per million cells (data not shown). Altogether, our platform shows great potential towards achieving a rapid, robust, and Th1-focused T cell response even in outbred populations.

Discussion

An ideal COVID-19 vaccine is expected to induce both humoral and cellular immunity, high titers of neutralizing antibodies and Th1-biased response to reduce potential risk of vaccine-associated enhancement of disease [42–44]. Using a well-established insect cell expression system, we have generated two versions of the SARS-CoV-2 S protein ectodomain and formulated them with a potent adjuvant, CoVaccine HT™. The vaccine candidates elicit both neutralizing antibody and cellular immunity with a balanced Th1/Th2 response in an outbred mouse model. The results obtained in this model may thus inform future vaccine development in animal models more closely related to humans [45]. These data support further preclinical and clinical develop-

ment of CoVaccine HT™ adjuvanted SARS-CoV-2 vaccines to mitigate the ongoing COVID-19 pandemic.

Stabilization of S proteins in the prefusion trimeric conformation results in increased expression, conformational homogeneity, and production of potent neutralizing antibody responses [16,25]. Current SARS-CoV-2 vaccines under development use either RBD or full-length S protein with or without modifications for stabilization of prefusion conformation as the major antigen targets [46–49]. Although RBD is a primary target for potent neutralizing antibodies, it lacks other neutralizing epitopes present on full-length S. This might suggest that full-length S-based vaccines would broaden the neutralizing repertoire and cross-neutralize circulating spike variants of concern. In the current work, we produced trimeric S ectodomains in which the furin cleavage site was mutated (SdTM) and that were further stabilized in the prefusion S form by removing the S2' protease cleavage site and introducing two proline substitutions (SdTM2P). The introduction of two prolines in the S2 subunit resulted in significantly greater production yield (~3-fold increase) in *Drosophila* S2 cells (unpublished data). Vaccination with adjuvanted SdTM or SdTM2P elicits comparable levels of IgG antibody and IFN-γ cellular responses; however, the SdTM2P vaccine generated a slightly higher level of neutralizing antibodies than SdTM, which was also reported in studies of other SARS-CoV-2 and MERS-CoV vaccines [16,22].

CoVaccine HT™ is a novel adjuvant that consists of a sucrose fatty acid sulfate ester (SFASE) immobilized on the oil droplets of a submicrometer emulsion of squalane in water (oil-in-water emulsion) [50]. It has been used for influenza virus and malaria vaccines and shown to enhance humoral and cellular protective immunity, in particular antibody response [51–55]. In addition, we have successfully utilized CoVaccine HT™ in our previous recombinant subunit EBOV, ZIKV and preliminary SARS-CoV-2 vaccine studies and have shown this adjuvant to elicit robust antibody responses [9–11,27]. Use of CoVaccine HT™ with SdTM and SdTM2P yielded significantly enhanced total IgG and neutralizing antibody responses after both the first and second dose as compared to protein alone which reached similar IgG concentrations after two doses as did a single dose of the adjuvanted formulations. Although not directly comparable, the elicitation of S- and RBD-specific IgG and neutralizing antibody titers, along with IFN-γ secreting cells, after each dose followed similar kinetics as other 2P-S subunit vaccine candidates formulated with saponin or CpG based adjuvants [56,57], while similarities in IgG and neutralizing antibody titers were observed compared with alum only-formulated candidates [58,59]. Interestingly antibody levels (total IgG or neutralizing) did not decrease with a decreased dose of adjuvant; instead, a pronounced increase in neutralizing antibody titers

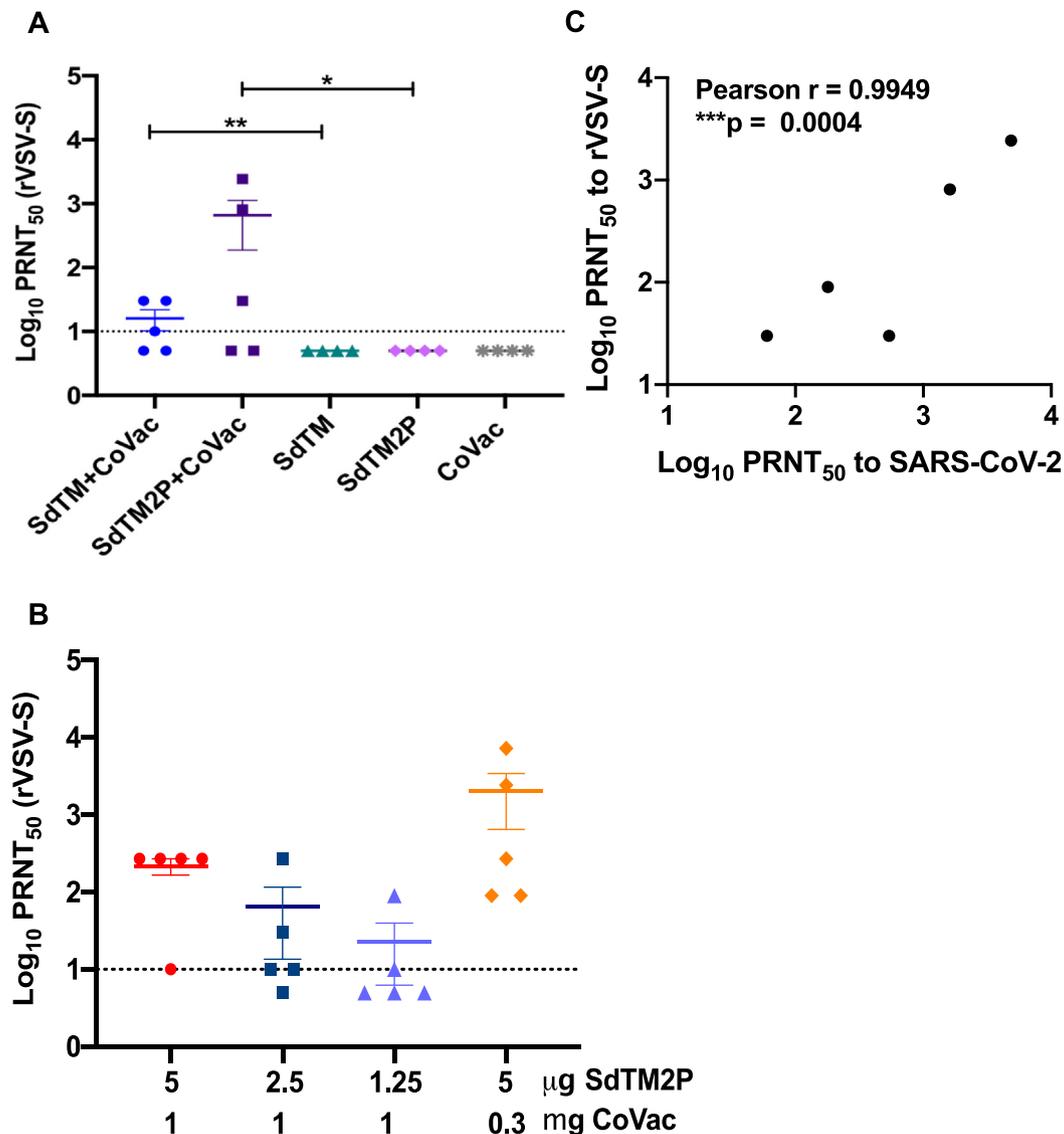


Fig. 2. The serum neutralization titers of mice immunized with recombinant subunit SARS-CoV-2 vaccines. The neutralization titers of sera obtained 2 weeks post boosting (day 35) from mice vaccinated with (A) SARS-CoV-2 SdTM or SdTM2P proteins (purified by hACE2 AC) adjuvanted with or without 1 mg of CoVaccine HTTM (CoVac) or (B) different dosages (5, 2.5, or 1.25 μ g) of SdTM2P (purified by mAb IAC) with CoVac (1 or 0.3 mg) was measured by a PRNT using rVSV-SARS-CoV-2-S. The data shown are log-transformed PRNT₅₀ values from individual animals, and mean \pm SEM of each group is indicated. Horizontal dashed lines represent the limit of detection. For the samples showing low (<50 %) or no neutralizing activity at the starting dilution (1:10) in the assay, a PRNT₅₀ of 5 (half of the limit of detection) was reported when the data were plotted. One sample t and Wilcoxon tests on log-transformed data were used to analyze the significant difference between adjuvanted and protein alone groups in panel A (* $p < 0.05$, ** $p < 0.01$). There are no significant differences between groups when analyzed by one-way ANOVA with Tukey's multiple comparisons test in panel B. (C) The correlation between the neutralization titers of pooled serum samples from the same group to rVSV-SARS-CoV-2 and those to WT SARS-SARS-CoV-2 was examined by Pearson correlation analysis. The PRNT₅₀ values are listed in Table 1.

was observed, indicating further opportunity for formulation optimization.

The decrease in neutralization capacity of the humoral response against the B.1.351 Beta and P.1 Gamma VOC is consistent with clinical data of vaccines using the pre-fusion stabilized spike [34,60] that are still protective with only a marginal decrease in efficacy [61–64]. Specifically, NVX-CoV2373 vaccine, a subunit vaccine in a Phase 1/2 clinical trial where the most common circulating strain is the B.1.351 Beta VOC showed sufficient protection against COVID-19 [65]. It is likely that a similar vaccine formulated with CoVaccine HTTM, based on our sustained neutralization results, may yield similar or better results in an appropriate challenge model.

In addition to improved kinetics, the addition of CoVaccine HTTM modulated the humoral response more towards Th1 type relative to protein alone, as indicated by higher levels of IgG2a and IgG2b. Antigen-specific IFN- γ production after splenocyte restimulation *ex vivo* was more variable, but also increased with the use of adjuvant, particularly after two doses, and was not strongly dependent on adjuvant concentration. These robust responses in outbred mice observed with relatively low antigen and adjuvant doses are particularly encouraging in light of our previous work which has demonstrated the successful thermostabilization of this adjuvant in combination with other glycoprotein antigens [66]. More recently, we have demonstrated that these recombinant subunits, in combination with CoVaccine HTTM, can be formulated as a

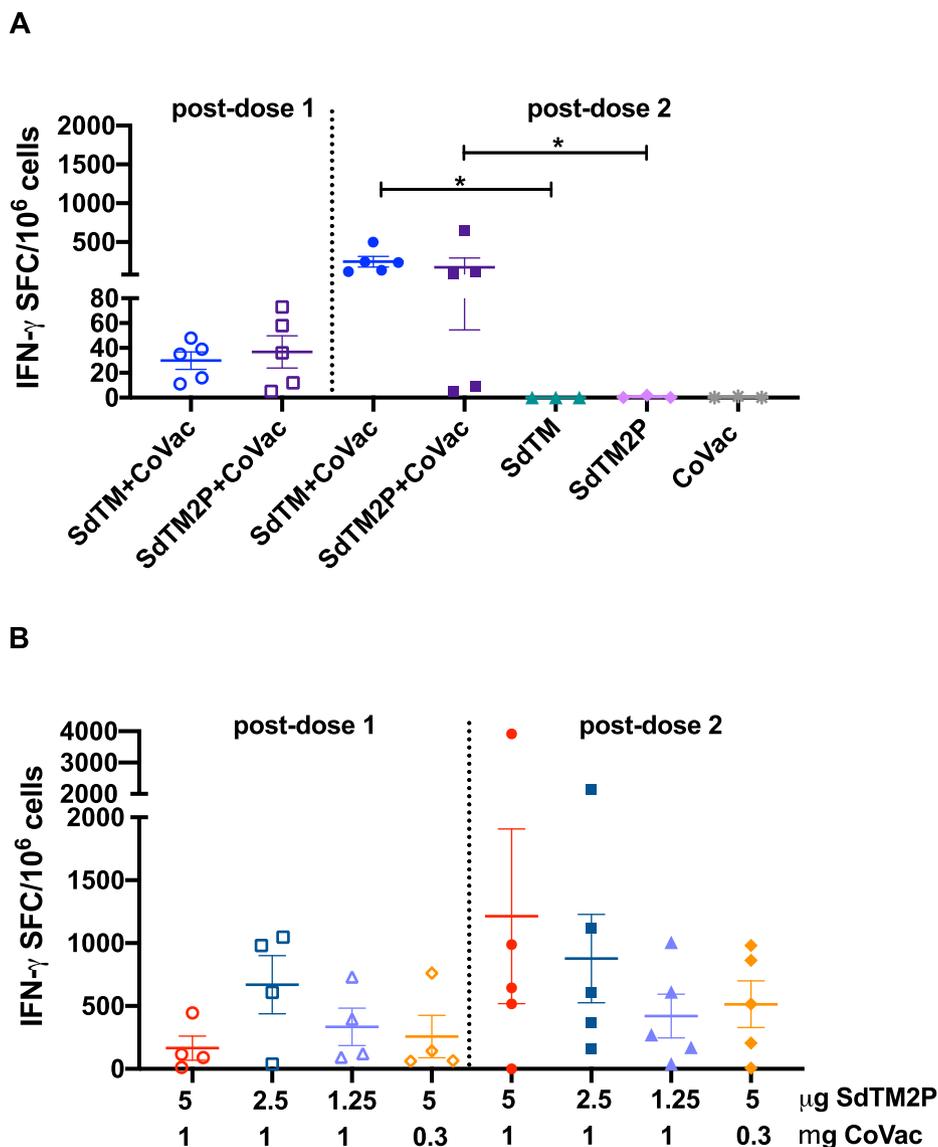


Fig. 4. Detection of IFN- γ secreting cells from mice immunized with SARS-CoV-2 vaccines. The splenocytes were obtained from mice ($n = 3$ or 5 per group) immunized with one dose (open symbols) or two doses (closed symbols) of (A) either $5 \mu\text{g}$ of SARS-CoV-2 SdTM or SdTM2P proteins (purified by hACE2 AC) adjuvanted with 1 mg of CoVaccine HTTM (CoVac) or (B) different dosages ($5, 2.5,$ or $1.25 \mu\text{g}$) of SdTM2P (purified by mAb IAC) with CoVac (1 or 0.3 mg). The cells were incubated for 24 h with medium or a peptide pool covering the S protein of SARS-CoV-2 ($10 \mu\text{g}/\text{mL}$), and the IFN- γ secreting cells were enumerated by FluoroSpot. The data are shown as the mean values of triplicate assays from individual animals using the number of spot forming cells (SFC) per 10^6 splenocytes after subtraction of the number of spots formed by cells in medium control wells (<5 spots). Bars represent mean \pm SEM of each group. Significant differences in numbers of IFN- γ secreting cells between groups receiving 2 doses of vaccines with or without adjuvant in panel A was determined by Mann-Whitney t test ($*p < 0.05$). There are no significant differences between groups given either one or two doses of vaccines when analyzed by one-way ANOVA with Tukey's multiple comparisons test in panel B.

in the collection, analysis and interpretation of data; in writing of the report; and in the decision to submit the article for publication.

CRediT authorship contribution statement

Chih-Yun Lai: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Albert To:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Teri Ann S. Wong:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Michael M. Lieberman:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **David E. Clements:**

Methodology, Resources, Writing – review & editing. **James T. Senda:** Methodology, Resources, Writing – review & editing. **Aquena H. Ball:** Investigation, Writing – original draft. **Laurent Pessaint:** Investigation. **Hanne Andersen:** Investigation. **Wakako Furuyama:** Resources. **Andrea Marzi:** Resources. **Oreola Donini:** Writing – original draft, Writing – review & editing. **Axel T. Lehrer:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Axel T. Lehrer and Oreola Donini are named inventors on a patent application covering a recombinant subunit vaccine for SARS-CoV-2. David E. Clements and James T. Senda, are current

employees of Hawaii Biotech Inc. Laurent Pessaint and Hanne Andersen are current employees of BIOQUAL, Inc. Oreola Donini is a current employee of Soligenix Inc. We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Please adjust the text as necessary, but if your standard text shown in the proof is typically applied in such cases, this will be fine for us.

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