# Title

A Novel SARS-CoV-2 Multitope Protein/Peptide Vaccine Candidate is Highly Immunogenic and Prevents Lung Infection in an Adeno Associated Virus Human Angiotensin-Converting Enzyme 2 (AAV hACE2) Mouse Model

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# Abstract

In this report, we describe the initial development and proof-of-concept studies for UB-612, the first multitope protein-peptide vaccine against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the pathogen responsible for the Coronavirus Disease of 2019 (COVID-19). UB-612 consists of eight components rationally designed for induction of high neutralizing antibodies and broad T cell responses against SARS-CoV-2: the S1-RBD-sFc fusion protein, six synthetic peptides (one universal peptide and five SARS-CoV-2-derived peptides), a proprietary CpG TLR-9 agonist, and aluminum phosphate adjuvant. Through immunogenicity studies in guinea pigs and rats, we optimized the design of protein/peptide immunogens and selected an adjuvant system, yielding a vaccine that provided excellent S1-RBD binding and high neutralizing antibody responses, robust cellular responses, and a Th1-oriented response at low doses of the vaccine. Our candidate vaccine was then advanced into challenge studies, in which it reduced viral load and prevented development of disease in a mouse challenge model and in nonhuman primates (NHP, immunogenicity part is completed, challenge is ongoing). A GLP-compliant toxicity study has shown a favorable safety profile for the vaccine. With the Phase 1 trial ongoing in Taiwan and additional trials planned worldwide, UB-612 is a highly promising and differentiated vaccine candidate for prevention of SARS-CoV-2 transmission and COVID-19 disease.


# Main

## Introduction

Since the appearance of the first cluster of cases of the new coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), in Wuhan China, in December 2019[1], the ongoing pandemic has caused over 60 milion confirmed cases and >1.4 million deaths worldwide[2]. As of November 25, 2020, more than 245,000 people have died from the coronavirus in the U.S, and the pace is likely to accelerate in the coming months. In response to this unprecedented public health crisis, researchers worldwide are developing hundreds of vaccines against SARS-CoV-2 using a wide range of vaccine platforms, some of which are already in late stages of efficacy trials. These include inactivated virus (Sinovac, Sinopharm), recombinant adenovirus-based vectors (CanSino, University of Oxford/AstraZeneca, Janssen, and Gamaleya), recombinant proteins (Novavax), and nucleic acids (Moderna's, Pfizer/BioNtech's and Curevac's mRNA vaccines and Inovio's DNA vaccine) approaches. Two mRNA vaccines developed by Pfizer/BioNtech and Moderna have shown to induce ~95% protective efficacy in large Phase 3 trials[3].

Like other coronaviruses, SARS-CoV-2 has a positive-sense single-stranded genomic RNA, approximately 30 kb in length, which is among the largest known viral RNA genomes. This genome encodes 29 proteins, of which four are structural: a spike (S), a membrane (M), an envelope (E), and a nucleocapsid (N) protein[4–6]. The S protein is a homotrimeric class I fusion glycoprotein that is divided into two functionally distinct parts (S1 and S2). The S1 protein bears the human angiotensin-converting enzyme 2 (hACE2) receptor binding domain (RBD) that directly engages the host cell receptors and therefore determines virus cell tropism and pathogenicity. The transmembare S2 subunit contains the fusion peptide, which mediates viral and host membrane fusion and thus facilitating viral entry[7].

Most of the vaccines in clinical trials target the full-length S protein only to induce a neutralizing antibody response. The induction of T cell responses would be limited compared to responses generated by natural multigenic SARS-CoV-2 infections.

The S1-RBD is a critical component of SARS-CoV-2. It is required for cell attachment and represents the principal neutralizing domain of the virus[8–10] of the highly similar SARS-CoV, providing a margin of safety not achievable with a full-length S antigen and eliminating the possibility of the potentially deadly side effects that led to withdrawal of an otherwise effective inactivated RSV vaccine[11,12]. Accordingly, the monoclonal antibodies for the treatment of newly diagnosed COVID-19, recently approved through FDA Emergency Use Authorization (Lilly's neutralizing antibody bamlanivimab, LY-CoV555 and REGN-COV2, casirivimab/imdevimab antibody cocktail), are all directed to S1-RBD[13]. Due to the clear advantages of a strong S1-RBD vaccine component, the primary immunogen for induction of neutralizing antibodies and a memory B cell response in UB-612, is S1-RBD-sFc; a recombinant protein made through a fusion of S1-RBD of SARS-CoV-2 to a single chain fragment crystallizable region (sFc) of a human IgG1. Genetic fusion of a vaccine antigen to a Fc fragment has been shown to promote antibody induction and neutralizing activity against HIV gp120 in rhesus macaques or Epstein Barr virus gp350 in BALB/c mice[14,15]. Moreover, engineered Fc has been used in many therapeutic antibodies as a solution to minimized non-specific binding, increase solubility, yield, thermostability, and *in vivo* half-life[16].

A neutralizing response against the S protein alone is unlikely to provide lasting protection against SARS-CoV-2 and its emerging variants with mutated B-cell epitopes[17]. A long-lasting cellular response could augment the initial neutralizing response (through memory B cell activation) and provide much greater duration of immunity as antibody titers wane. Recent studies have demonstrated that IgG response to S declined rapidly in >90% of SARS-CoV-2 infected individuals within 2-3 months[8]. In contrast, memory T cells to SARS were shown to endure 11-17 years after 2003 SARS outbreak[18,19]. S is a critical antigen for elicitation of humoral immunity which mostly contains CD4+ epitopes[20]. Other antigens are needed to raise/augment cellular immune responses to clear SARS-CoV-2 infection. The vast majority of reported CD8+ T cell epitopes in SARS-CoV-2 proteins are located in ORF1ab, N, M, and ORF3a regions; only 3 are in S, with only 1 CD8+ epitope being located in the S1-RBD[21]. The smaller M and N structural proteins are recognized by T cells of patients who successfully controlled their infection[21,22]. In a study of nearly 3,000 people in the UK, it was found that individuals with higher numbers of T cells were more protected against SARS-CoV-2 compared to those with low T cell responses, suggesting that T cell immunity may play a critical role in preventing COVID-19[23].

To provide immunogens to elicit T cell responses, Th/CTL epitopes from highly conserved sequences derived from S, N and M proteins of SARS-CoV and SARS-CoV-2[24–31]were identified after extensive literature search. Five peptides within these regions were selected and subject to further designs. Each selected peptide contains Th or CTL epitopes with prior validation of MHC I or II binding and exhibits good manufacturability characteristics (optimal length and amenability for high quality synthesis). These rationally designed Th/CTL peptides were further modified by addition of a Lys-Lys-Lys tail to each respective peptide's N-terminus to improve peptide solubility and enrich positive charge for use in vaccine formulation[32].

To enhance the immune response, we added our proprietary peptide UBITh®1a to the peptide mixture. UBITh®1a is a proprietary synthetic peptide with an original framework sequence derived from the measles virus fusion protein (MVF). This sequence was further

modified to exhibit a palindromic profile within the sequence to allow accommodation of multiple MHC class II binding motifs within this short peptide of 19 amino acids. A Lys-Lys-Lys sequence was added to the N terminus of this artificial Th peptide as well to increase its positive charge thus facilitating the peptide's subsequent binding to the highly negatively charged CpG oligonucleotide molecule to form immunostimulatory complexes through "charge neutralization". In previous studies, attachment of UBITh®1a to a target "functional B epitope peptide" derived from a self-protein rendered the self-peptide immunogenic, thus breaking immune tolerance[33]. The Th epitope of UBITh®1 has shown this stimulatory activity whether covalently linked to a target peptide or as a free charged peptide, administered together with other designed target peptides, that are brought together through the "charge neutralization" effect with CpG1, to elicit site-directed B or CTL responses. Such immunostimulatory complexes have been shown to enhance otherwise weak or moderate response of the companion target immunogen[34]. CpG1 is designed to bring the rationally designed immunogens together through "charge neutralization" to allow generation of balanced B cells (induction of neutralizing antibodies) and Th/CTL responses in a vaccinated host. In addition, activation of TLR-9 signaling by CpG is known to promote IgA production and favor Th1 immune response[35]. UBITh®1 peptide is incorporated as one of the Th peptides for its "epitope cluster" nature to further enhance the SARS-CoV-2 derived Th and CTL epitope peptides for their antiviral activities.

Finally, to further improve the immune response, UB-612 is formulated with aluminum phosphate (Adju-Phos®) adjuvant. Aluminum phosphate serves as a Th2 oriented adjuvant via the nucleotide binding oligomerization domain (NOD) like receptor protein 3 (NLRP3) inflammasome pathway. Additionally, it has pro-phagocytic and repository effects with a long record of safety and the ability to improve immune responses to target proteins in many vaccine formulations[36,37].

In this report, we describe the selection of S1-RBD-sFc protein among three candidates having different structures within the Fc-fusion protein design category, by vaccinating guinea pigs (GP) with three CHO cells-transiently expressed proteins. The lead candidate was chosen, based on having the highest neutralization and ACE2:RBD inhibition titers, and further formulated with Th/CTL peptides (UB-612)[32], with its immunogenicity and efficacy confirmed in rats, mice and rhesus monkeys. The results of a GLP repeated dose toxicology study in rats indicate that the UB-612 vaccine product is safe and well tolerated and also induced potent neutralizing antibodies.

## Results

### *Construction and in vitro Characterization of S1-RBD-sFc*

Three S1-RBD-based protein antigen sequences (aa340-539) were designed: S1-RBD-sFc (single chain Fc), S1-RBDa-sFc (RBD domain modified to reduce a Cys-disulfide bond for better domain folding), and S1-RBD-Fc (double chain Fc) (structure of S1-RBD-sFc illustrated in Figure 1B). These synthetic genes were expressed in Chinese Hamster Ovary (CHO) cells by transient transfection to generate proteins for initial studies. Each protein was used for studies in guinea pigs, which led to selection of S1-RBD-sFc as the lead B cell immunogen candidate as described in the following section.

After selection of S1-RBD-sFc as the lead candidate, a stable cell line was generated through transfection of CHO cells followed by selection and DHFR amplification. A stable high-expressing clone was isolated, and a cell bank was produced using standard stable CHO methods. The protein was produced in a suspension culture and purified. The

purified protein was then characterized. Peptide mapping, N- and C-terminal amino acid sequencing, and analysis of disulfide bonding and glycosylation reveal that the expressed and purified protein conforms to the predicted characteristics (data not shown). Size-exclusion chromatography (SEC), analytical ultracentrifugation, and capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) experiments demonstrated that S1-RBD-Fc exists in two major isoforms, termed S1-RBD-sFc-1 and S1-RBD-sFc2, corresponding to N-linked and O-linked glycoforms of the protein (data not shown). Because the RBD of SARS-CoV-2 binds to hACE2, measurement of binding to hACE2 is a relevant method to demonstrate that S1-RBD-Fc is in a structure representing that of SARS-CoV-2 spike protein. The binding activity of the vaccine was tested in an hACE2 ELISA and was demonstrated to bind hACE2 with an EC50 of 8.477 ng/mL, indicative of high affinity (Figure 1C). The molecular mass of S1-RBD-sFc protein is about 50 kDa. S1-RBD-sFc protein contains 431 amino acid residues with 12 cysteine residues (Cys6, Cys31, Cys49, Cys61, Cys102, Cys150, Cys158, Cys195, Cys246, Cys306, Cys352 and Cys410), forming 6 pairs of disulfide bonds (Cys6-Cys31, Cys49-Cys102, Cys61-Cys195, Cys150-Cys158, Cys246-Cys306 and Cys352-Cys410).

***Modification of human IgG Fc portion.***

S1-RBD-sFc consists of the RBD (aa340-539 of SARS-CoV-2 S protein), linked with a human IgG1 sFc at the C-terminus. The RBD domain functions as a high-affinity ligand for hACE2 cell receptors[7]. The IgG1 sFc domain was engineered to contain mutations, C220S, C226S, C229S, and N297H, in order to eliminate the disulfide bonds and N-glycan, respectively. S1-RBD-sFc contains 12 cysteine residues, forming 6 pairs of disulfide bonds. There is one N-glycosylation site $Asn_{13}$ on the RBD domain and two O-glycosylation sites $Ser_{211}$ and $Ser_{224}$ on a sFc fragment. Glycosylation of an IgG Fc fragment on a conserved asparagine residue, $Asn_{297}$ (EU-index numbering), is an essential factor for the Fc-mediated effector functions such as complement dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC)[38,39]. The Fc fragment in S1-RBD-sFc is designed for purification by protein A affinity chromatography. In addition, the glycosylation site at $Asn_{297}$ of the heavy chain was removed through mutation to His (N297H)[40,41] to prevent the depletion of target hACE2 through Fc-mediated effector functions.

***Down-Selection of RBD Design through Testing in Guinea Pigs***

A single protein construct was selected through immunogenicity testing in guinea pigs (GPs). Three groups of GPs (N=5/group) were vaccinated at 0 and 3 weeks post initial immunization (WPI) intramuscularly (IM) with three S1-RBD-based protein immunogens formulated with ISA50 V2 adjuvant. Sera were drawn at three time points (0, 3, and 5 wpi), and tested for immunogenicity through measurement of binding antibodies (BAbs) by ELISA. As shown in Figure 2A, all constructs elicited high BAb responses to SARS-CoV-2 S1 protein at 3- and 5-weeks post initial immunization (WPI). Of the three constructs tested, S1-RBD-sFc induced the highest immune responses, with geometric mean titer (GMT) nearly 5 $\log_{10}$ at 3 weeks post immunization (WPI) and 6 $\log_{10}$ at 5 WPI. The difference between S1-RBD-sFc and S1-RBDa-sFc at 3 WPI was statistically significant ($p \leq 0.05$), indicating that all constructs were highly immunogenic with S1-RBD-sFc apparently holding a slight advantage in terms of BAb responses. At 5 WPI, however, no significant difference was notable for the S1-RBDa-sFc vs. S1-RBD-Fc ($p > 0.99$) and the S1-RBD-sFc vs. S1-RBD-Fc ($p = 0.20$).

The function of anti-RBD antibodies was quantified both as inhibition of RBD binding to hACE2 and as neutralization of live SARS-CoV-2. Both measurements are important and

complementary. The major mechanism of action of anti-RBD antibodies is the inhibition of binding of RBD to hACE2, therefore, an hACE2 binding assay would serve as a surrogate measurement of antibody neutralization. Cell-based neutralization assays provide a clearer and more direct, but lower-throughput, measurement of the neutralization potential of anti-RBD antibodies. Both types of tests were used to demonstrate the functional activity of antibodies raised by our protein antigens.

The ability of RBD-Fc-elicited antibodies to inhibit SARS-CoV-2 binding to hACE2 was investigated using two methods, an ELISA and a cell-based assay. In the ELISA, hACE2 was coated onto plates, and individual sera were tested for their ability to prevent binding of SARS-CoV-2 S-1 RBD protein to the coating antigen. The mean ID50 inhibition (50% GMT) values were statistically ($p \leqq 0.05$) the highest for antibodies raised by S1-RBD-sFc (7,984), followed by S1-RBDa-sFc (1,950) and S1-RBD-Fc (1,939), indicating that all antigens elicited antibodies capable of inhibiting hACE2 inhibition, with S1-RBD-sFc raising the most potent responses. In the cell-based assay, HEK293 cells expressing hACE2 were treated with mixtures of pooled guinea pig sera and S1-protein(Fc tagged), then assayed by flow cytometry (FACS) by staining cells with fluorescently labeled anti-human IgG Fc protein antibody. In this test, the GMT ID50 values for antibodies raised were 202 for S1-RBD-sFc, 69.2 for S1-RBDa-sFc, and 108 for S1-RBD-Fc vaccine, again showing functional activity for antibodies elicited by all candidates with S1-RBD-Fc having the highest activity. Results of the ELISA and cell-based assays are provided in Figures 3A, B and 3C, D, respectively.

Neutralizing activity of the elicited antibodies was tested using a live virus Cytopathic Effect (CPE) assay. Again, the sera from S1-RBD-sFc demonstrated superior activity, with neutralization titers at five weeks post initial immunization (WPI) more than twice as high as those from the other two groups, protecting the cells from viral infection at serum dilution folds of at least 504-1024 at 3 WPI and >8,192 at 5 WPI (Table 1). Further confirmation of the neutralizing potency of the antibodies was provided by a separate CPE study with anti-SARS-CoV-2 N protein antibody and immunofluorescent visualization. Again, a complete neutralization of SARS-CoV-2 ($VNT_{100}$) was observed at a 1:32,768-fold dilution of animal sera in samples from animals immunized with S1-RBD-sFc fusion protein at 5 WPI (Figure 4).

**Table 1. Titers of neutralizing antibodies in GP sera after 1 dose (at 3 WPI) or two doses (at 5 WPI) by CPE Assay[a]**

| Group # | Immunogens in ISA50 V2 | Neutralizing antibody titer guinea pig immune sera | | |
|---|---|---|---|---|
| | | 0 WPI | 3 WPI | 5 WPI |
| G1 | S1-RBD-sFc | <4 | 512-1024 | >8192 |
| G2 | S1-RBDa-sFc | <4 | 128 | 4096 |
| G3 | S1-RBD-Fc | <4 | 256 | 4096 |

[a:] The assay was conducted in BSL-3 lab at Academia Sinica, Taipei.

We compared neutralizing titers in sera from guinea pigs immunized with S1-RBD-sFc against those in convalescent sera of COVID-19 patients. Using the S1-RBD:ACE2 binding inhibition ELISA (also termed as qNeu ELISA), we compared responses in guinea pigs against those in convalescent sera from Taiwanese COVID-19 patients after

discharge from hospitalization. The results, given in Figure 5, demonstrated that guinea pig immune sera diluted 1,000-fold (3 WPI) or 8,000-fold (5 WPI) exhibited comparable or higher inhibition of S1-RBD:ACE2 binding than by the convalescent sera of 10 patients diluted at 20-fold, illustrating that the sera of guinea pigs contained ≥50-fold higher antibody titers than human convalescent sera.

To further verify the neutralizing titers obtained by the CPE assay and IFA, 10 samples (positive and negatives) were blind coded and sent to Dr. Alexander Bukreyev's laboratory at the University of Texas Medical Branch (UTMB) in Galveston, TX. These were tested in a replicating virus neutralization assay and the $VNT_{50}$ titer for each sample was calculated. The results showed a strong correlation (r=0.9400) between the two assays performed at UTMB and Academia Sinica (Figure 6).

***Immunogenicity Studies in Rats***

The initial immunogenicity assessment in guinea pigs established the humoral immunogenicity of our RBD-based protein and allowed selection of S1-RBD-sFc as the B cell component of our vaccine against SARS-CoV2.

In a second set of experiments conducted in rats, our proprietary Th/CTL peptides and CpG1 were added to the S1-RBS-sFc B cell component for further assessment of optimal formulations and adjuvants and establishment of the cellular immunity components of our vaccine.

The guinea pig experiments were tested with three protein candidates with a single dosing regimen (200 µg prime, 100 µg boost, ISA 50 adjuvant), allowing for a rigorous comparison of the respective candidate constructs. In our second set of experiments conducted in rats, we varied immunogen doses and adjuvants to allow selection of an optimal adjuvant based on S1-RBD binding antibody titers and balanced Th1/Th2 responses. S1-RBD-sFc was formulated with five Th/CTL peptides selected from S2, M and N proteins of SARS-COV-2 and our proprietary universal Th peptide (UBITh®1a)[32] to generate our multitope protein-peptide vaccine candidate (Figure 1A). We then combined our candidate vaccine with two different adjuvant systems, ISA51/CpG3 and Adju-Phos®/CpG1. These vaccine-adjuvant combinations were administered to rats IM on 0 WPI (prime) and 2 WPI (boost) with a wide dose range of 10 to 300 µg per injection. The animals were bled at 0, 2 (i.e., after 1 dose), 3 and 4 WPI (i.e., 1 and 2 weeks after the 2$^{nd}$ dose) for antibody titer analyses.

Results of BAb testing at all time points demonstrated that vaccines formulated with both adjuvant systems elicited similar levels of anti S1-RBD ELISA titers across all doses ranging from 10 to 300 µg, indicative of an excellent immunogenicity of the vaccine formulations even with low quantities of the primary protein immunogen (Figure 7A). In addition, a 100-µg dose of S1-RBD-sFc without the synthetic peptide components stimulated high S1-RBD binding activity similar to previous guinea pig studies (data not shown).

In the S1-RBD:ACE2 binding inhibition ELISA test, doses of 10 and 30 µg induced as strong inhibitory activity as the high doses at 100 and 300 µg at 4 WPI (Figure 7B). The most potent inhibitory activity was seen with the lowest dose of S1-RBD-sFc protein (10 µg) formulated with rationally designed peptides and the Adju-Phos®/CpG1 adjuvant. In the replicating virus neutralization assay against the Taiwanese SARS-CoV-2 isolate (as discussed above for guinea pig studies), the 4 WPI immune sera induced by UB-612 did not show a significant dose-dependent effect. However, low doses of adjuvanted

protein,10 and 30 μg, could neutralize viral infection at $VNT_{50}$ of >10,240 dilution fold (Figure 7C). The rat immune sera at 6 WPI from each vaccinated dose group were assayed, a. in comparison with a set of convalescent sera of COVID-19 patients for titers in S1-RBD:ACE2 binding inhibition ELISA, expressed in blocking level of μg/mL; and b. by a SARS-CoV-2 CPE assay in Vero-E6 cells, expressed as $VNT_{50}$. As shown in Figure 7D, all doses of the vaccine formulations elicited neutralizing titers in rats that are significantly higher than those in convalescent patients by S1-RBD:ACE2 binding ELISA and higher (but not achieving statistical significance due to the spread in the patient data and the low number of animals) by $VNT_{50}$.

To address the issue related to Th1/Th2 response balance, cellular responses in vaccinated rats were evaluated using ELISpot. Rats were dosed at 0 and 2 WPI with doses of protein in the range of 1 to 100 μg. Splenocytes were then collected at 4 WPI and restimulated in vitro at 2ug/well either with the Th/CTL peptide pool plus S1-RBD or with the Th/CTL peptide pool alone. A dose-dependent trend in IFN-γ secretion was observed in splenocytes, while little secretion of IL-4 was seen (Figure 8A)**.** The results indicated that UB-612 was highly immunogenic and induced a Th1-prone cellular immune response as shown by the high ratios of IFN-γ/IL-4 or IL-2/IL-4[42]. High ratios of IL-2/IL-4 were also observed in the presence of the Th/CTL peptide pool (Figure 8B) and for restimulation with individual peptides, which induced little IL-4 secretion (Figure 8C).

***Challenge Studies in Transduced Mice***

The initial challenge study of UB-612 was performed in the AAV/hACE2 transduced BALB/c mouse model established by Dr. Tau, Mi-Hua at Academia Sinica in Taiwan; adaptations of this model are also reported by other investigators[43]. Groups of 3 mice were vaccinated at study 0 and 2 WPI with UB-612 containing 3, 9 or 30 μg of protein and formulated with Adju-Phos®/CpG1. The mice were infected with adeno-associated virus (AAV) expressing hACE2 at 4 WPI and challenged 2 weeks later with $10^6$ $TCID_{50}$ of SARS-CoV-2 by the intranasal (IN) route (Figure 9A). Efficacy of the vaccine was measured using lung viral loads and body weight measurements. As shown in Figure 9B, vaccination with 30 μg of UB-612 significantly reduced lung viral loads (~3.5 $\log_{10}$ viral genome copies/ug RNA or ~ 5-fold $TCID_{50}$/mL of infectious virus) compared to saline group ($p < 0.05$ as measured by paired t test). As shown in Figure 9C, vaccination with middle and high doses led to clear reduction in lung pathology. Vaccination with 3 or 9 ug of UB-612 reduced live virus detection by cell culture method ($TCID_{50}$) to below of the level of detection (LOD, Fig 9). But it did not appear to reduce viral loads significantly when measured by RT-PCR. Similarly, body weight measurements showed a significant difference between the high-dose group and the control group (data not shown). In sum, despite the lack of an statistical power (N=3 mice) in this study, it appears that the highest dose at 30ug per dose could have had the maximum protective efficacy when one combines the lack of live virus detection and the lack of inflammatory cell infiltrations as well as lack of immunopathology in the lungs altogether.

***Immunogenicity and Challenge Studies in Rhesus Macaques***

Based on an established model using rhesus macaques (RM)[44,45], an immunization study of UB-612 by IM injection was initiated with RM (N = 4/group) receiving 0, 10, 30 or 100 μg of UB-612 at 0 and 4 WPI. Immunogenicity measurements indicated that the serum IgG binding to S1-RBD was increased over baseline in all animals with binding titers reaching around 3 logs at 5 and 7 WPI (Figure 10A). Strong neutralizing antibody responses were induced, with the 30 μg dose being most potent (Figure 10B). ELISpot

analysis indicated that UB-612 activated antigen-specific IFN-γ-secreting T cells in a dose-dependent manner (Figure 10C). T cell responses were highest at the 100 μg dose level.

***Toxicity Study in preparation for Clinical Trials***

To enable clinical trials, UB-612 was tested in a GLP-compliant repeat-dose toxicology study in Sprague-Dawley rats. The study included a 300 ug dose, 3 times higher than that of the highest dose intended for clinical use. Although the schedule of 2 injections did not exceed that intended for clinical use, this is acceptable according to the WHO guidelines[46]. The study was also designed to evaluate the immunogenicity of the UB-612 vaccine. One hundred and sixty (160) rats were randomly divided into 8 groups (80 males and 80 females) of which 40 rats were included in the satellite immunogenicity study. The low- and high dose groups were inoculated with UB-612 at 100 μg/animal (0.5 mL) and 300 μg/animal (0.5 mL) respectively; control groups were injected either with saline (0.9% saline) or adjuvant (UB-612 Placebo) at the same dose volume. The first ten animals/sex/group were designated for the terminal necropsy after two weeks of dosing at 2 WPI (Day 18) and the remaining 20 animals/sex/group were designated for the 4-week recovery necropsy after the last dosing at 4 WPI (Day 44). Under the experimental conditions, rats received IM injections into one hind limb muscle (quadriceps femoris and gastrocnemius, left side for the first dose and right side for the second dose) at multiple sites once every two weeks for 2 consecutive weeks, total 2 doses at 0 and 2 WPI (on Days 1 and 15).

Treatment with UB-612 at dose levels of up to 300 μg/animal at weeks 1 and 3 was well tolerated with no signs of systemic toxicity. Neither test article-related mortality nor moribundity was noted throughout the study. No UB-612-related abnormal findings were noted in clinical observations (including injection site observations) throughout the study. Neither erythema nor edema were noted at injection sites, and the Draize score was 0 for all observation time points. Similarly, no UB-612 related changes in body weight, food consumption, body temperature, hematology, chemistries (other than AG ratio), ophthalmoscopic examinations or urinalysis were observed, and no statistically significant changes were noted in CD3+, CD3+CD4+, CD3+CD8+, and the ratio of CD3+CD4+/CD3+CD8. Statistically significant increases were seen in fibrinogen, IFN-γ, and IL-6, while decreases in albumin/globulin ratio were observed; these results are consistent with an acute phase response to a vaccine, and all resolved by the end of the recovery period. Histopathological examinations of epididymides, skin, liver, prostate and mammary gland, revealed minimal inflammatory cell infiltrations with no visible lesions or abnormalities.

Immunogenicity of UB-612 measured in satellite groups showed that the vaccine was able to induce substantial levels of anti-SARS-CoV-2 S1-RBD IgG in animals receiving two doses of 100 μg/animal or 300 μg/animal at 2 and 4 WPI (a 14-day interval) (data not shown). The S1-RBD binding IgG titers rose modestly over time after the boost at 2 WPI (Day 15), which reached around 2.6 $\log_{10}$ and 3.3 $\log_{10}$ in rats immunized with UB-612 at 100 μg/animal and 300 μg/animal, respectively, at 6 WPI (Day 44). The findings observed in this study are as expected for a vaccine designed to stimulate immune responses resulting in production of high titers of antibodies. Anti-SARS-CoV-2 S1-RBD IgG titers, subtype IgG and serum cytokine production by ELISA were performed to determine the Th1/Th2 responses. On analyses of S1-RBD-specific IgG subclasses, the patterns and induction levels of Th2-related subclass $IgG_1$ anti-SARS-CoV-2 S1-RBD were comparable to what was observed in total IgG anti-SARS-CoV-2 S1-RBD. Only slight induction of Th1-related subclass $IgG_{2b}$ anti-SARS-CoV-2 S1-RBD was detected in rats vaccinated with

UB-612 at 6 WPI (Day 43). However, the serum cytokine pattern measured by ELISA indicated a Th1/Th2 balanced response (data not shown).

Clinical trials of UB-612 have begun in Taiwan. The first study, entitled "Phase 1, Open-Label Study to Evaluate the Safety, Tolerability, and Immunogenicity of UB-612 Vaccine in Healthy Adult Volunteers", was initiated in Taiwan in September 2020. This trial includes three dose groups (N=20 per group) of UB-612 (10, 30, or 100 μg) given at days 1 and 29 (2 dose regimen). The primary endpoint is the occurrence of adverse events within seven days of vaccination; secondary endpoints include adverse events during the six-month follow-up period, standard laboratory safety measures, antigen-specific antibody titers, seroconversion rates, T cell responses and increase of neutralizing antibody titers.

## Discussion

Successful vaccines against a number of other viral diseases have typically protected through elicitation of neutralizing antibody responses, and neutralizing antibodies are likely to be critical for any successful COVID-19 vaccine. However, neutralizing antibodies may fully protect when levels are high, but not sufficient for full protection over time. Under this circumstance, T cell responses may be important to maintaining protection. Falling neutralizing titers also raise the possibility of antibody-dependent enhancement (ADE) of infection or concerns over Vaccine Associated Enhanced Respiratory Disease (VAERD) by SARS-CoV-2[12,47,48]. UB-612 was developed specifically to address the need for a vaccine that elicits a strong, but ADE-free (by avoiding amino acid residues 597-603, located in the S2 subunit, which were implicated in ADE of SARS-CoV in vitro and in NHP[11]) neutralizing response while also eliciting robust and long-lasting T cell responses.

In this report, we described the initial development and proof-of-concept studies for UB-612, the first multitope protein/peptide-based vaccine against SARS-CoV-2, the pathogen responsible for COVID-19.

Initially, we developed three RBD-sFc fusion protein vaccine candidates. Engineered Fc has been used safely in many commercialized therapeutic antibodies including TNF-alpha, to improve their solubility, half-life, yields and thermostabilities[39].

However, we deimmunized our single chain Fc construct by introducing mutation to change the Asparagine (N) at position 297 to Histidine (H) (N297H), which removes the N-link glycosylation site and abrogates Fc-mediated effector functions while retaining its ability to bind to protein A for purification purposes. We then tested these candidates and down selected to a single candidate through immunogenicity testing in GPs. Immunogenicity results showed robust S1-RBD binding antibody responses and functional activity including neutralization of live SARS-CoV-2 and inhibition of hACE2:RBD binding. Of the three candidates tested, S1-RBD-sFc (S1-RBD fused to a single-chain Fc) gave the strongest responses in all measurements. S1-RBD-sFc was slightly more immunogenic than the other two constructs in S1-RBD binding antibody assays, but the strength of the S1-RBD-sFc immunogen became abundantly clear when functional activity of elicited antibodies was tested. Function of anti-RBD antibodies was quantified as inhibition of viral binding to hACE2 and as neutralization of live SARS-CoV-2. In both tests, all antigens elicited functional antibodies, with S1-RBD-sFc raising the most potent responses. Based on these results, the S1-RBD-sFc protein was selected as the lead candidate for our B cell component of the vaccine.

With S1-RBD binding and neutralizing antibody results indicating superiority of S1-RBD-sFc in all measurements tested, we obtained further confirmation of the immunogenicity of this vaccine through a comparison of neutralizing titers in sera from GPs immunized with this vaccine against those in convalescent sera of COVID-19 patients. The results demonstrated that highly diluted GP immune sera (e.g. 1:1000 from one dose or 1:8000 from 2 dose immunizations) exhibited comparable or higher inhibition of S1-RBD:ACE2 binding than by the convalescent sera of 10 patients diluted 20-fold. This finding is of clinical significance since it suggests that an S1-RBD-sFc-based vaccine will be capable of eliciting sufficient neutralizing antibodies to prevent SARS-CoV-2 infection.

In sum, the results from the immunogenicity testing indicated that all three vaccine formulations were immunogenic, with S1-RBD-sFc having clear advantages in terms of S1-RBD binding antibody titer, inhibition of ACE2 binding by SARS-CoV-2 S1-RBD protein, and neutralization of live SARS-CoV-2.

S1-RBD-sFc was then combined with rationally designed Th/CTL peptides, derived from S2, M and N structural proteins of SARS-CoV-2, to generate the final unadjuvanted vaccine candidate, which was studied in Sprague Dawley rats to compare two adjuvant combinations (ISA 51VG/CpG3 and Adju-Phos®/CpG1).

In the initial rat study, which tested the two vaccine-adjuvant combinations with a wide dose range of 10 to 300 μg per injection, binding antibody results demonstrated that vaccines formulated with both adjuvant systems elicited similar binding titers across all doses, indicating excellent immunogenicity of the vaccine even with very low quantities of the primary protein immunogen. As in the earlier guinea pig studies, however, functional antibody assays demonstrated clear differences between candidate vaccine formulations. These tests showed the equivalency in immunogenicity between the two adjuvant combinations while confirming the excellent immunogenicity of very low doses of the protein vaccine. Due to the long safety record and the ability to improve immune responses to target proteins of Alum based adjuvant in many vaccine formulations[36,37], Adju-Phos®/CpG1 was chosen to be the adjuvant in our final vaccine formulation. Neutralization results indicated that even very low doses of adjuvanted protein (10 and 30 μg) showed excellent neutralizing immunogenicity. All doses of protein elicited neutralizing titers significantly higher than those in convalescent patients by S1-RBD:ACE2 binding ELISA and higher (but not achieving statistical significance due to the spread in the patient data and the low number of animals) by $VNT_{50}$. Currently, we are performing efficacy experiments using AAV hACE2 C57/Bl6 mouse model to demonstrate that the presence of the Th/CTL peptides in the vaccine product may be indispensable.

Th1 orientation of immune response against SARS-CoV-2 is potentially important to avoid ADE or VAERD, due to prior experience with SARS and MERS coronaviruses as well as a commercial formalin inactivated RSV vaccine (inducing a Th2 biased response), leading to death of several vaccinated children who were later exposed to live RSV[12]. Despite that the short-term protection data from SARS-CoV-2 manufactures have not shown any indication of ADE or VAERD potential in animals or humans, FDA has recommended that any vaccines for COVID-19 should provide data for induction of a Th1 biased immune responses in animals before proceeding to FIH trials[49]. Therefore, the Th1/Th2 immune balance is a characteristic important to the safety of any novel vaccine against COVID-19. To address this issue, T cell immunity of the vaccinated animals were assessed in rats upon restimulation of splenocytes harvested from vaccinated animals with the respective B and T cell vaccine components. High ratios of IFN-g/IL4 and IL-2/IL-4 were also observed in the presence of the Th/CTL peptide pool and for restimulation with individual

peptides, which induced little IL-4 secretion (data not shown). Thus, UB-612 vaccination can induce a robust Th1-prone cellular immune response, likely due to presence of CpG1[50], even under the influence of a Th2-biased alum-containing adjuvant system[36,51].

Due to the novelty of SARS-CoV-2, established "gold standard" models are not yet available, and researchers are working quickly to develop and validate animal models. Wild-type mice are not a suitable host for SARS-CoV-2; however, mice expressing hACE2 are susceptible to infection and disease from SARS-CoV-2[52]. A transient model of this type, using the AAV/hACE2 transduced BALB/c or C57BL/6 mice and intranasal (IN) challenge, was established by Dr. Tau, Mi-Hua at Academia Sinica in Taiwan; adaptations of this model are also reported by other investigators[43]. In this mouse model, productive infection with SARS-CoV-2 leads to high viral loads in lungs and weight loss, which can be used together with other clinical symptoms to assess safety, (e.g., lack of ADE or VAERD) and efficacy of vaccine candidates including UB-612.

We used the AAV/hACE2 transduced BALB/c mice to test the protective efficacy of UB-612. Mice were infected with AAV hACE2 at 4 WPI and challenged 2 weeks later with 106 TCID50 of SARS-CoV-2 by the IN route. Efficacy of the vaccine was measured using lung viral loads and body weight measurements. Results demonstrated the ability of UB-612 to reduce lung viral load and weight loss without induction of VAERD or immunopathology in the lungs; these results were statistically significant at the highest 30ug dose tested and showed a dose dependent protective response. The mouse challenge results therefore confirmed that the responses raised by UB-612 are in fact protective in vivo.

While SARS-CoV-2 does not cause a lethal COVID-19-like disease in monkeys, the virus is capable of causing illness in macaques, perhaps more so in rhesus than in cynomolgus macaques (RM). In both species, the disease is generally mild, self-limiting and overcome within 2 weeks[53–55]. Nonetheless, NHP testing of COVID-19 vaccines is extremely valuable because NHP provide the model closest to humans. UB-612 was tested in an RM model with animals receiving 3 vaccinations at three dose levels. Immunogenicity results demonstrated that S1-RBD binding antibodies were raised in all vaccinated animals and that the immune sera could induce potent neutralization effects. Currently, RM have been challenged by the intratracheal route and efficacy will be determined in the near future.

In GLP toxicity testing, UB-612 (inoculated to rats at a dose 3x higher than the highest dose intended to be used in humans) showed a very benign safety profile with no vaccine-related abnormal clinical observations, changes in body or organ weight, food consumption, or any other measure potentially indicative of a toxicity signal. The observed changes were consistent with acute phase response to a vaccine, and all resolved by the end of the recovery period. The FIH clinical trials of UB-612 have begun in Taiwan. This Phase 1 dose-escalation study is designed to evaluate the safety, tolerability and immunogenicity of 2 IM injections at 28-day intervals of UB-612 at three different dose levels (10, 30 and 100 μg) to healthy volunteers aged 20-55 years. Based on safety and immunogenicity profiles, the optimal vaccination regimens will be selected to proceed into expanded worldwide clinical testing with trials in the United States, India and Brazil scheduled for initiation in late 2020 or early 2021. An innovative feature of these future trials is the use of viral shedding as an efficacy endpoint. This endpoint will measure the ability of UB-612 to prevent viral transmission much more directly than other measurements such as viral load.

In summary, we have developed and demonstrated proof of concept for UB-612, a novel multitope protein-peptide vaccine being rapidly advanced in clinical trials for prevention of SARS-CoV-2 infection and COVID-19. We showed extremely high levels of neutralizing antibodies and Th1 prone immune response induced by the vaccine that protected animals, challenged by a high dose of SARS-CoV-2, without induction of immunopathology in the lungs.

## Materials and Methods

### *Vaccine Construction and Preparation*

To construct the vector expressing the recombinant fusion protein, the cDNA sequence encoding the S1-RBD-sFc protein was synthesized, digested, and then ligated into Freedom® pCHO 1.0 vector (Life Technology) to obtain the pCHO S1-RBD-sFc expression vector. The cDNA sequence was confirmed by DNA sequencing.

The S1-RBD-sFc protein was expressed in transient transfections of CHO cells for guinea pig and rat immunogenicity studies. Three variants of cDNA fragment for S1-RBD-sFc, S1-RBDa-sFc and S1-RBD-Fc fusion proteins were designed for transient expression in ExpiCHO-S system for target protein production. The cell culture was harvested after 12–14 days post-transfection, clarified by centrifugation and 0.22-μm filtration, and purified by protein A chromatography. The purity of the fusion proteins was determined on SDS gel, and protein concentration was determined according to the optical density (OD) of UV absorbance at a wavelength of 280 nm. To formulate S1-RBD-based protein vaccines at 200 μg/mL, equal volume of the immunogen (S1-RBD sFc, S1-RBDa-sFc or S1-RBD-Fc) in aqueous phase at 400 μg/mL and sterile water-in-oil adjuvant MontanideTM ISA50 V2 (Seppic) were mixed using two syringes attached to 3-way Stopcock and emulsified until no phase separation was seen. The emulsified vaccines were stored at 4°C before use.

For challenge studies and the rat toxicology study, the protein was expressed from a stable clone generated from the expression vector in CHO cells by standard transfection, cloning, and amplification processes. The protein was purified by multi-step column chromatography. Peptides and CpG1 were produced synthetically. Adju-Phos® was purchased ready to use.

### *Animal Procedures for Immunogenicity Studies*

*Immunogenicity studies in guinea pigs*

Male Hartley guinea pigs (200-250 gm/BW) were obtained from National Laboratory Animal Center (NLAC), Taiwan, and maintained in the laboratory animal center of UBIA. All procedures on animals were performed in accordance with the regulations and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at UBIAsia. The IACUC number is AT-2010. The guinea pigs were vaccinated intramuscularly at weeks 0 and 3 with Montanide™ ISA50 V2-adjuvanted S1-RBD-based proteins. The animals received the primary dose of 200 μg of the vaccine (four injection sites, 0.25 mL/site) at week 0 and were boosted with 100 μg of the vaccine (two injection sites, 0.25 mL/site) at week 3. The immune sera from guinea pigs (n = 5 for each protein immunogen) were collected at weeks 0, 3, and 5.

*Adjuvant selection in rats*

A total of 33 male Sprague Dawley rats at 8-10 weeks of age (300-350 gm/BW) were purchased from BioLASCO Taiwan Co., Ltd. After a 3-day acclimation, animals were randomly assigned to 11 groups. All procedures on animals were performed in accordance

with the regulations and guidelines reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at UBI Asia. The IACUC number is AT-2024. The rats were vaccinated intramuscularly at weeks 0 (prime) and 2 (boost) with different doses ranging from 10 to 300 μg of UB-612 formulated in Montanide™ ISA 51 VG/CpG3 or Adju-Phos®/CpG1 adjuvant. The immune sera from rats (n = 3 for each dose group) were collected at weeks 0, 2, 3, and 4 for assessment of antigenic and functional activities. Local irritation analysis was conducted via a modified Draize technique once daily within 24, 48 and 72 hrs after each vaccination. Additional abnormal clinical observations or lesions such as lameness, abscesses, necrosis or local inflammation were recorded.

*Rat Th1/Th2 balance study*

A total of 12 male Sprague Dawley rats at 8-10 weeks of age (300-350 gm/BW) were purchased from BioLASCO Taiwan Co., Ltd. After a 3-day acclimation, animals were randomly assigned to 4 groups. All procedures on animals were performed in accordance with the regulations and guidelines reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at UBIAsia. The IACUC number is AT-2028. The rats were vaccinated intramuscularly at weeks 0 (prime) and 2 (boost) with different doses ranging from 1 to 100 μg of UB-612 formulated in Adju-Phos®/CpG1 adjuvant. The immune sera from rats (n = 3 for each dose group) were collected at weeks 0, 2, 3, and 4 for assessment of antigenic activities.

*Immunogenicity studies in NHP*

The study was conducted at JOINN Laboratories (Beijing) in rhesus macaques aged approximately 3-6 years. Animals were housed individually in stainless steel cages, an environmentally monitored, and well-ventilated room (conventional grade) maintained at a temperature of 18-26°C and a relative humidity of 40-70%. Animals were quarantined and acclimatized for at least 14 days. The general health of the animals was evaluated and recorded by a veterinarian within three days upon arrival. Detailed clinical observations, body weight, body temperature, electrocardiogram (ECG), hematology, coagulation and clinical chemistry were performed on monkeys. The data were reviewed by a veterinarian before being transferred from the holding colony. Based on pre-experimental body weights obtained on Day -1, all animals were randomly assigned to respective dose groups using a computer-generated randomization procedure. All animals in Groups 1 to 4 was given either control or test article via intramuscular (IM) injection. Doses were administered to the quadriceps injection of one hind limbs. Monkeys were observed at least twice daily (AM and PM) during the study periods for clinical signs which included, but not limited to mortality, morbidity, feces, emesis, and the changes in water and food intake. Animals were bled at regular intervals for the immunogenicity studies described below.

Rhesus macaques (3-6 years old) were divided into four groups and injected intramuscularly with high dose (100 μg/dose), medium dose (30 μg/dose), low dose (10 μg/dose) vaccine and physiological saline, respectively. All grouped animals were immunized at three times (days 0, 28 and 70) before challenged with $10^6$ TCID50/ml SARS-CoV-2 virus by intratracheal routes (performed on day 82). Macaques were euthanized and lung tissues were collected at 7 days post challenge. At days 3, 5, 7 dpi, the throat swabs were collected. Blood samples were collected on days 0, 14, 28, 35, 42, 70 and 76 days post immunization, and 0, 3, 5, 7 days post challenge for neutralizing antibody test of SARS-CoV-2. Lung tissues were collected at 7 days post challenge and used for RT-PCR assay and histopathological assay. Analysis of lymphocyte subset

percent (CD3+, CD4+ and CD8+) and key cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-6) were also performed in collected blood samples on days 0 and 3 post challenge, respectively.

*Toxicology Studies*

A total of 160 rats (80/sex) were randomly assigned to 8 groups based on the body weights obtained on Day -1 (1 days prior to the first dosing, the first dosing day was defined as Day 1), of which 120 rats were assigned to Groups 1, 2, 3 and 4 (15/sex/group) for the toxicity study, and 40 rats to Groups 5, 6, 7 and 8 (5/sex/group) for the satellite study. Rats were treated with Saline Injection for Groups 1 and 5 as negative control, UB-612 Placebo for Groups 2 and 6 as adjuvant control, and UB-612 at doses of 100, 300 μg/animal for Groups 3 and 7 as well as Groups 4 and 8, respectively. Rats were treated via intramuscular injection into the one-side hind limbs muscle (quadriceps femoris and gastrocnemius, left side for the first dose and right side for the second dose) at multiple sites once every two weeks for 2 consecutive weeks, total 2 doses (on Days 1 and 15). The dose volume was 0.5 mL/animal. Clinical observations (including injection sites observation), body weight, food consumption, body temperature, ophthalmoscopic examinations, hematology, coagulation, clinical chemistry, urinalysis, T lymphocyte subpopulation, number of T lymphocyte spots secreting IFN-γ by peripheral blood mononuclear cells (PBMCs), cytokines, and immunogenicity, neutralizing antibody titer and $IgG_{2b}/IgG_1$ ratio analysis were performed during the study. The first 10 animals/sex/group in Groups 1 to 4 were designated for the terminal necropsy after 2 weeks of dosing (Day 18) and the remaining 5 animals/sex/group were designated for the 4-week recovery necropsy after the last dosing (Day 44). All animals in Groups 1 to 4 were given complete necropsy examinations, and then the organ weights, macroscopic and microscopic examinations were evaluated.

***Animal Procedures for Challenge Studies***

*Animal procedures for BALB/C challenge studies*

A total of 12 male BALB/C at 8-10 weeks of age were purchased from BioLASCO Taiwan Co., Ltd. After a 3-day acclimation, animals were randomly assigned to 4 groups. All procedures on animals were performed in accordance with the regulations and guidelines reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at UBI Asia. The IACUC numbers are AT2032 and AT2033.

The mice were vaccinated by IM route at weeks 0 (prime) and 2 (boost) with 3, 9, or 30 μg of UB-612 formulated in Adju-Phos®/CpG1 adjuvant. The immune sera from mice were collected at weeks 0, 3 and 4 for assessment of immunogenic and functional activities by the assay methods described below.

AAV6/CB-hACE2 and AAV9/CB-hACE2 were produced by AAV core facility in Academia Sinica. 8-10 weeks old BALB/C mice were anaesthetized by intraperitoneal injection of a mixture of Atropine (0.4 mg/ml)/Ketamine (20 mg/ml)/Xylazine (0.4%). The mice were then intratracheally (IT) injected with $3 \times 10^{11}$ vg of AAV6/hACE2 in 100 μL saline. To transduce extrapulmonary organs, $1 \times 10^{12}$ vg of AAV9/hACE2 in 100 μl saline were intraperitoneally injected into the mice.

Two weeks after AAV6/CB-hACE2 and AAV9/CB-hACE2 transduction, the mice were anesthetized and intranasally challenged with $1 \times 10^4$ PFU of the SARS-CoV-2 virus (hCoV-19/Taiwan/4/2020 TCDC#4 obtained from National Taiwan University, Taipei, Taiwan) in a volume of 100 μL. The mouse challenge experiments were evaluated and approved by the IACUC of Academia Sinica. Surviving mice from the experiments were sacrificed using

carbon dioxide, according to the ISCIII IACUC guidelines. All animals were weighed after the SARS-CoV-2 challenge once per day.

### *Immunoassay*

*ELISA for quantification of serum binding antibodies to SARS-CoV-2 S1-RBD*

96-well ELISA plates were coated with 2 µg/mL UBP Recombinant S1-RBD-His protein antigen (100 µL/well in coating buffer, 0.1 M sodium carbonate, pH 9.6) and incubated overnight (16 to 18 hrs) at 4°C. One hundred µL/well of serially diluted serum samples (10-fold diluted from 1:100 to 1:100,000, total 4 dilutions) in 2 replicates were added and plates were incubated at 37°C for 1 hr. The plates were washed six times with 200 µL Wash Buffer (solution of phosphate buffered saline, pH 7.0-7.4 with 0.05% Tween 20 as surfactant). Bound antibodies were detected with standardized preparation of HRP-rProtein A/G (1:101 of Horseradish peroxidase-conjugated rProtein A/G dissolved in HRP-stabilizer) at 37°C for 30 min, followed by six washes with Wash Buffer. Finally, 100 µL/well of TMB (3,3',5,5'-tetramethylbenzidine) prepared in Substrate Working Solution (citrate buffer containing hydrogen peroxide) was added into each well and incubated at 37°C for 15 min in the dark, and the reaction was stopped by adding 100 µL/well of Stop Solution (sulfuric acid solution, $H_2SO_4$, 1.0 M). The absorbance at 450 nm was measured by an ELISA plate reader (Molecular Device, Model: SpectraMax M2e). The UBI® ELISA Titer Calculation Program was used to calculate the relative titer. The anti-S1-RBD antibody level was expressed as $\log_{10}$ of an end point dilution for a test sample (SoftMax Pro 6.5, Quadratic fitting curve, Cut-off value 0.5).

*ELISA for binding inhibition of S1-RBD and human ACE2*

96-well ELISA plates were coated with 2 µg/mL ACE2-ECD-Fc antigen (100 mL/well in coating buffer, 0.1M sodium carbonate, pH 9.6) and incubated overnight (16 to 18 hrs) at 4°C. The plates were washed 6x with Wash Buffer (25-fold solution of phosphate buffered saline, pH 7.0-7.4 with 0.05% Tween 20 (250 µL/well/wash) using an Automatic Microplate Washer. Extra binding sites were blocked by 200 µL/well of blocking solution (5 N HCl, Sucrose, Triton X-100, Casein, and Trizma Base). Two-fold serial dilutions (from 1:20 to 1:12,800) of immune serum or a positive control (diluted in a buffered salt solution containing carrier proteins and preservatives) were mixed with 1:100 dilution of S1-RBD-HRP conjugate (horseradish peroxidase-conjugated recombinant protein S1-RBD-His), incubated for 30±2 min at 25±2°C, washed and TMB substrate (3,3',5,5'-tetramethylbenzidine diluted in citrate buffer containing hydrogen peroxide) was added. The reaction was stopped by adding stop solution (diluted sulfuric acid, $H_2SO_4$, solution, 1.0 M) and the absorbance of each well was read at 450 nm within 10 min using the Microplate reader (VersaMax).

*Flow cytometry assay for hACE binding inhibition*

Human ACE2-transfected HEK293 cells (prepared in-house) were collected and washed with FACS buffer, 2% FBS (GIBCO, CN: 10099-148) in 1X PBS. One hundred µL of 20 µg/mL 2019-nCoV Spike Protein S1 (Fc Tag) (Sino Biological, CN: 40591-V02H) was mixed with 100 µL of antisera dilutions (serially 5-fold diluted from 1:5 to 1:3,125, total 5 dilutions) at 25° C for 1 hr. The mixture (200µL) was then added to the transfected cells (cell no. $2\times10^5$) followed by incubation at room temperature for 1 hr. Cells were washed with FACS buffer and incubated with diluted (1:200) anti-human IgG Fc protein antibody (FITC) (Bethyl Laboratories, CN: A80-104F) on ice for additional 30 min. After washing, the cells were analyzed in a FACSCanto II flow cytometry (BD Biosciences) using BD FACSDiva software.

*Neutralization of live SARS-CoV2 by immune sera by CPE assay*

Vero-E6 cells were expanded and their concentrations were adjusted to $1.5x10^5$ viable cells/mL of culture medium (DMEM containing 10% FBS). The 96-well microtiter plates were seeded with $1.5x10^4$ cells/100 µL/well. The plates were incubated at 37°C in a CO2 incubator overnight. The next day, serum samples were diluted (1:5) starting with 72 µL of serum sample + 288 µL of dilution medium (Dulbecco's Modified Eagle Medium, DMEM, containing 5% FBS) to yield the first dilution. Then, 7x2-fold serial dilutions were made with dilution medium (dilution points were adjusted according to the characteristics of the sample). The challenge virus (SARS-CoV-2-TCDC#4, Taiwanese strain) was prepared at 100 $TCID_{50}$ in 50 µL of culture medium, incubated with 50 µL volume of each serum dilution (50 µL) (in triplicates) for 1 hr at 37°C, before adding to Vero-E6 cells in triplicates. Medium only was co-incubated with an equal volume of 100 $TCID_{50}$ of the viruses for 1 hr at 37°C and used as 100% infected control. The plates were incubated at 37°C in a $CO_2$ incubator for 4 days. Cells were then fixed overnight with 100 µL of 10% formaldehyde prepared in phosphate buffered saline, pH 7.0-7.4, added into each well. The next day formaldehyde solution was discarded by inverting the plate, 100 µL of crystal 0.5% violet staining solution was added into each well and incubated at room temperature for 1 hr. The infection rate was quantified by ELISA reader and image analysis. The infection rate of medium only at a challenge dose of 100 $TCID_{50}$ virus was set at 100% and each serum dilution with more than 50% infection was scored as infected. The 50% protective titer was determined by the Reed and Muench method[56,57].

*Neutralization assessment by immunofluorescence*

Vero-6E cell monolayers in 96-well plates from the CPE assay (described above) were also processed for visualization by immunofluorescence assay (IFA): the cells were stained with anti-SARS-CoV-2 N protein antibody and detected with anti-human IgG-488. The nuclei were counter stained with DAPI.

*ELISpot for measurement of cellular responses*

Spleens from vaccinated rats at 4 WPI were collected in Lymphocyte-conditioned medium (LCM; RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin) and processed into single cell suspensions. Cell pellets were resuspended in 5 mL of RBC lysis buffer for 3 min at room temperature (RT), and RPMI-1640 medium containing penicillin/streptomycin was then added to stop the reaction. After centrifugation, cell pellets resuspended in LCM were used in ELISpot assay. ELISpot assays were performed using the Rat IFN-γ ELISpot$^{PLUS}$ kit (MABTECH, Cat. No.: 3220-4APW), Rat IL-4 T cell ELISpot kit (U-CyTech, Cat. No.: CT081) and Rat IL-2 ELISpot Kit (R&D Systems, Cat. No.: XEL502). ELISpot plates precoated with capture antibody were blocked with LCM for at least 30 min at RT. 250,000 rat splenocytes were plated into each well and stimulated with S1-RBD-His protein plus Th/CTL peptide pool, S1-RBD-His protein, Th/CTL peptide pool, or each single Th/CTL peptide for 18-24 hrs at 37°C. Cells were stimulated with a final concentration of 1 μg of each protein/peptide per well in LCM. The spots were developed based on manufacturer's instructions. LCM and ConA were used for negative and positive controls, respectively. Spots were scanned and quantified by AID iSpot reader. Spot-forming unit (SFU) per million cells was calculated by subtracting the negative control wells.

***Real-time RT-PCR for SARS-CoV-2 RNA quantification***

To measure the RNA levels of SARS-CoV-2, specific primers targeting 26,141 to 26,253 regions in the envelope (E) gene of the SARS-CoV-2 genome were used by Taqman real-time RT-PCR method that described in the previous study (Corman, et al. 2020). Forward

primer E-Sarbeco-F1 (5'-ACAGGTACGTTAATAGTTAATAGCGT-3') and the reverse primer E-Sarbeco-R2 (5'-ATATTGCAGCAGTACGCACACA-3'), in addition to the probe E-Sarbeco-P1 (5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ-3') were used. A total of 30 μL RNA solution was collected from each sample using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. 5 μL of RNA sample was added in a total 25 μL mixture using Superscript III one-step RT-PCR system with Platinum Taq Polymerase (Thermo Fisher Scientific, USA). The final reaction mix contained 400 nM forward and reverse primers, 200 nM probe, 1.6 mM of deoxy-ribonucleoside triphosphate (dNTP), 4 mM magnesium sulphate, 50 nM ROX reference dye and 1 μL of enzyme mixture from the kit. The cycling conditions were performed with a one-step PCR protocol: 55°C for 10 min for cDNA synthesis, followed by 3 min at 94°C and 45 amplification cycles at 94°C for 15 sec and 58°C for 30 sec. Data were collected and calculated by Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). A synthetic 113-bp oligonucleotide fragment was used as a qPCR standard to estimate copy numbers of viral genome. The oligonucleotides were synthesized by Genomics BioSci and Tech Co. Ltd. (Taipei, Taiwan).

### *Median tissue culture infectious dose (TCID50) assays*

Mouse lung tissues were weighed and homogenized in 1 mL of DMEM with 1% penicillin/streptomycin using a homogenizer. After centrifugation at 13,000 rpm for 10 min, supernatant was harvested for live virus titration (TCID50 assay). Briefly, serial 10-fold dilutions of each sample were inoculated in a Vero-E6 cell monolayer in quadruplicate and cultured in DMEM with 1% FBS and penicillin/streptomycin. The plates were observed for cytopathic effects for 4 days. TCID50 was interpreted as the amount of virus that caused cytopathic effects in 50% of inoculated wells. Virus titers were expressed as TCID50/ml.

### *Histopathological analysis of lung tissues*

After fixation with 10% formaldehyde for one week, the lung was trimmed, processed, embedded, sectioned and stained with Hematoxylin and Eosin (H&E), followed by microscopic examination. To score the lung histopathology, lung section was divided into 9 equal square areas using a 3 × 3 grid. Lung tissue of every area was scored using a scoring system. The average of scores of these 9 areas was used to represent the score of the animal.

The scoring system was as follows: 0, Normal, no significant finding; 1, Minor inflammation with slight thickening of alveolar septa and sparse monocyte infiltration; 2, Apparent inflammation, alveolus septa thickening with more interstitial mononuclear inflammatory infiltration; 3, Diffuse alveolar damage (DAD), with alveolus septa thickening, and increased infiltration of inflammatory cells; 4, DAD, with extensive exudation and septa thickening, shrinking of alveoli, restricted fusion of the thick septa, obvious septa hemorrhage and more cell infiltration in alveolar cavities; 5, DAD, with massive cell filtration in alveolar cavities and alveoli shrinking, sheets of septa fusion, and hyaline membranes lining the alveolar walls.

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## Figures

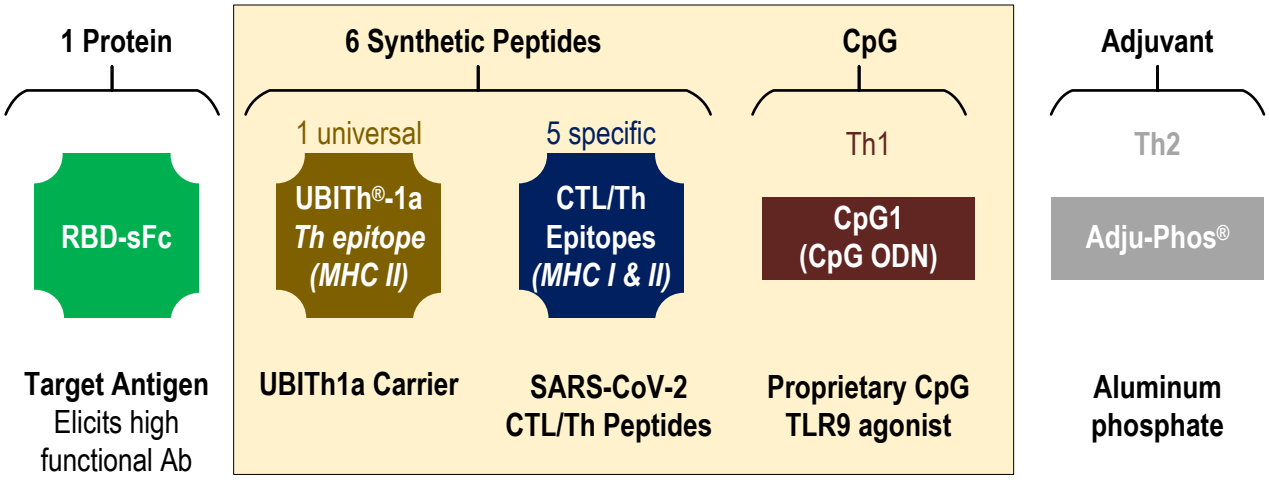


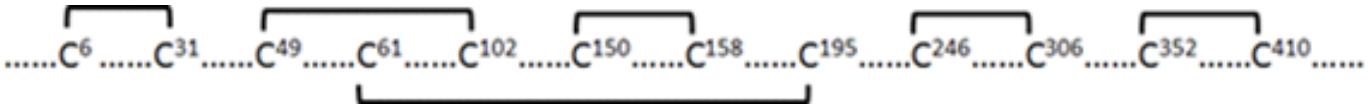

**Figure 1. Components of the UB-612 Multitope vaccine.** UB-612 vaccine construct contains an S1-RBD-sFc fusion protein for the B cell epitopes, plus five synthetic Th/CTL peptides for class I and II MHC molecules derived from SARS-CoV2 S2, M, and N proteins, and the UBITh1a peptide. These components are mixed with CpG1 which binds the positively (designed) charged peptides by dipolar interactions and also serves as an adjuvant, which is then bound to Adju-Phos adjuvant to constitute the UB-612 vaccine drug product.

**A**

```
1   NITNLCPFGE VFNATRFASV YAWNRKRISN CVADYSVLYN SASFSTFKCY GVSPTKLNDL CFTNVYADSF
71  VIRGDEVRQI APGQTGKIAD YNYKLPDDFT GCVIAWNSNN LDSKVGGNYN YLYRLFRKSN LKPFERDIST
141 EIYQAGSTPC NGVEGFNCYF PLQSYGFQPT NGVGYQPYRV VVLSFELLHA PATVCGPKKS EPKSSDKTHT
211 SPPSPAPELL GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ
281 YHSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR DELTKNQVSL
351 TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH
421 YTQKSLSLSP G
```

N: N-linked glycosylation site  S: O-linked glycosylation site

**B**




**C**

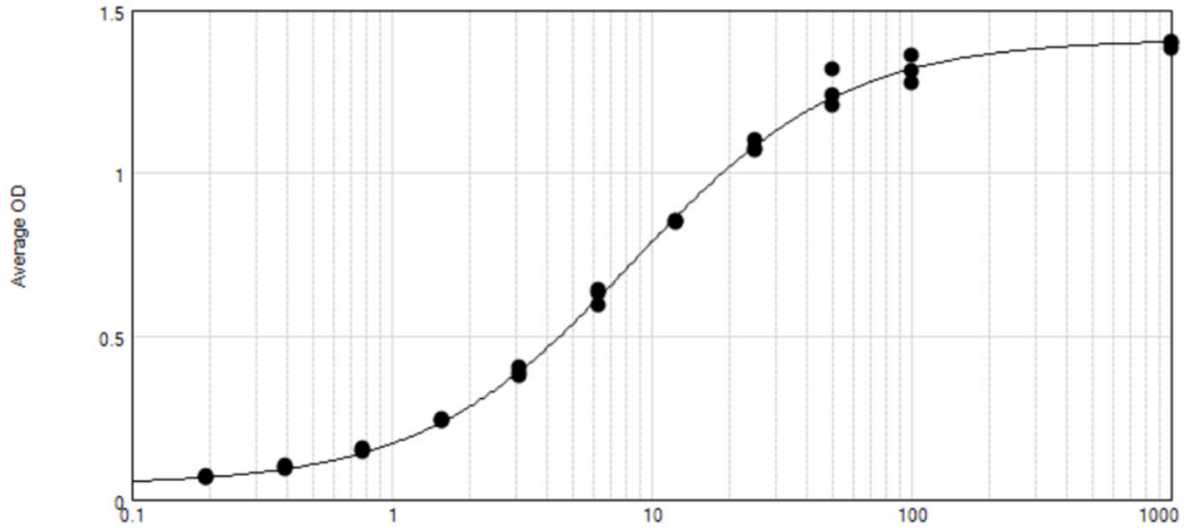



**Figure 2. Structure and hACE2 binding of S1-RBD-sFc.**

A. Sequence of S1-RBD-sFc. S1-RBD-sFc protein is a glycoprotein consisting of one N-linked glycan (Asn13) and two O-linked glycans (Ser211 and Ser224).Light blue shading indicates RBD of SARS-CoV-2 and no shading is for sFc fragment of an IgG1. The substitution of His297 for Asn297 (EU-index numbering) in single chain Fc, His282 in S1-RBD-sFc, is indicated by underline. S1-RBD-sFc protein contains 431 amino acid residues including 12 cysteine residues (Cys6, Cys31, Cys49, Cys61, Cys102, Cys150, Cys158, Cys195, Cys246, Cys306, Cys352 and Cys410), forming 6 pairs of disulfide bonds (Cys6-Cys31, Cys49-Cys102, Cys61-Cys195, Cys150-Cys158, Cys246-Cys306 and Cys352- Cys410), which are shown as orange lines.

B. Summary of disulfide bonding in S1-RBD-sFc

C. hACE2 binding ability of S1-RBD-sFc. X axis, concentration (ng/mL); Y axis, optical density

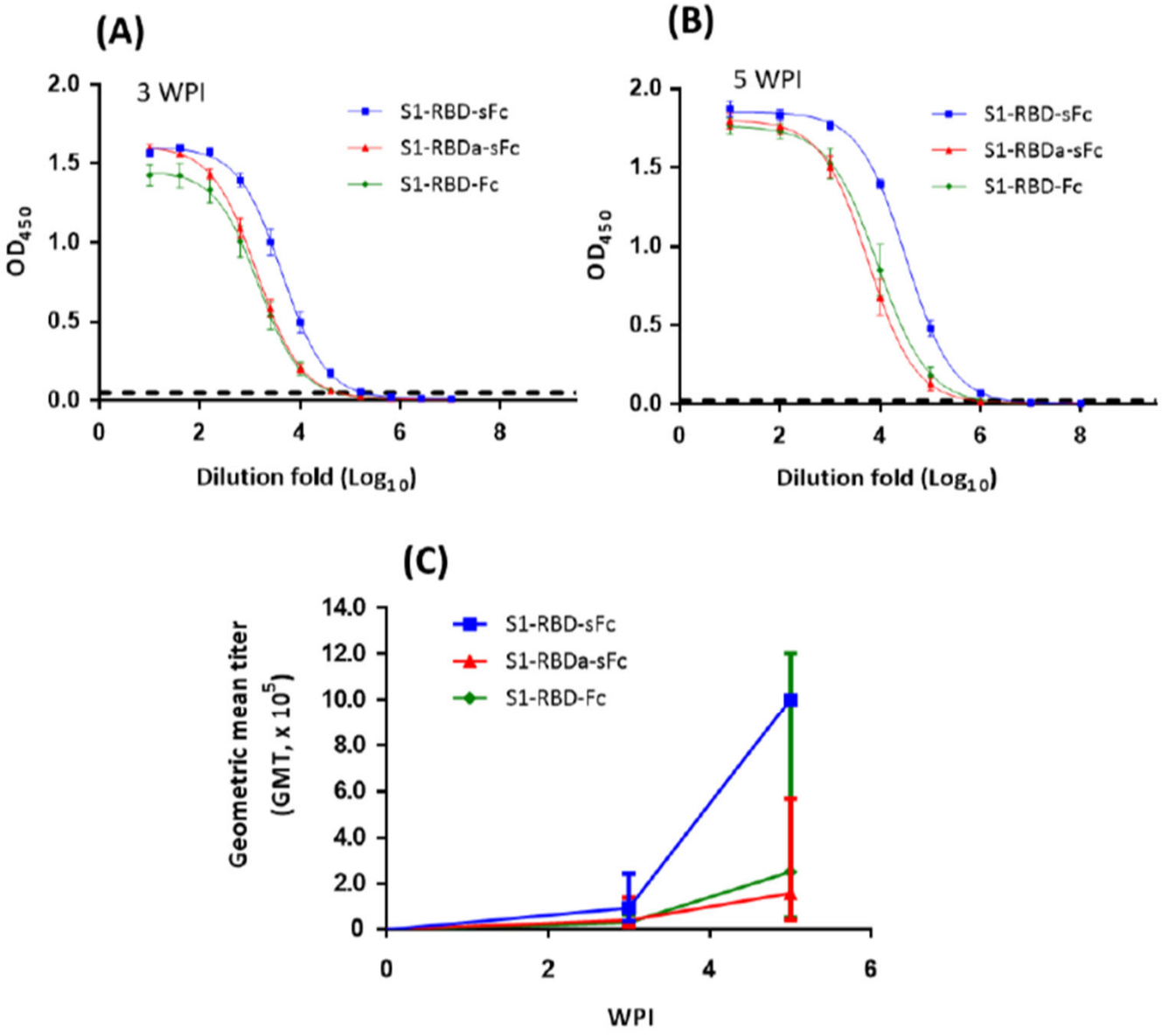



**Figure 3. Direct binding antibody titers of guinea pig immune sera to S1 protein by ELISA.** Guinea pigs were immunized with S1-RBD-sFC, S1-RBDa-sFC, or S1-RBD-Fc (n = 5 each group) at weeks 0 and 3 via intramuscular route. Immune sera were collected at 0, 3, and 5 weeks post initial immunization (WPI). Anti-S1 binding antibodies were detected by ELISA.

A. Anti-S1 antibody binding curves ($OD_{450}$) of immune sera at 3 WPI (mean ± standard error)

B. Anti-S1 antibody binding curves ($OD_{450}$) of immune sera at 5 WPI (mean ± standard error)

C. Geometric mean titer (GMT) values for binding antibodies at 3 and 5 WPI. Results shown as GMT ± 95% confidence interval

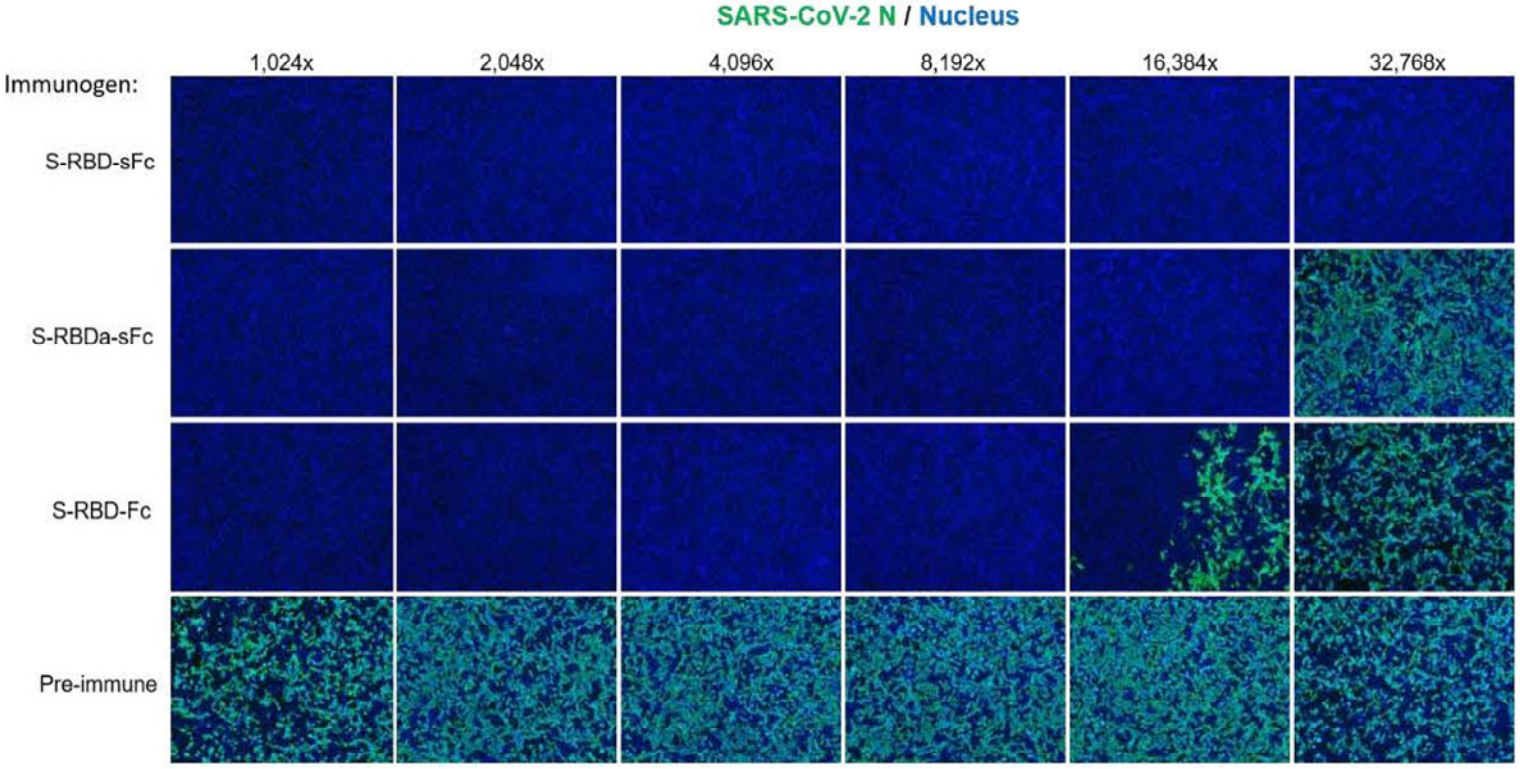



**Figure 4. Potent neutralization of live SARS-CoV-2 by immune sera.** Immune sera collected at 5 WPI from GP vaccinated at 0 and 3 WPI with S1-RBD-sFc, S1-RBDa-sFc, and S1-RBD-Fc with Montanide ISA 50 V2 were analyzed. The monolayers of Vero-E6 cells infected with virus-serum mixtures were assessed by immunofluorescence (IFA). Cells were stained with human anti-SARS-CoV-2 N protein antibody and detected with anti-human IgG-488 (green). The nuclei were counter stained with DAPI (4',6-diamidino-2-phenylindole) (blue).

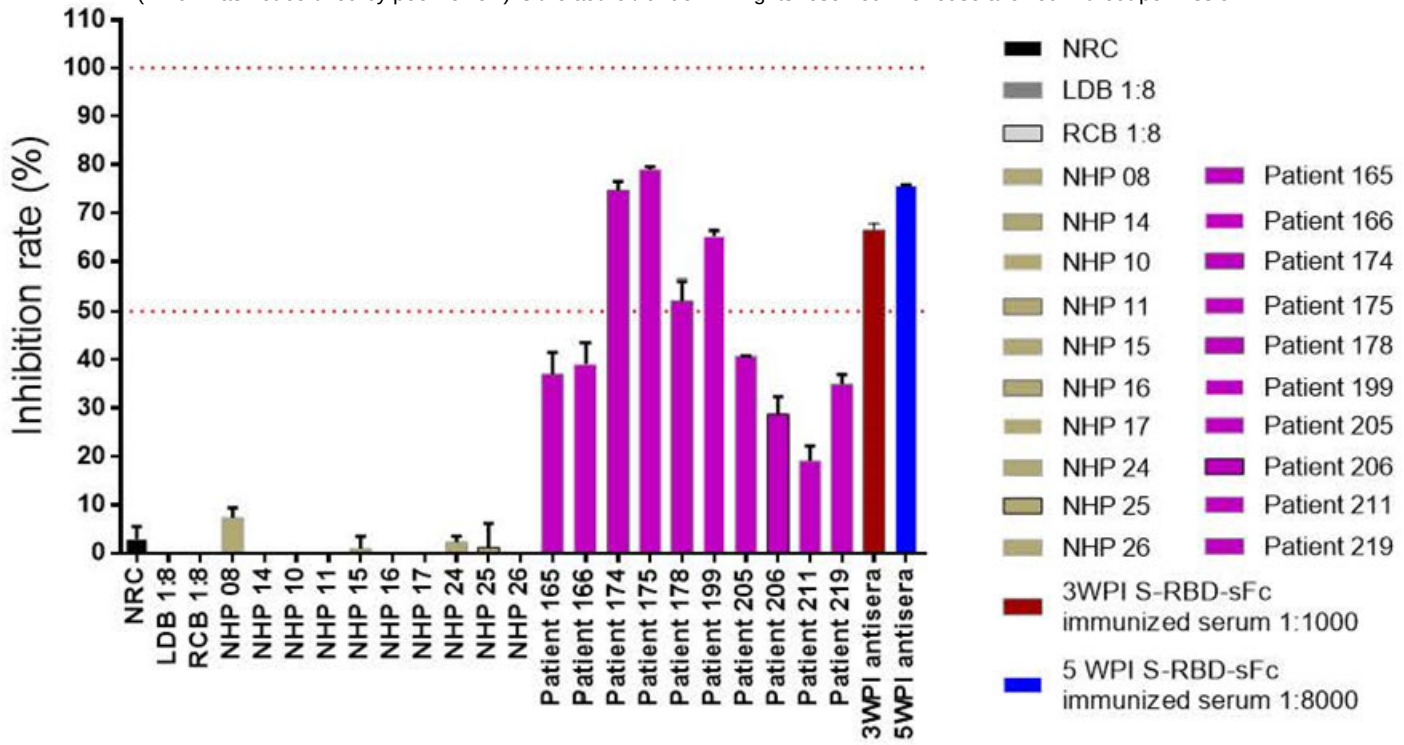



**Figure 5. Comparative S1-RBD:ACE2 binding inhibition by GP sera and convalescent sera.** SARS-CoV-2 inhibition rates were evaluated with human serum samples from normal healthy persons (NHP, n=10) and virologically diagnosed COVID-19 patients (n=10) tested at a 1:20 dilution. Pooled immune sera from S1-RBD-sFc vaccinated GP collected at 3 WPI and 5 WPI, were tested at 1:1000 and 1:8000 dilutions, respectively.

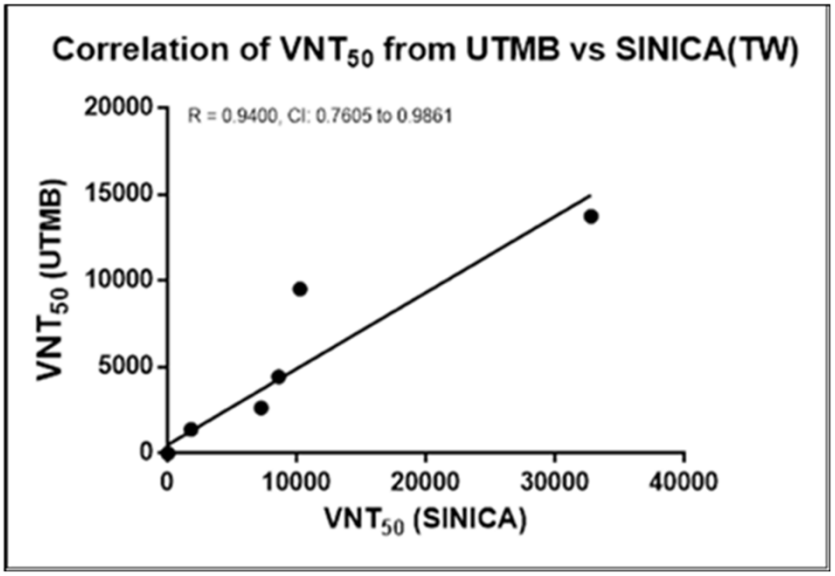



**Figure 6. Neutralization tests on blinded serum samples at UTMB.** Neutralization was assessed with a recombinant SARS-CoV-2 expressing Neon Green protein (ic-SARS-CoV-2-mNG) using the fluorescent signal as a readout for viral replication. The limit of detection of the assay is 1:20 and negative samples were assigned a 1:10 titer. As a positive control, plasma from a convalescent COVID-19 human patient was included. There was a strong correlation (R=0.94) in this assay with the neutralization titers obtained at Academia Sinica.

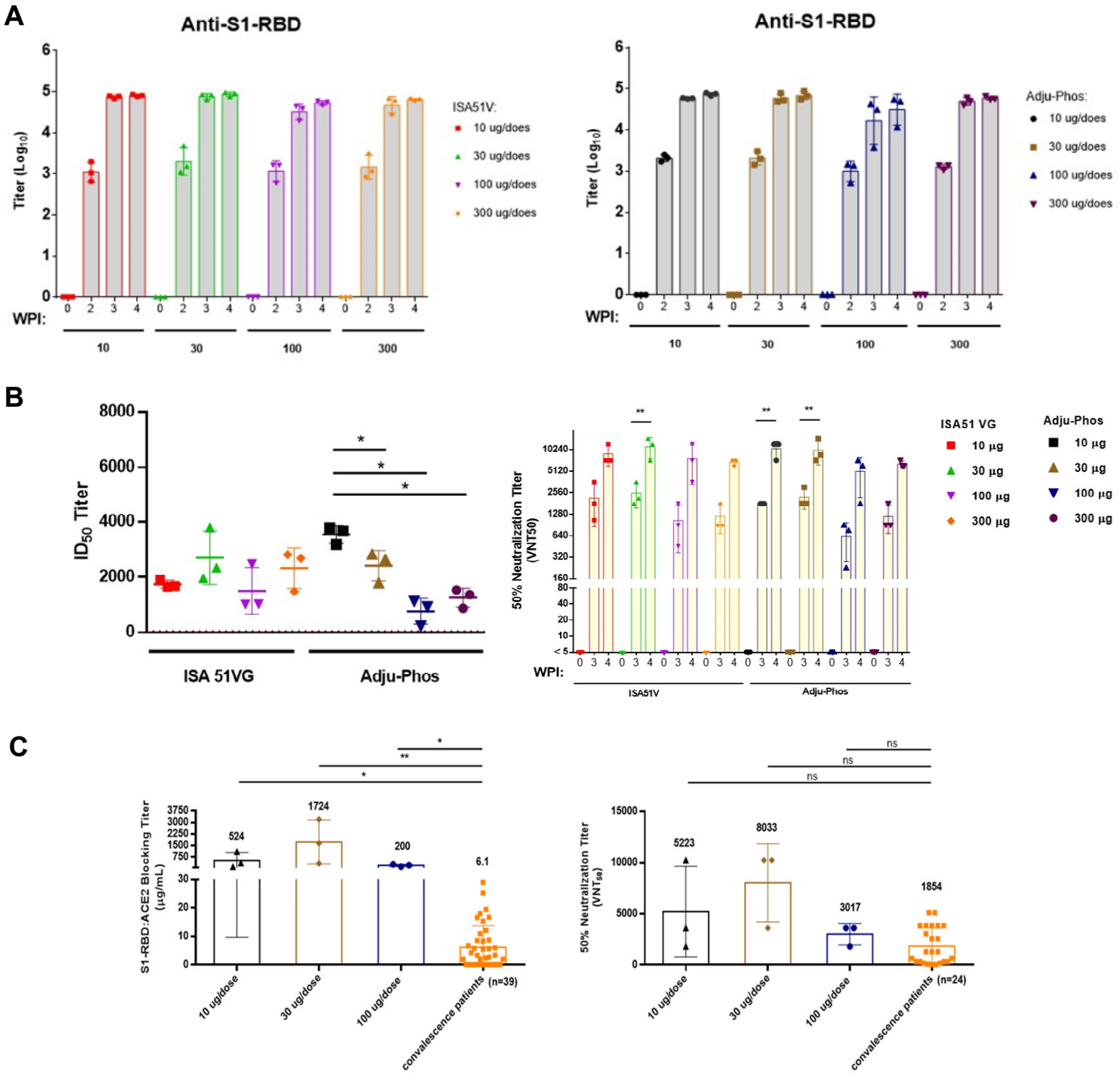



**Figure 7. Humoral immunogenicity testing in rats.**

A. Immunogenicity of UB-612 adjuvanted with ISA51/CpG3 or Adju-Phos/CpG1. Sprague Dawley rats were immunized at weeks 0 and 2 with UB-612 vaccine (at a dose range of 10-300 of S1-RBD-sF, formulated with synthetic designer peptides and adjuvants). Immune sera at 0, 2, 3, and 4 WPI were assayed for direct binding to S1-RBD protein on ELISA

B. hACE binding inhibition by antibodies from rats immunized with UB-612 adjuvanted with ISA51/CpG3 or Adju-Phos/CpG1. Samples taken 4 WPI from rats immunized at weeks 0 and 2 with UB-612 vaccine.

C. Potent neutralization of live SARS-CoV-2 by rat immune sera. Neutralization titers expressed as VNT50.

D. RBD:ACE2 Inhibiting (Panel A) and Neutralizing Antibody (Panel B) Titers of Sera from UB-612 Vaccinated Rats Higher than Titers in Convalescent COVID-19 Patients. * $p \leqq 0.05$, ** $p \leqq 0.01$ and NS (not significant) (Kruskal-Wallis ANOVA with Dunn's multiple comparisons test)

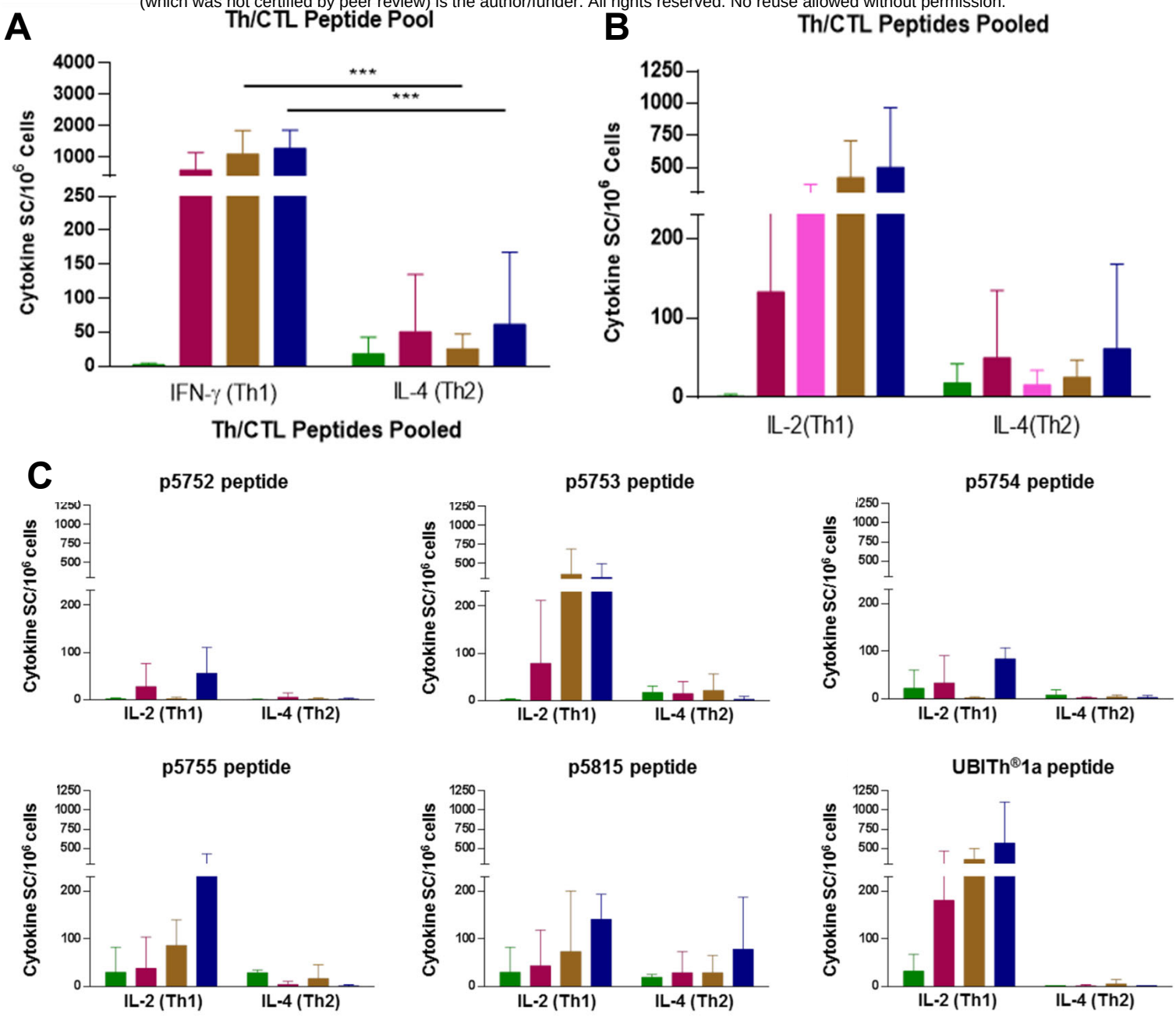



**Figure 8. Cellular immunogenicity testing in rats (ELISpot detection of IFN-γ, IL-2 and IL-4 secreting cells in UB-612 immunized rats)** Groups of rats were immunized with UB-612 on 0 and 2 WPI. Splenocytes were collected at 4 WPI and stimulated with Th/CTL peptide pool or individual Th/CTL peptides used in the UB-612 vaccine composition. IFN-γ, IL-2 and IL-4-secreting splenocytes were determined by ELISpot analysis. Cytokine-secreting cells (SC) per million cells was calculated by subtracting the negative control wells. Bars represent the mean  SD (n = 3). The secretion of IFN-γ or IL-2 was observed to be significantly higher than that of IL-4 in 30 and 100 ug group (*** $p < 0.005$ using Least Square Mean and paired wise comparison) but they were not statistically different in 1 or 3 ug dose groups.

A. ELISpot detection of IFN-γ and IL-4 responses from cells stimulated with Th/CTL peptide pool

B. ELISpot detection of IL-2 and IL-4 responses from cells stimulated with Th/CTL peptide pool

C. ELISpot detection of IL-2 and IL-4 responses from cells stimulated with individual peptides

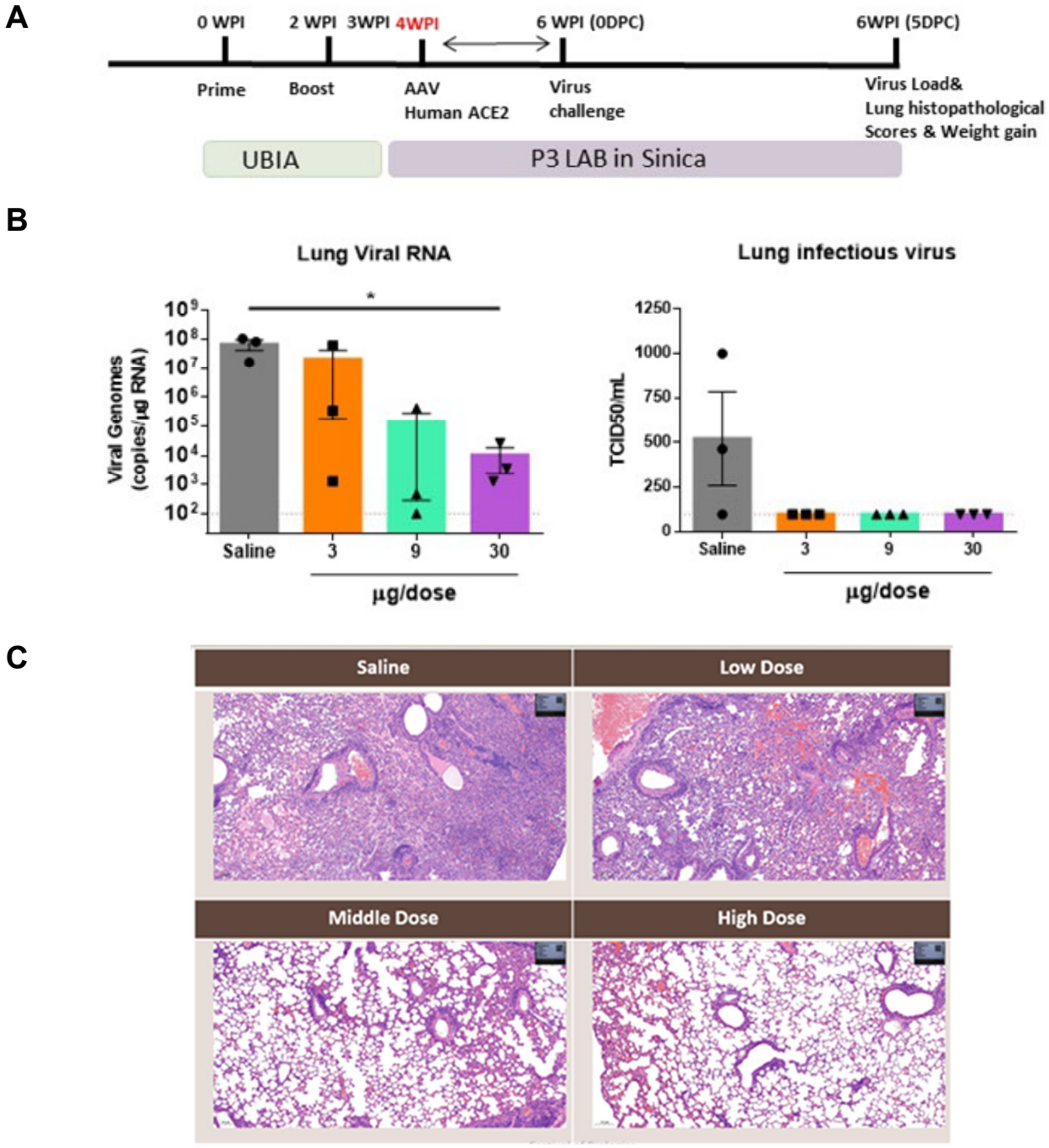



**Figure 9. Challenge testing in mice.** Results from live SARS-CoV-2 challenge in hACE-transduced mice.

A. Immunization and challenge schedule.

B. Viral load data. SARS-CoV-2 titers by RT-PCR and $TCID_{50}$ from mice challenged with live virus.

C. Lung pathology. Stained sections of lungs isolated from from mice challenged with live virus

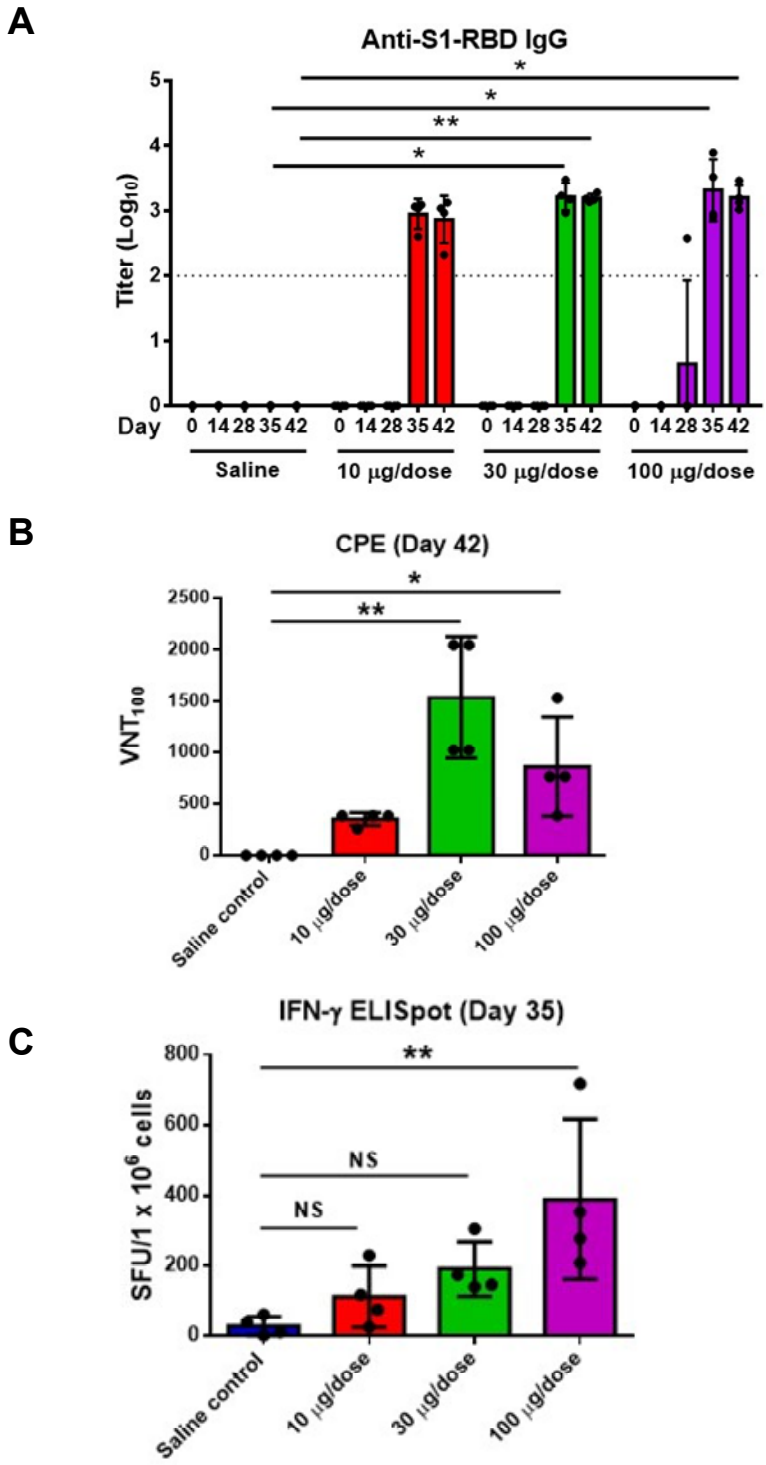



**Figure 10. Immunogenicity results in rhesus macaques.**

A. Direct binding of RM immune sera to S1-RBD on ELISA. ELISA-based serum antibody titer (mean $Log_{10}$ SD) was defined as the highest dilution fold with $OD_{450}$ value above the cutoff value. * $p \leqq 0.05$, ** $p \leqq 0.01$

B. Potent neutralization of live SARS-CoV-2 by RM immune sera. Immune sera collected at Day 42 from rhesus monkeys (RM) vaccinated at weeks 0 and 4 were assayed inSARS-CoV-2 infected Vero-E6 cells for cytopathic effect (CPE).

C. ELISpot analysis of RM PBMC cells stimulated with Th/CTL peptide pool. PBMCs were collected at Day 35 and stimulated with Th/CTL peptide pool. IFN-γ-secreting cells were determined by ELISpot analysis. ** $p \leqq 0.01$.