Research Article

Title: Receptor-binding domain recombinant protein RBD219-N1C1 on alum-CpG induces broad protection against SARS-CoV-2 variants of concern

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Abstract (Max. of 300 words)

We conducted preclinical studies in mice using a yeast-produced SARS-CoV-2 RBD219-N1C1 subunit vaccine candidate formulated with aluminum hydroxide (alum) and CpG deoxynucleotides. This vaccine formulation is similar to one that entered advanced phase 3 clinical development in India. We compared the immune response of mice vaccinated with RBD219-N1C1/alum to mice vaccinated with RBD219-N1C1/alum+CpG. We also evaluated mice immunized with RBD219-N1C1/alum+CpG and boosted with RBD219-N1C1/alum. Mice were immunized twice intramuscularly at a 21-day interval. Compared to two doses of the RBD219-N1C1/alum formulation, the RBD219-N1C1/alum+CpG vaccine induced a stronger and more balanced Th1/Th2 cellular immune response, with high levels of neutralizing antibodies against the original Wuhan isolate of SARS-CoV-2 as well as the B.1.1.7 (Alpha), B.1.351 (Beta) and B.1.617.1 (Kappa) variants. Notably, the sera from mice that received two 7 µg doses of RBD219-N1C1/alum+CpG showed more than 18 times higher neutralizing antibody titers against B.1.351, than the WHO International Standard for anti-SARS-CoV-2 immunoglobulin NIBSC 20/136. Interestingly, a booster dose did not require the addition of CpG to induce this effect. The data reported here reinforces that the RBD219-N1C1/alum+CpG vaccine formulation is suitable for inducing broadly neutralizing antibodies against SARS-CoV-2 including three variants of concern, B.1.1.7 (Alpha), B.1.351 (Beta), and B.1.617.1 (Kappa).

Keywords: COVID-19, Subunit vaccine, TLR9 adjuvant, Virus neutralization

Introduction

More than a year into the COVID-19 pandemic, the world has now recorded over 3.8 million deaths and more than 175 million infections affecting 192 global regions [1]. With unprecedented efforts from governments, non-profits, the academic and the industry sector, vaccines have become available to everybody in countries like the United Kingdom or the United States, however, the majority of the low- and middle-income countries (LMICs) still lack access to COVID-19 vaccines [2]. For example, the vaccination rate in the United States exceeds Nigeria or South Africa by more than 125-fold [3]. Consequently, we must realize that overall, only a small fraction of the people in the world have yet been fully vaccinated; in countries like India, Indonesia, the Philippines, or even Canada less than 12% of the population have been fully vaccinated [4]. In addition, unequal access to vaccines and continued circulation of the virus has resulted in more and more SARS-CoV-2 variants emerging globally [5]. Some of these variants have raised concerns for their ability to at least, partially evade the immune response acquired after natural infection with SARS-CoV-2 or even after vaccination with the first-generation COVID-19 vaccines [6]. There is an urgent need to bridge these gaps in vaccine equity and global distribution.

While mRNA, viral-vector vaccines, and whole-inactivated virus vaccines have been crucial tools in the first round of controlling COVID-19, they have limitations concerning cost, production capacity, as well as distribution and storage. Recombinant protein-based vaccines are therefore expected to play an important role in providing expanded coverage to populations that have yet to receive their immunizations [7]. Their advantages include ease of production, absence of freezer-chain requirements, and decades-long track records of safety with durable protection. For that reason, we and other groups have focused on recombinant protein platforms for our

portfolio of neglected disease ‘antipoverty’ vaccines to prevent schistosomiasis, hookworm, Chagas disease, and other parasitic infections affecting LMICs [8, 9].

The receptor-binding domain (RBD) of the viral spike protein is one of the main targets for COVID-19 vaccines and our studies found that RBD-based subunit vaccines represent an effective strategy for the development of vaccines against SARS [10] and MERS [11]. For COVID-19, RBD-based vaccines are also under evaluation. For instance, an RBD-ferritin-nanoparticle vaccine (RFN), expressed in mammalian cell lines and adjuvanted with QS-21 elicited SARS-CoV-2 neutralizing antibodies and a Th1-biased T-cell response in rhesus macaques. Moreover, cross-neutralization with this vaccine candidate against the B.1.351 (Beta) variant decreased by only 2-fold, compared to the original virus isolate [12]. Likewise, an aluminum hydroxide-adjuvanted RBD-dimer (ZF2001) recombinantly produced in Chinese hamster ovary cell lines, recently completed Phase 1/2 studies in China, and was shown to be well-tolerated and able to produce neutralizing antibody titers exceeding the levels found in convalescent serum, albeit, ideally, in a three-dose regimen [13].

We previously demonstrated that a yeast-expressed RBD219-N1C1 protein, formulated with aluminum hydroxide (alum), stimulates virus-neutralizing antibodies, and induces primarily a Th2 cellular immune response in mice [14]. As the next step, we optimized the alum-based formulation by reducing the protein doses as well as by incorporating in the formulation, at point of injection, a synthetic oligodeoxynucleotide [15] that mimics the effect of CpG motifs in bacterial DNA, stimulating TLR9 receptors to induce a stronger and more balanced Th1/Th2 response [16].

In humans, the Class B CpG1018 has a strong track record in the clinic and is part of the formulation of HEPLISAV-B, a licensed hepatitis B vaccine. Related to COVID-19, CpG1018 has

been used to induce robust protection in a non-human primate study as part of an RBD-nanoparticle vaccine [17], and several CpG1018-adjuvanted protein vaccines have now entered the clinic [18]. As an equivalent to CpG1018, the Class B CpG1826 [19], has been shown in mice to strongly activate B cells and elicits TLR9-dependent NF- κ B signaling, and only weakly stimulates IFN- α secretion [20].

Here, we report the result of preclinical studies in mice using the yeast-produced SARS-CoV-2 RBD219-N1C1 subunit vaccine candidate formulated with aluminum hydroxide (alum) and CpG deoxynucleotide. We compared the humoral response, the vaccine-induced neutralizing antibody titers, and the cellular immune responses of mice vaccinated with RBD219-N1C1/alum to mice vaccinated with RBD219-N1C1/alum+CpG. In addition, we also evaluated the option to prime the mice with RBD219-N1C1/alum+CpG and boost with RBD219-N1C1/alum alone. This vaccine formulation is similar to one that is currently entering advanced phase 3 clinical development in India.

Materials and Methods

Sources of recombinant protein RBD219-N1C1

Recombinant protein SARS-CoV-2 RBD219-N1C1) was designed and produced originally at Baylor College of Medicine, using previously published methods [21, 22]. Lot# S2RBD110620JXL-1 (formulated with alum) was used as a reference protein. Using the same master cell bank, the recombinant protein was produced at scale by Biological E (Hyderabad, India) and shipped to BCM where it was used in the preclinical studies (lot#BC07/001/20).

Vaccine formulations

SARS-CoV-2 RBD219-N1C1 proteins were diluted in 20 mM Tris, 100 mM NaCl, pH 7.5 (TBS buffer) before mixing with alum (Alhydrogel®, aluminum hydroxide, Catalog # 250-843261 EP, Croda Inc., UK). CpG1826 (Invivogen, USA) was added at point of injection.

Table 1: Vaccine formulation table

Vaccine groups	RBD219-N1C1	Alum	CpG1826
Alum	0 µg	100 µg aluminum	0 µg
CpG	0 µg	0 µg aluminum	20 µg
Alum+CpG	0 µg	100 µg aluminum	20 µg
2.3 µg RBD/Alum	2.3 µg	100 µg aluminum	0 µg
7 µg RBD/Alum	7 µg	100 µg aluminum	0 µg
2.3 µg RBD /Alum+CpG	2.3 µg	100 µg aluminum	20 µg
7 µg RBD/Alum+CpG	7 µg	100 ug aluminum	20 µg

Preclinical Design

All animal experiments were performed under a Baylor College of Medicine Institutional Animal Care and Use Committee approved protocol. Female BALB/c mice, age 11-13 weeks old, were immunized two (2) times intramuscularly at 21-day intervals with formulations shown in Table 1, and then euthanized two (2) weeks after the second vaccinations.

Serological Antibody Measurements by ELISA

To examine SARS-CoV-2 RBD-specific antibodies in the mouse sera, indirect ELISAs were conducted as published elsewhere [14]. Briefly, plates were coated with 0.2 µg/well SARS-CoV-2 RBD219-N1C1. Mouse serum samples were 3-fold diluted from 1:200 to 1: 11,809,800 in 0.1% BSA in assay buffer (0.05% Tween-20 in 1x PBS). Assay controls included a 1:200 dilution of pooled naive mouse sera and assay buffer as blanks. 100 µL/well of either 1:6000 goat anti-mouse IgG HRP, 1:6000 goat anti-mouse IgG1 HRP or 1:6000 goat anti-mouse IgG2a HRP in assay buffer was added. After incubation plates were washed five times followed by the addition of 100 µL/well 4°C TMB substrate. Plates were incubated for 15 minutes at room temperature (RT) while protected from light. After incubation, the reaction was stopped by adding 100 µL/well 1 M HCl. The absorbance at a wavelength of 450 nm was measured using a Biotek Epoch 2 spectrophotometer

The titer cutoff value was calculated using the following formula: Titer cutoff = average of negative control + 3 * standard deviation of the negative control. Then, for each sample, the titer was determined as the lowest dilution of each mouse sample with an average O.D. value above the titer cutoff. When a serum sample did not show any signal at all and a titer could not be calculated, an arbitrary baseline titer value of 67 was assigned to the serum sample. All data were plotted in Graphpad Prism 6 as individual samples and the geometric mean.

Cytokine secretion in re-stimulated splenocytes by Luminex

A single-cell suspension from mouse splenocytes was prepared according to methods published elsewhere[14]. Briefly, spleens were dissociated using GentleMACS Octo Dissociator. Pelleted splenocytes were resuspended in 1 mL ACK lysing buffer by pipetting to lyse red blood cells.

After 1 minute at RT, the reaction was stopped by the addition of 40 mL PBS. Tubes were centrifuged for 5 minutes at 300 x g at RT. Then supernatant was discarded and splenocytes were resuspended in 5 mL 4°C cRPMI (RPMI 1640 + 10% HI FBS + 1x pen/strep). Splenocyte suspensions were transferred through a 40 µm filter to a new 50-mL conical tube to obtain a single-cell suspension. For the *in vitro* cytokine release assay, splenocytes were seeded in a 96-well culture plate at 1×10^6 live cells in 250 µL cRPMI. Splenocytes were (re-)stimulated with 10 µg/mL RBD219-N1-C1 protein, PMA/Ionomycin (positive control), or media (negative control) for 48 hours at 37° C 5% CO₂. After incubation, 96-well plates were centrifuged to pellet the splenocytes down and supernatant was transferred to a new 96-well plate. A Milliplex Mouse Th17 Luminex kit (EMD Millipore) with analytes IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17A, IFN-γ, and TNF-α was used to quantify the cytokines secreted in the supernatant by the re-stimulated splenocytes according to a previously published method [23]. The readout was performed using a MagPix Luminex instrument. Raw data was analyzed using Bio-Plex Manager software, and further analysis was done with Excel and Prism. For the visualization of the individual cytokine results in the heatmap, the median was calculated of the cytokine concentrations of each group.

Pseudovirus assay for determination of neutralizing antibodies

The pseudovirus used is a non-replicating lentiviral particle that has the SARS-CoV-2 spike protein on its membrane and can express a luciferase gene after infection. Using in vitro grown human 293T-hACE2 cells, infected cells were quantified based on the expression of luciferase [14].

The plasmids used for the pseudovirus production are the luciferase-encoding reporter plasmid (pNL4-3.lucR-E-, [24]), Gag/Pol-encoding packaging construct (pΔ8.9, [25]), and codon-optimized SARS-CoV-2 spike protein expression plasmids (pcDNA3.1-CoV-2 S gene) based on

clone p278-1 [26]. Pseudovirions were generated by transfection of 293T cells as we previously described [14]. Pseudovirus containing supernatants were recovered after 48 hours and passed through a 0.45 μ m filter and saved at -80°C until used for neutralization studies.

In order to test the different pseudovirus variants, the following RBD mutations were introduced into a spike expression clone that includes the D614G mutation: N501Y (mimics the B.1.1.7 strain), L452R-E484Q (mimics B.1.617.1), and K417N, E484K, N501Y (mimics the B.1.351 strain). Mutations were generated sequentially by overlap extension PCR in 20 μ L reactions using Phusion Green DNA polymerase master mix (ThermoFisher) according to the manufacturer's protocol, 2 ng of the spike plasmid clone as template, and 500 nM of each PCR primer of a set in Table 2. The basic PCR cycling protocol was denaturation at 98°C for 30 sec followed by 25 cycles of 98°C for 10 sec, 30 sec at the annealing temperature, and extension at 72°C for 4 min 40 sec. A final elongation step at 72°C for 6 min was included. The annealing temperature for each primer set was determined using the ThermoFisher web-based Tm Calculator for Phusion DNA polymerase. PCR products were digested with DpnI for 1 hour at 37°C and NEB 10 beta cells were transformed and grown for plasmid isolation. The mutations in the spike coding region were confirmed by Sanger sequencing.

Table 2. PCR primers for mutagenesis

Mutation	Primer	Sequence
K417N	Spike-1251T-F	5'-AGGCAATATCGCCGACTACAACTAC-3'
	Spike-1251A-R	5'-GGCGATATTGCCTGTCTGTCCAG-3'
L452R	Spike-1355G-F	5'-ATTACCGGTACCGGCTGTTCCG-3'
	Spike 1355C-R	5'-CGGTACCGGTAATTGTAGTTGCCGC-3'
E484Q	Spike-1450C-F	5'-GGCGTGCAAGGCTTCAACTGCTA-3'
	Spike1450G-R	5'-GAAGCCTTGACACGCCGTTACAAGG-3'

E484K	Spike-1450A-F	5'-GGCGTGAAAGGCTTCAACTGCTA-3'
	Spike-1450T-R	5'-GAAGCCTTTCACGCCGTTACAAGG-3'
N501Y	Spike-1501T-F	5'-CCCACATATGGCGTGGGCTATCA-3'
	Spike-1501A-R	5'-CGCCATATGTGGGCTGAAAGCC-3'
D614G	Spike 1835G-F	5'-TACCAGGGCGTGAAGTGTACCGAAGTG-3'
	Spike-1852C-R	5'-TCACGCCCTGGTACAGCACTGCC-3'

185

186 Ten microliters of pseudovirus were incubated with serial dilutions of the serum samples for 1

187 hour at 37°C. Next, 100 µL of sera-pseudovirus were added to 293T-hACE2 cells in 96-well poly-

188 D-lysine coated culture plates. Following 48 hours of incubation in a 5% CO₂ environment at

189 37°C, the cells were lysed with 100 µL of Promega Glo Lysis buffer for 15 minutes at RT. Finally,

190 50 µL of the lysate were added to 50 µL luc substrate (Promega Luciferase Assay System). The

191 amount of luciferase was quantified by luminescence (relative luminescence units (RLU)), using

192 the Luminometer (Biosynergy H4). The percentage (%) virus inhibition was calculated as

$$193 \quad \% \text{ virus inhibition} = 1 - \frac{\text{Log10}(\text{sample}) - \text{Log10}(\text{uninfected cells})}{\text{Log10}(\text{infected cells}) - \text{Log10}(\text{uninfected cells})} \times 100$$

194 Serum from vaccinated mice was also compared by their 50% inhibitory dilution (IC₅₀), defined

195 as the serum dilution at which the virus infection was reduced by 50% compared with the negative

196 control (virus + cells).

197 SARS-CoV-2

198 SARS-CoV-2 (USA-WA1/2020) was obtained from the University of Texas Medical Branch

199 World Reference Center for Emerging Viruses and Arboviruses. SARS-CoV-2 virus Beta variant

200 in lineage B.1.351 was obtained from BEI Resources through the Africa Health Research

201 Institute (NR-54009 BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-

19/South Africa/KRISP-K005325/2020, NR-54009, contributed by Alex Sigal and Tulio de Oliveira.). The virus was grown on Vero CCL81, aliquoted, and titrated by standard plaque assay on Vero E6 cells (*Cercopithecus aethiops*, Vero 76, clone E6, ATCC CRL-1586) as previously described (https://wwwnc.cdc.gov/eid/article/26/6/20-0516_article). The viral stock sequence was verified using Oxford Nanopore sequencing technologies using the NEBNext ARCTIC protocol.

Plaque reduction neutralization test (PRNT)

Vero E6 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. The assay was performed in duplicate using 12-well tissue culture plates in a biosafety level 3 facility. Serial dilutions of each serum sample were incubated with 55 plaque-forming units of SARS-CoV-2 or the Beta variant for 1 h at 37 °C. The virus-serum mixtures were added onto pre-formed Vero E6 cell monolayers and incubated for 1 h at 37 °C in a 5% CO₂ incubator. The cell monolayer was then overlaid with 1% agarose:2X MEM:complete DMEM in a 1:1:1 ratio and incubated for 46 hours, after which time the plates were fixed and stained. Antibody titers were defined as the highest serum dilution that resulted in a >50% (IC₅₀) reduction in the number of virus plaques. This method has previously been extensively validated on infected and control sera.

Results

Preclinical studies were performed in eleven to thirteen-week-old female BALB/c mice, using the yeast-produced SARS-CoV-2 RBD219-N1C1 subunit vaccine candidate. Formulations were prepared with two protein amounts (2.3 and 7 μ g). Similar to previously published experiments, we adsorbed the protein on aluminum hydroxide (alum) containing 100 μ g aluminum per dose [14], and added, 20 μ g CpG1826 just before injection. The animals were immunized twice, intramuscularly on days 0 and 21. On day 35, the study was terminated, and serum and spleen samples were collected. Serum was tested for total anti-RBD IgG, and IgG subtypes, IgG2a and IgG1. Cytokines secreted into the supernatant upon restimulation of splenocytes were measured. Sera were further evaluated for neutralizing antibodies against the original SARS-CoV-2 and the variants-of-concern.

RBD219-N1C1/alum + CpG triggers a robust humoral immune response in mice.

Mice that received the vaccine antigen formulated with alum alone showed above baseline IgG titers in all groups (**Figure 1A**). The magnitude of the response was dose-dependent, as evidenced by the observation that mice that had received 7 μ g of RBD antigen showed mean IgG titers approximately 100x higher than mice that only received 2.3 μ g of the RBD antigen. The addition of 20 μ g CpG to the vaccine formulation significantly raised geometric mean titers by an additional factor of approximately 100 (when comparing formulations with a constant amount of 7 μ g of RBD antigen). In addition, we note that the intra-cohort variation in the response of individual mice was minimized through the addition of CpG.

We did not observe any statistically significant differences between the amount of antigen delivered and the mean IgG titers in the CpG-immunized groups. As expected for vaccines

adjuvanted with alum alone, IgG1 titers were greater than IgG2a titers (**Figure 1B**). The addition of CpG compensated for the alum-induced Th2-bias, producing a balanced IgG2a to IgG1 antibody response.

RBD219-N1C1/alum + CpG elicits a strong cellular immune response in mice

Cytokines secreted into the supernatant from splenocytes restimulated using recombinant SARS-CoV-2 RBD219-N1C1 protein were analyzed in a Luminex assay (**Figure 1C**). As expected for vaccines adjuvanted with alum alone, a Th2 cytokine response was induced, while the addition of CpG to the formulation raised a Th1/Th2 balanced cytokine response, with increased levels of anti-inflammatory cytokines IL-4 and IL-10, antiviral interferon IFN-g, and proinflammatory cytokines IL-6 and IL-2. We also observed that cytokine secretion increased when the RBD219-N1C1 protein doses increased from 2.3 to 7 µg.

RBD219-N1C1/alum + CpG elicits a strong neutralizing antibody response in mice against the original Wuhan SARS-CoV-2

Sera were tested for neutralizing antibodies in a pseudovirus assay. **Figure 1D** shows the IC50 values measured by a neutralization assay using a lentiviral SARS-CoV-2 pseudovirus with a spike protein closely mimicking the sequence of the spike protein of the first virus isolated in Wuhan, China (Genbank entry MN908947.3). We detected no neutralizing antibodies in the sera of mice immunized twice with 2.3 µg RBD/alum, and only 3 out of 8 mice immunized with two doses of the 7 µg RBD/alum showed neutralizing titers. In the latter group, we also observed a widespread response difference between the individual mice.

The addition of 20 µg CpG to the vaccine formulation significantly raised the mean neutralizing titers by a factor of approximately 100. Notably, sera from mice that received 7 µg RBD/alum+CpG showed on average 5.5 times higher neutralizing antibody titers compared to the NIBSC 20/136 human convalescent plasma standard. There was no statistical difference between the amount of neutralizing antibodies when the RBD219-N1C1 protein was increased from 2.3 to 7 µg, however, we did observe a reduction in the geometric mean titers (GMT) and the intra-cohort variation (Comparing 7 µg RBD/Alum+CpG GMT=4631 95% confidence interval 3293-6514, to 2.3 µg RBD/Alum+CpG GMT=2012 95% confidence interval 595-6808).

A booster dose may not require the addition of CpG to elicit strong humoral and cellular immune responses in mice

We evaluated the need for CpG to be included in the boost vaccination strategy. As can be seen in **Figures 2A-B**, when the second dose of RBD219-N1C1/alum+CpG was replaced with a formulation containing RBD219-N1C1/alum alone (same formulation without CpG), no significant drop in the antibody titers were observed when compared to two doses of RBD219-N1C1/alum+CpG. The cytokine profile was also similar to the one observed with the second dose containing CpG. Finally, we also detected similar amounts of neutralizing antibodies (**Figure 2D**), indicating that a boost without CpG may be sufficient to induce a protective immunological response.

The RBD219-N1C1/alum+CpG vaccine elicits robust neutralizing antibodies effective against the B.1.1.7 (Alpha), B.1.351 (Beta), and B.1.617.1 (Kappa) SARS-CoV-2 variants

To determine if the vaccine would cross-protect against different variants of concern and a variant of interest of the SARS-CoV-2 virus, we evaluated the neutralizing antibodies from selected pooled sera against four pseudoviruses: (i) Original Wuhan SARS-CoV-2 isolate, (ii) B.1.1.7 (Alpha) SARS-CoV-2 variant, (iii) B.1.617.1 (Kappa) SARS-CoV-2 variant and (iv) B.1.351 (Beta) SARS-CoV-2 variant (**Figure 3A, Supplementary Figure 1, Supplementary Table 1**).

Sera from mice vaccinated with the CpG adjuvanted vaccine candidate maintained the ability to neutralize pseudoviruses which include key mutations, mimicking important variants of interest and concern. Human convalescent plasma (WHO International Standard for anti-SARS-CoV-2 immunoglobulin, NIBSC code: 20/136 [27]) showed reduced neutralization ability against the variant pseudoviruses, while the RBD219-N1C1/alum+CpG vaccine groups showed a reduction to a much lesser extent. Comparing the average IC₅₀ values for the Wuhan pseudovirus, the 7 µg RBD219-N1C1/alum+CpG vaccine showed 5.5 times higher values, while the IC₅₀ values for the B.1.351 pseudovirus (K417N, E484K, N501Y, D614G) are up to 20-fold higher (**Figure 3A**). The data indicates that broader protection is offered by the antibodies produced through the RBD219-N1C1/alum+ CpG vaccine versus the antibodies induced by natural infection with the original Wuhan virus.

To verify the results obtained by the pseudovirus experiments, we also evaluated the virus-neutralizing ability of the pooled mouse sera, using isolated live viruses in a plaque reduction

neutralization assay (PRNT) (**Figure 3B, Supplementary Figure 2, Supplementary Table 2**).

The results of the live virus assay were concordant with the results found in the pseudovirus assay (**Figure 3A, B**). For example, against the B.1.351 live virus, the IC₅₀ values for the mouse serum obtained after two vaccinations with 7 µg RBD219-N1C1/alum+CpG are 18-fold higher than the human convalescent plasma standard (**Figure 3B**). This increased protection against B.1.351 is similar to the 20-fold increased protection calculated from the pseudovirus data. The equivalent results of the two isolated live strains of SARS-CoV-2 demonstrate that our pseudovirus-based neutralization assay can be used as a valid alternative to more rapidly screen for neutralizing activity of the serum antibodies against the spike protein of various SARS-CoV2 variants of interest.

In summary, the data reported here reinforces that the RBD219-N1C1/alum+CpG vaccine formulation is potentially suitable for broad neutralization against SARS-CoV-2 including variants-of-concern.

Discussion

Here we show the results of preclinical studies in mice of a recombinant protein vaccine candidate, SARS-CoV-2 RBD219-N1C1, produced in yeast and formulated with aluminum hydroxide (alum) and CpG deoxynucleotides, a formulation comparable to one that has currently entered phase 3 clinical development in India.

The ability of this vaccine in a two-dose scheme to broadly neutralize the original Wuhan SARS-CoV-2, as well as cross-neutralize against the B.1.1.7 (Alpha), B.1.617.1 (Kappa), and B.1.351

(Beta) variants of the virus may resemble similar findings in humans previously infected with COVID-19 and boosted through mRNA spike protein immunization [28].

The RBD antigen, adjuvanted with alum and CpG, elicited neutralization titers against the Wuhan SARS-CoV-2 in a pseudovirus assay, which were significantly higher than those provided by sera from mice vaccinated with RBD/alum or NIBSC 20/136 human convalescent plasma standard. In addition, the RBD219-N1C1/alum+CpG vaccine also provided superior protective antibodies against the emerging variants of concern [29]. Human convalescent plasma showed a 5.4-fold reduction of the neutralization of the B.1.351 pseudovirus ($IC_{50}=95$) compared to the Wuhan pseudovirus ($IC_{50}=508$). By contrast, sera from mice vaccinated with 7 μ g RBD219-N1C1/alum/CpG only showed a 1.49-fold reduction ($IC_{50}=1894$ and $IC_{50}=2187$), respectively, potentially indicating broader protection offered by the vaccine compared to natural infection. Furthermore, when comparing our neutralizing data to the results of (pre)clinical studies from other SARS-CoV-2 vaccines, we observe that our vaccine candidate is among the leading vaccines which induce high neutralizing antibody titers against SARS-CoV-2 and all tested variants [30, 31].

This improved protection against the variants of concern was also seen with live viruses. Against B.1.351 live virus, the IC_{50} neutralization values of mouse serum obtained after two vaccinations with 7 μ g RBD219-N1C1/alum+CpG ($IC_{50}PRNT=1678$) are 18-fold higher than the human convalescent plasma standard ($IC_{50}PRNT=90$). This again emphasizes the broader protection offered through the antibodies produced by a recombinant protein-based vaccine versus the antibodies induced by a natural infection.

Multiple vaccines containing CpG adjuvants [32] have undergone evaluation in human clinical trials, including vaccines for cancer, allergy, and infectious diseases. Most recently, a phase 1

study of the SARS-CoV-2 trimeric spike protein with alum+CpG showed excellent tolerability and immunogenicity [33]. While our RBD219-N1C1 antigen formulated only with alum elicited neutralizing antibody titers against SARS-CoV-2 that exceeded titers of the human convalescent plasma standard [14], there was a high level of variability observed, together with a strong bias for Th2-type immunity.

Such findings stimulated efforts to examine CpG formulations, leading to our current COVID-19 vaccine – RBD219-N1C1 on alum+CpG – which is now entering phase 3 trials.

We further evaluated the possibility of obtaining high neutralizing antibody titers with a single dose of the CpG adjuvanted vaccine, followed by a boost with RBD/alum. While the addition of CpG to the formulation of the prime vaccine resulted in an approximately 100-fold increase in antibodies, including CpG in the formulation of the boost vaccine induced only a minimal additional immunological effect. Therefore, potential booster vaccines may not need the addition of CpG to the formulations, thus reducing supply chain stress and reducing cost.

Further studies are in progress to examine whether the cross-protection of the vaccine corresponds to the enhanced neutralizing breadth phenomenon noted with previous COVID-19 infection followed by mRNA vaccine boosters [28]. Additionally, an important shortcoming of this initial investigation is that we did not address the duration of protection achieved after boosting with only RBD/alum. While antibody titers and cytokine profiles were in line with those observed in mice that received two vaccinations, we do not know if that will also hold after an extended period. Additional long-term studies are underway to evaluate the duration of the response. In parallel studies, we are also expanding our evaluation of a one-dose approach, which is of great interest to the scientific community since it would improve the ease of delivery as well as confidence and compliance with the necessary vaccination protocol.

Even though there are many vaccines under development against COVID-19, there remains an urgent need to provide additional tools against a virus that continues to spread, specifically in LMICs. There are several requirements that a COVID-19 vaccine for impoverished populations must meet. The vaccine must be able to be produced on a large scale. Moreover, storage and delivery logistics of the vaccine must be compatible with the infrastructure in LMICs (i.e. without the need for ultralow temperature storage). The yeast-produced RBD219-N1C1 vaccine uses a production platform very similar to that used for the hepatitis B vaccine and fulfills these criteria. It can be produced at a low cost, with high yields, and by manufacturers that have previous experience with yeast-based recombinant protein vaccines.

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Author Contributions

JP contributed to the design and planning of the experiments, prepared the vaccine formulations, quantified neutralizing antibodies, processed all data, prepared the figures, and drafted the manuscript.

US contributed to the design and planning of the experiments, the evaluation, and the interpretation of the data. US also drafted parts of the original manuscript and edited subsequent versions of the manuscript.

BZ and JW established the expression of recombinant protein RBD-219-N1C1 in yeast. ZL and JL performed the fermentation and purification of recombinant protein RBD219-N1C1.

WHC, RTK, and RA contributed to the characterization of the recombinant protein RBD219-N1C1.

LV contributed to the design and the planning of the experiments, the execution and data analysis of ELISA and Luminex experiments, and the review and editing of the manuscript.

BK contributed to the design and planning of the experiments and performed all animal manipulations.

PG contributed to the design and planning of the experiments, the evaluation, and the interpretation of the data.

BL and JTK prepared the plasmids for the pseudovirus-based neutralization assays.

MJV and CP have executed the pseudovirus experiments and processed data.

SR executed the live SARS-CoV-2 virus assays inside the BSL-3

MR and VP contributed to the design and planning of the experiments and managed the production of RBD219-N1C1 at Biological E, Limited (Hyderabad, India).

FC and BS conceptualized and designed the experiments.

MEB and PJH conceptualized and designed the experiments and managed the process of manuscript review and editing.

All authors contributed equally and provided critical feedback, reference sources, and critical revisions for intellectual content, and verified the information presented here.

Competing Interests

The authors declare they are developers of the RBD219-N1C1 technology, and that Baylor College of Medicine recently licensed it to Biological E, an Indian manufacturer, for further advancement and licensure. The research conducted in this paper was performed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Information

Supplementary Information was added as a separate document.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

References

- [1] Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *The Lancet Infectious diseases*. 2020;20:533-4.
- [2] Hotez PJ, Narayan KMV. Restoring Vaccine Diplomacy. *JAMA*. 2021.
- [3] Zimmer C, Corum J, Wee S-L. Coronavirus Vaccine Tracker. *New York Times* 2021.
- [4] Holder J. Tracking Coronavirus Vaccinations Around the World. *The New York Times* 2021.
- [5] Mascola JR, Graham BS, Fauci AS. SARS-CoV-2 Viral Variants-Tackling a Moving Target. *JAMA*. 2021;325:1261-2.
- [6] Garcia-Beltran WF, Lam EC, Denis KS, Nitido AD, Garcia ZH, Hauser BM, et al. Circulating SARS-CoV-2 variants escape neutralization by vaccine-induced humoral immunity. *medRxiv : the preprint server for health sciences*. 2021.
- [7] Pollet J, Chen WH, Strych U. Recombinant protein vaccines, a proven approach against coronavirus pandemics. *Adv Drug Deliv Rev*. 2021;170:71-82.
- [8] Hotez PJ, Bottazzi ME, Strych U. New Vaccines for the World's Poorest People. *Annu Rev Med*. 2016;67:405-17.
- [9] Bottazzi ME, Hotez PJ. "Running the Gauntlet": Formidable challenges in advancing neglected tropical diseases vaccines from development through licensure, and a "Call to Action". *Human vaccines & immunotherapeutics*. 2019;15:2235-42.
- [10] Chen WH, Tao X, Agrawal A, Algaissi A, Peng BH, Pollet J, et al. Yeast-Expressed SARS-CoV Recombinant Receptor-Binding Domain (RBD219-N1) Formulated with Alum Induces Protective Immunity and Reduces Immune Enhancement. *Vaccine*. 2020;22:31232-9.
- [11] Nyon MP, Du L, Tseng CK, Seid CA, Pollet J, Naceanceno KS, et al. Engineering a stable CHO cell line for the expression of a MERS-coronavirus vaccine antigen. *Vaccine*. 2018;36:1853-62.
- [12] King HAD, Gordon Joyce M, Naouar IE, Ahmed A, Cincotta CM, Subra C, et al. Efficacy and breadth of adjuvanted SARS-CoV-2 receptor-binding domain nanoparticle vaccine in macaques. *bioRxiv*. 2021.

[13] Yang S, Li Y, Dai L, Wang J, He P, Li C, et al. Safety and immunogenicity of a recombinant tandem-repeat dimeric RBD-based protein subunit vaccine (ZF2001) against COVID-19 in adults: two randomised, double-blind, placebo-controlled, phase 1 and 2 trials. *The Lancet Infectious diseases*. 2021.

[14] Pollet J, Chen W-H, Versteeg L, Keegan B, Zhan B, Wei J, et al. SARS-CoV-2 RBD219-N1C1: A yeast-expressed SARS-CoV-2 recombinant receptor-binding domain candidate vaccine stimulates virus neutralizing antibodies and T-cell immunity in mice. *Human vaccines & immunotherapeutics*. 2021:1-11.

[15] Campbell JD. Development of the CpG Adjuvant 1018: A Case Study. In: Fox C, editor. *Vaccine adjuvants : methods and protocols*: Humana Press, New York; 2016. p. 15-27.

[16] Mirotti L, Alberca Custodio RW, Gomes E, Rammauro F, de Araujo EF, Garcia Calich VL, et al. CpG-ODN Shapes Alum Adjuvant Activity Signaling via MyD88 and IL-10. *Front Immunol*. 2017;8:47.

[17] Arunachalam PS, Walls AC, Golden N, Atyeo C, Fischinger S, Li C, et al. Adjuvanting a subunit COVID-19 vaccine to induce protective immunity. *Nature*. 2021.

[18] McGill_COVID19_Tracker_Team. COVID-19 VACCINE TRACKER. 2021.

[19] Switaj T, Jalili A, Jakubowska AB, Drela N, Stoksik M, Nowis D, et al. CpG immunostimulatory oligodeoxynucleotide 1826 enhances antitumor effect of interleukin 12 gene-modified tumor vaccine in a melanoma model in mice. *Clin Cancer Res*. 2004;10:4165-75.

[20] Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. *Vaccine*. 2014;32:6377-89.

[21] Lee J, Liu Z, Chen WH, Wei J, Kundu R, Adhikari R, et al. Process development and scale-up optimization of the SARS-CoV-2 receptor binding domain-based vaccine candidate, RBD219-N1C1. *Appl Microbiol Biotechnol*. 2021.

[22] Chen WH, Wei J, Kundu RT, Adhikari R, Liu Z, Lee J, et al. Genetic modification to design a stable yeast-expressed recombinant SARS-CoV-2 receptor binding domain as a COVID-19 vaccine candidate. *Biochim Biophys Acta Gen Subj*. 2021:129893.

[23] Versteeg L, Le Guezennec X, Zhan B, Liu Z, Angagaw M, Woodhouse JD, et al. Transferring Luminex(R) cytokine assays to a wall-less plate technology: Validation and comparison study with plasma and cell culture supernatants. *J Immunol Methods*. 2017;440:74-82.

[24] He J, Choe S, Walker R, Di Marzio P, Morgan DO, Landau NR. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J Virol*. 1995;69:6705-11.

[25] Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature biotechnology*. 1997;15:871-5.

[26] Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol*. 2020;5:562-9.

[27] Kristiansen PA, Page M, Bernasconi V, Mattiuzzo G, Dull P, Makar K, et al. WHO International Standard for anti-SARS-CoV-2 immunoglobulin. *Lancet*. 2021;397:1347-8.

[28] Wang Z, Musecksch F, Schaefer-Babajew D, Finkin S, Viant C, Gaebler C, et al. Vaccination boosts naturally enhanced neutralizing breadth to SARS-CoV-2 one year after infection. *bioRxiv*. 2021.

[29] Earle KA, Ambrosino DM, Fiore-Gartland A, Goldblatt D, Gilbert PB, Siber GR, et al. Evidence for antibody as a protective correlate for COVID-19 vaccines. *Vaccine*. 2021.

[30] Spencer AJ, Morris S, Ulaszewska M, Powers C, Kailath R, Bissett C, et al. The ChAdOx1 vectored vaccine, AZD2816, induces strong immunogenicity against SARS-CoV-2 B.1.351 and other variants of concern in preclinical studies. *bioRxiv*. 2021:2021.06.08.447308.

[31] Stamatatos L, Czartoski J, Wan YH, Homad LJ, Rubin V, Glantz H, et al. mRNA vaccination boosts cross-variant neutralizing antibodies elicited by SARS-CoV-2 infection. *Science*. 2021.

[32] Kayraklioglu N, Horuluoglu B, Klinman DM. CpG Oligonucleotides as Vaccine Adjuvants. *Methods Mol Biol*. 2021;2197:51-85.

[33] Richmond P, Hatchuel L, Dong M, Ma B, Hu B, Smolenov I, et al. Safety and immunogenicity of S-Trimer (SCB-2019), a protein subunit vaccine candidate for COVID-19 in healthy adults: a phase 1, randomised, double-blind, placebo-controlled trial. *Lancet*. 2021;397:682-94.

Figure Legends

Figure 1: Humoral and Cellular response analysis of immunized mice. **A)** Total IgG titers at day 35, measured by ELISA. **B)** IgG2a/IgG1 titer ratios. Geometric mean and 95% confidence intervals are shown. **C)** Heatmap of secreted cytokine levels measured in the supernatants from splenocytes re-stimulated with the SARS-CoV-2 RBD protein. Median values were calculated within each treatment group for each cytokine and plotted. **D)** IC50 values measured by a neutralization assay using a lentiviral SARS-CoV-2 Wuhan pseudovirus. Mann-Whitney tests were performed to evaluate for statistical significance between different groups. $p > 0.12$ (ns), $p < 0.033$ (*), $p < 0.002$ (**), $p < 0.001$ (***)).

Figure 2: Comparison of RBD219-N1C1-specific immune responses after one dose with CpG and a boost without CpG versus two vaccine doses with CpG. Geometric mean and 95% confidence intervals are shown. **A)** Total IgG titers measured from mouse sera against RBD protein, **B)** IgG2a/IgG1 titer ratios from mouse sera against RBD protein, **C)** Heatmap of secreted cytokines measured in supernatants from splenocytes re-stimulated with RBD protein. **D)** IC50 values measured by a neutralization assay using a lentiviral Wuhan SARS-CoV-2 pseudovirus. Mann-Whitney tests were performed to evaluate for statistical significance between different groups. $p > 0.12$ (ns), $p < 0.033$ (*), $p < 0.002$ (**), $p < 0.001$ (***)).

Figure 3: Neutralizing activity against different SARS-CoV-2 variants **A)** Neutralization of various Pseudovirus mimicking different variants of concern: Wuhan SARS-CoV-2 (blue), B.1.1.7 (Alpha) SARS-CoV-2 variant (red), B.1.351 (Beta) SARS-CoV-2 (green) and B.1.617.1 (Kappa) SARS-CoV-2 variant (purple). **B)** Live virus neutralization (PRNT assay): Original SARS-CoV-2 (blue), B.1.351 (Beta) SARS-CoV-2 variant (green).

Supplementary Material

Supplementary Figure 1: Neutralization of SARS-CoV-2 Pseudovirus mimicking different variants. Datapoints represent averages (n=4)

Supplementary Table 1: IC50 values of pseudovirus neutralization assays

Supplementary Figure 2: Live Virus Neutralization. A) Plaque reduction neutralization test (PRNT) with select serum samples using the live virus strain USA_WA1/2020, isolated before the emergence of key mutations, and PRNT with select serum samples using a live virus strain of the (B.1.351 variant (Alpha). Datapoints represent averages (n=2).

Supplementary Table 2: IC50 values of live virus neutralization assays

A) Total IgG titers

B) IgG2a/IgG1 titer ratios

C) Cytokine release profile of restimulated splenocytes

D) IC50 values measured by a pseudovirus neutralization assay

A) Total IgG titers

B) IgG2a/IgG1 titer ratios

C) Cytokine release profile of restimulated splenocytes

D) IC50 values measured by a pseudovirus neutralization assay

A) Pseudovirus assay neutralization

B) Live virus neutralization