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

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

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

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

85 In humans, the Class B CpG1018 has a strong track record in the clinic and is part of the
86 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-κB 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).

109 **Vaccine formulations**

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

113 **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

114

115

116 **Preclinical Design**

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

121

122 **Serological Antibody Measurements by ELISA**

123 To examine SARS-CoV-2 RBD-specific antibodies in the mouse sera, indirect ELISAs were
124 conducted as published elsewhere [14]. Briefly, plates were coated with 0.2 µg/well SARS-CoV-
125 2 RBD219-N1C1. Mouse serum samples were 3-fold diluted from 1:200 to 1: 11,809,800 in 0.1%
126 BSA in assay buffer (0.05% Tween-20 in 1x PBS). Assay controls included a 1:200 dilution of
127 pooled naive mouse sera and assay buffer as blanks. 100 µL/well of either 1:6000 goat anti-mouse
128 IgG HRP, 1:6000 goat anti-mouse IgG1 HRP or 1:6000 goat anti-mouse IgG2a HRP in assay
129 buffer was added. After incubation plates were washed five times followed by the addition of 100
130 µL/well 4°C TMB substrate. Plates were incubated for 15 minutes at room temperature (RT) while
131 protected from light. After incubation, the reaction was stopped by adding 100 µL/well 1 M HCl.
132 The absorbance at a wavelength of 450 nm was measured using a Biotek Epoch 2
133 spectrophotometer

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

140

141 **Cytokine secretion in re-stimulated splenocytes by Luminex**

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

145 After 1 minute at RT, the reaction was stopped by the addition of 40 mL PBS. Tubes were
146 centrifuged for 5 minutes at 300 x g at RT. Then supernatant was discarded and splenocytes were
147 resuspended in 5 mL 4°C cRPMI (RPMI 1640 + 10% HI FBS + 1x pen/strep). Splenocyte
148 suspensions were transferred through a 40 µm filter to a new 50-mL conical tube to obtain a single-
149 cell suspension. For the *in vitro* cytokine release assay, splenocytes were seeded in a 96-well
150 culture plate at $1x10^6$ live cells in 250 µL cRPMI. Splenocytes were (re-)stimulated with 10 µg/mL
151 RBD219-N1-C1 protein, PMA/Ionomycin (positive control), or media (negative control) for 48
152 hours at 37° C 5% CO_2. After incubation, 96-well plates were centrifuged to pellet the splenocytes
153 down and supernatant was transferred to a new 96-well plate. A Milliplex Mouse Th17 Luminex
154 kit (EMD Millipore) with analytes IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17A, IFN-γ, and TNF-
155 α was used to quantify the cytokines secreted in the supernatant by the re-stimulated splenocytes
156 according to a previously published method [23]. The readout was performed using a MagPix
157 Luminex instrument. Raw data was analyzed using Bio-Plex Manager software, and further
158 analysis was done with Excel and Prism. For the visualization of the individual cytokine results in
159 the heatmap, the median was calculated of the cytokine concentrations of each group.

160 **Pseudovirus assay for determination of neutralizing antibodies**

161 The pseudovirus used is a non-replicating lentiviral particle that has the SARS-CoV-2 spike
162 protein on its membrane and can express a luciferase gene after infection. Using in vitro grown
163 human 293T-hACE2 cells, infected cells were quantified based on the expression of luciferase
164 [14].

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

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

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

184 **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'-GAAGCCTTGCACGCCGTTACAAGG-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'-TACCAGGGCGTGAACTGTACCGAAGTG-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 $$\%\ virus\ inhibition = 1 - \frac{Log10(sample) - Log10(uninfected\ cells)}{Log10(infected\ cells) - Log10(uninfected\ cells)} x100$$

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-

202 19/South Africa/KRISP-K005325/2020, NR-54009, contributed by Alex Sigal and Tulio de
203 Oliveira.). The virus was grown on Vero CCL81, aliquoted, and titrated by standard plaque assay
204 on Vero E6 cells (Cercopithecus aethiops, Vero 76, clone E6, ATCC CRL-1586) as previously
205 described (https://wwwnc.cdc.gov/eid/article/26/6/20-0516_article). The viral stock sequence
206 was verified using Oxford Nanopore sequencing technologies using the NEBNext ARCTIC
207 protocol.

208 **Plaque reduction neutralization test (PRNT)**

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

219

220 **Results**

221 Preclinical studies were performed in eleven to thirteen-week-old female BALB/c mice, using the
222 yeast-produced SARS-CoV-2 RBD219-N1C1 subunit vaccine candidate. Formulations were
223 prepared with two protein amounts (2.3 and 7 µg). Similar to previously published experiments,
224 we adsorbed the protein on aluminum hydroxide (alum) containing 100 µg aluminum per dose
225 [14], and added, 20 µg CpG1826 just before injection. The animals were immunized twice,
226 intramuscularly on days 0 and 21. On day 35, the study was terminated, and serum and spleen
227 samples were collected. Serum was tested for total anti-RBD IgG, and IgG subtypes, IgG2a and
228 IgG1. Cytokines secreted into the supernatant upon restimulation of splenocytes were measured.
229 Sera were further evaluated for neutralizing antibodies against the original SARS-CoV-2 and the
230 variants-of-concern.

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

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

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

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

245

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

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

254

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

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

264 The addition of 20 µg CpG to the vaccine formulation significantly raised the mean neutralizing
265 titers by a factor of approximately 100. Notably, sera from mice that received 7 µg
266 RBD/alum+CpG showed on average 5.5 times higher neutralizing antibody titers compared to the
267 NIBSC 20/136 human convalescent plasma standard. There was no statistical difference between
268 the amount of neutralizing antibodies when the RBD219-N1C1 protein was increased from 2.3 to
269 7 µg, however, we did observe a reduction in the geometric mean titers (GMT) and the intra-cohort
270 variation (Comparing 7 µg RBD/Alum+CpG GMT=4631 95% confidence interval 3293-6514,
271 to 2.3 µg RBD/Alum+CpG GMT=2012 95% confidence interval 595-6808).

272

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

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

283

284

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

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

292

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

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

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

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

319

320 **Discussion**

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

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

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

329 The RBD antigen, adjuvanted with alum and CpG, elicited neutralization titers against the Wuhan
330 SARS-CoV-2 in a pseudovirus assay, which were significantly higher than those provided by sera
331 from mice vaccinated with RBD/alum or NIBSC 20/136 human convalescent plasma standard. In
332 addition, the RBD219-N1C1/alum+CpG vaccine also provided superior protective antibodies
333 against the emerging variants of concern [29]. Human convalescent plasma showed a 5.4-fold
334 reduction of the neutralization of the B.1.351 pseudovirus (IC50=95) compared to the Wuhan
335 pseudovirus (IC50=508). By contrast, sera from mice vaccinated with 7 µg RBD219-
336 N1C1/alum/CpG only showed a 1.49-fold reduction (IC50=1894 and IC50=2187), respectively,
337 potentially indicating broader protection offered by the vaccine compared to natural infection.
338 Furthermore, when comparing our neutralizing data to the results of (pre)clinical studies from
339 other SARS-CoV-2 vaccines, we observe that our vaccine candidate is among the leading vaccines
340 which induce high neutralizing antibody titers against SARS-CoV-2 and all tested variants [30,
341 31].

342 This improved protection against the variants of concern was also seen with live viruses. Against
343 B.1.351 live virus, the IC50 neutralization values of mouse serum obtained after two vaccinations
344 with 7 µg RBD219-N1C1/alum+CpG (IC50PRNT=1678) are 18-fold higher than the human
345 convalescent plasma standard ((IC50PRNT= 90). This again emphasizes the broader protection
346 offered through the antibodies produced by a recombinant protein-based vaccine versus the
347 antibodies induced by a natural infection.

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

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

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

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

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

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

382

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392 **Author Contributions**

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

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

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

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

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

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

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

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

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

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

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

414 FC and BS conceptualized and designed the experiments.

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

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

419 **Competing Interests**

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

424

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.

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508

509

510 **Figure Legends**

511 **Figure 1: Humoral and Cellular response analysis of immunized mice**. **A)** Total IgG titers at
512 day 35, measured by ELISA. **B)** IgG2a/IgG1 titer ratios. Geometric mean and 95% confidence
513 intervals are shown. **C)** Heatmap of secreted cytokine levels measured in the supernatants from
514 splenocytes re-stimulated with the SARS-CoV-2 RBD protein. Median values were calculated
515 within each treatment group for each cytokine and plotted. **D)** IC50 values measured by a
516 neutralization assay using a lentiviral SARS-CoV-2 Wuhan pseudovirus. Mann-Whitney tests
517 were performed to evaluate for statistical significance between different groups. $p > 0.12$(ns), $p <$
518 0.033 (*),$p < 0.002$ (**), $p<0.001$ (***).

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

527 **Figure 3: Neutralizing activity against different SARS-CoV-2 variants A)** Neutralization of
528 various Pseudovirus mimicking different variants of concern: Wuhan SARS-CoV-2 (blue), B.1.1.7
529 (Alpha) SARS-CoV-2 variant (red), B.1.351 (Beta) SARS-CoV-2 (green) and B.1.617.1 (Kappa)
530 SARS-CoV-2 variant (purple). **B)** Live virus neutralization (PRNT assay): Original SARS-CoV-
531 2 (blue), B.1.351 (Beta) SARS-CoV-2 variant (green).

532 **Supplementary Material**

533

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

536

537 **Supplementary Table 1: IC50 values of pseudovirus neutralization assays**

538

539

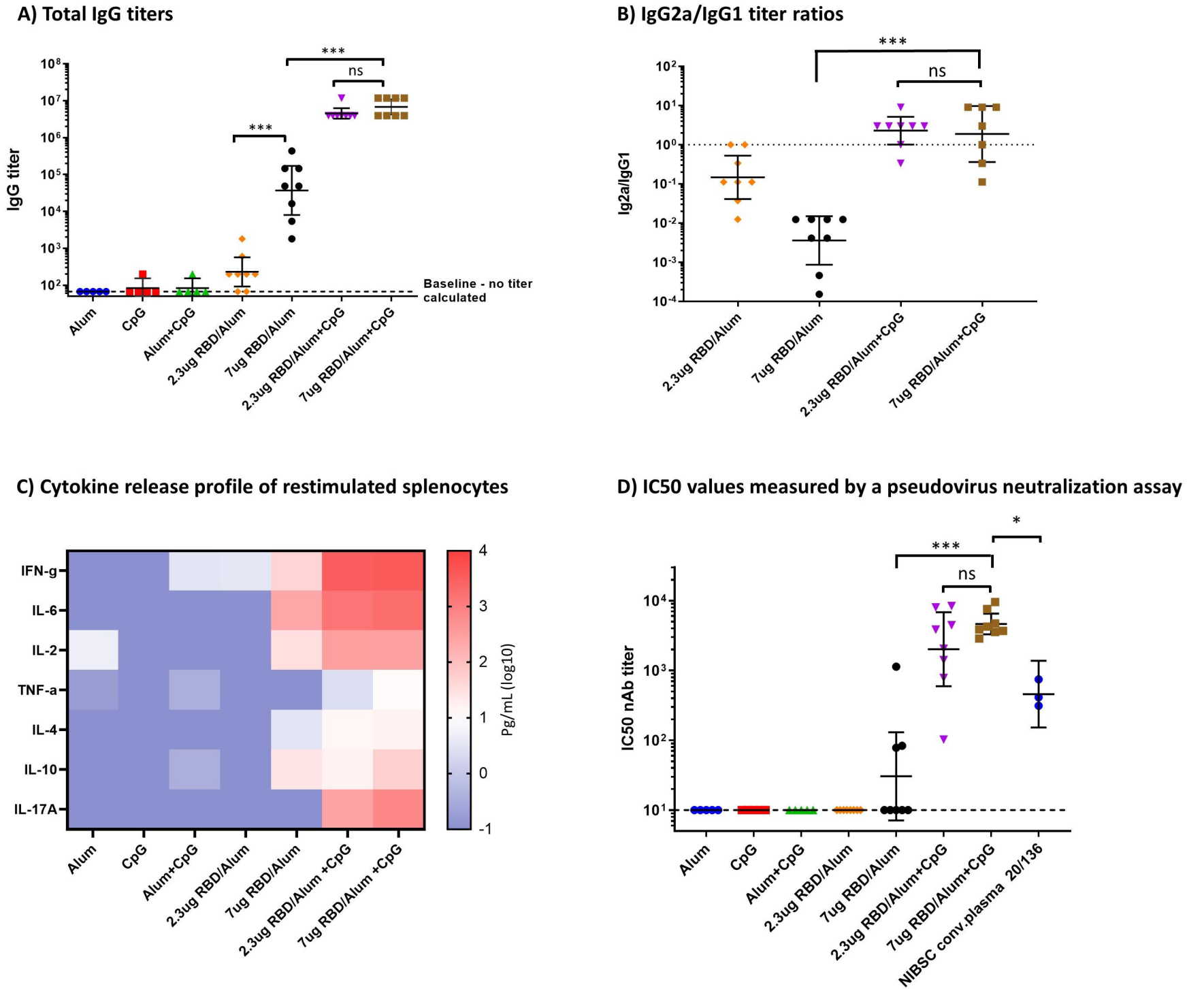
540 **Supplementary Figure 2: Live Virus Neutralization.** A) Plaque reduction neutralization test
541 (PRNT) with select serum samples using the live virus strain USA_WA1/2020, isolated before the
542 emergence of key mutations, and PRNT with select serum samples using a live virus strain of the
543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

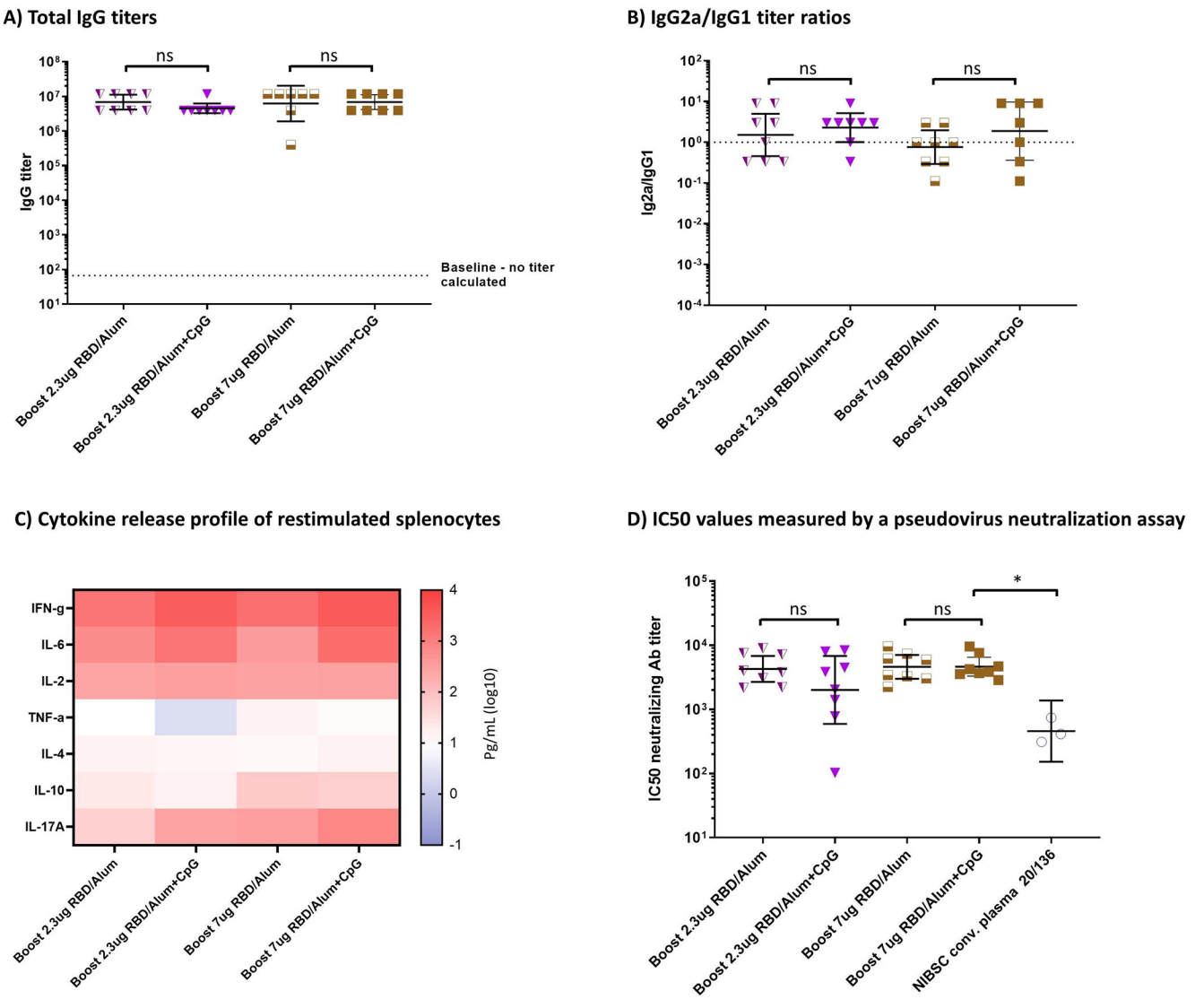
544

545 **Supplementary Table 2: IC50 values of live virus neutralization assays**

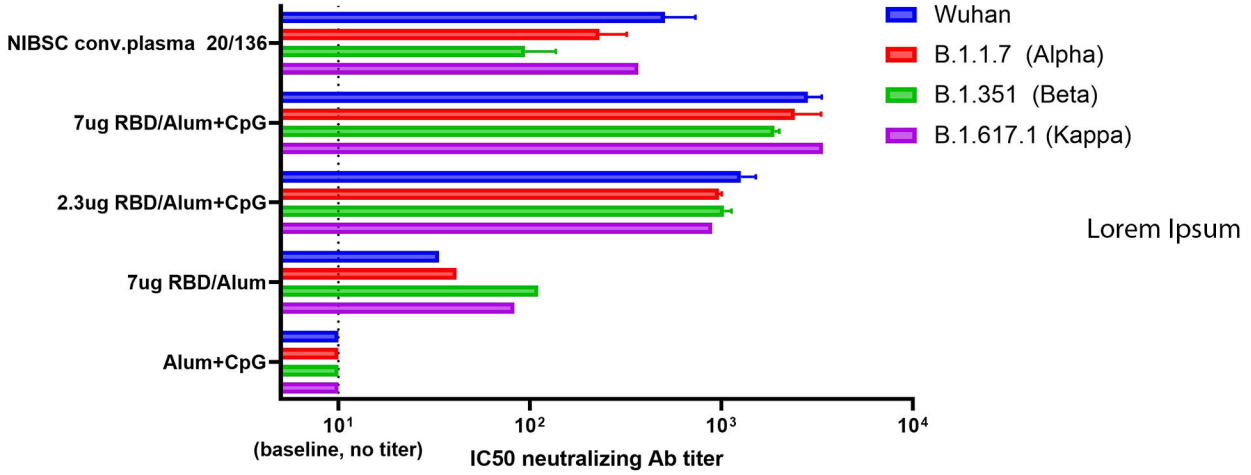
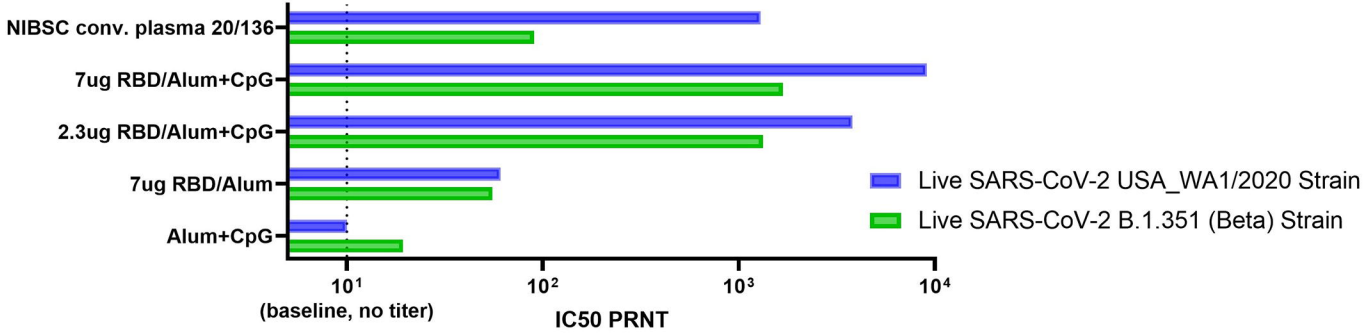
546

547


A) Total IgG titers
IgG titer
Baseline - no titer calculated
Alum
CpG
Alum+CpG
2.3ug RBD/Alum
7ug RBD/Alum
2.3ug RBD/Alum+CpG
7ug RBD/Alum+CpG
***
ns
B) IgG2a/IgG1 titer ratios
Ig2a/IgG1
C) Cytokine release profile of restimulated splenocytes
IFN-g
IL-6
IL-2
TNF-a
IL-4
IL-10
IL-17A
Pg/mL (log10)
D) IC50 values measured by a pseudovirus neutralization assay
IC50 nAb titer
NIBSC conv.plasma 20/136
*


A) Total IgG titers
IgG titer
ns
ns
Baseline - no titer calculated
Boost 2.3ug RBD/Alum
Boost 2.3ug RBD/Alum+CpG
Boost 7ug RBD/Alum
Boost 7ug RBD/Alum+CpG
B) IgG2a/IgG1 titer ratios
Ig2a/IgG1
ns
ns
Boost 2.3ug RBD/Alum
Boost 2.3ug RBD/Alum+CpG
Boost 7ug RBD/Alum
Boost 7ug RBD/Alum+CpG
C) Cytokine release profile of restimulated splenocytes
IFN-g
IL-6
IL-2
TNF-a
IL-4
IL-10
IL-17A
Pg/mL (log10)
Boost 2.3ug RBD/Alum
Boost 2.3ug RBD/Alum+CpG
Boost 7ug RBD/Alum
Boost 7ug RBD/Alum+CpG
D) IC50 values measured by a pseudovirus neutralization assay
IC50 neutralizing Ab titer
ns
ns
*
Boost 2.3ug RBD/Alum
Boost 2.3ug RBD/Alum+CpG
Boost 7ug RBD/Alum
Boost 7ug RBD/Alum+CpG
NIBSC conv. plasma 20/136

A) Pseudovirus assay neutralization



B) Live virus neutralization