

Title Page

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

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3 **Jeroen Pollet^{1,2}, Wen-Hsiang Chen^{1,2}, Leroy Versteeg¹, Brian Keegan¹, Bin Zhan^{1,2}, Junfei**
4 **Wei¹, Zhuyun Liu¹, Jungsoon Lee¹, Rahki Kundu¹, Rakesh Adhikari¹, Cristina Poveda¹,**
5 **Maria-Jose Villar Mondragon¹, Ana Carolina de Araujo Leao¹, Joanne Altieri Rivera¹, Portia**
6 **M. Gillespie¹, Ulrich Strych^{1,2}, Peter J. Hotez^{1,2,3,4,*}, Maria Elena Bottazzi^{1,2,3*}**

7 ¹ Texas Children's Hospital Center for Vaccine Development, Houston, TX, USA

8 ² Departments of Pediatrics and Molecular Virology & Microbiology, National School of Tropical
9 Medicine, Baylor College of Medicine, Houston, TX, USA

10 ³ Department of Biology, Baylor University, Waco, TX, USA

11 ⁴ James A. Baker III Institute for Public Policy, Rice University, Houston, TX, USA

12 *** Correspondence:**

13 Corresponding Authors

14 bottazzi@bcm.edu; hotez@bcm.edu

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16

17 **Abstract**

18 There is an urgent need for an accessible and low-cost COVID-19 vaccine suitable for low- and
19 middle-income countries. Here we report on the development of a SARS-CoV-2 receptor-binding
20 domain (RBD) protein, expressed at high levels in yeast (*Pichia pastoris*), as a suitable vaccine
21 candidate against COVID-19. After introducing two modifications into the wild-type RBD gene to
22 reduce yeast-derived hyperglycosylation and improve stability during protein expression, we show
23 that the recombinant protein, RBD219-N1C1, is equivalent to the wild-type RBD recombinant
24 protein (RBD219-WT) in an *in vitro* ACE-2 binding assay. Immunogenicity studies of RBD219-
25 N1C1 and RBD219-WT proteins formulated with Alhydrogel[®] were conducted in mice, and, after
26 two doses, both the RBD219-WT and RBD219-N1C1 vaccines induced high levels of binding IgG
27 antibodies. Using a SARS-CoV-2 pseudovirus, we further showed that sera obtained after a two-dose
28 immunization schedule of the vaccines were sufficient to elicit strong neutralizing antibody titers in
29 the 1:1,000 to 1:10,000 range, for both antigens tested. The vaccines induced IFN- γ , IL-6, and IL-10
30 secretion, among other cytokines. Overall, these data suggest that the RBD219-N1C1 recombinant
31 protein, produced in yeast, is suitable for further evaluation as a human COVID-19 vaccine, in
32 particular, in an Alhydrogel[®] containing formulation and possibly in combination with other
33 immunostimulants.

34 **1 Introduction**

35 The number of coronavirus disease 19 (COVID-19) cases globally is readily approaching the 50-
36 million-person mark, with over 1.2 million deaths. In response to the pandemic, an international
37 enterprise to develop effective and safe vaccines is underway. There are many ways to categorize the
38 more than 100 potential COVID-19 vaccine candidates¹, but one approach is to divide them as those
39 employing new technologies for production, but that have not yet been licensed for use, versus
40 traditional vaccine production approaches with prior experience in licensed vaccines². The Operation
41 Warp Speed (OWS) initiative in the United States³ and other similar efforts in other parts of the
42 world⁴ initially seemed to focus on approaches employing new platforms, including several
43 messenger RNA (mRNA)-based vaccines as well as non-replicating adenovirus vector vaccines³.
44 Among the more established or traditional approaches, whole-inactivated virus vaccines on
45 aluminum oxy-hydroxide have been developed in China⁵, as have several recombinant protein
46 vaccine candidates⁶⁻⁸. Each of these approaches offers both distinct advantages and disadvantages in
47 terms of production, scale-up, potential efficacy and safety, and delivery.

48 We have previously reported on recombinant protein-based coronavirus vaccine candidates,
49 formulated with Alhydrogel[®] to prevent severe acute respiratory syndrome (SARS)⁹⁻¹¹ and Middle
50 East Respiratory Syndrome (MERS)¹². In both cases, the receptor-binding domain (RBD) of the
51 SARS or MERS spike proteins was used as the target vaccine antigen. In a mouse model, the SARS-
52 CoV RBD219-N1/Alhydrogel[®] vaccine induced high titers of virus-neutralizing antibodies and
53 protective immunity against a mouse-adapted SARS-CoV virus challenge. It was also found to
54 minimize or prevent eosinophilic immune enhancement compared to the full spike protein⁹.

55 The RBD of SARS-CoV-2 has likewise attracted interest from several groups now entering
56 clinical trials with RBD-based vaccines^{7,13-17}. Our approach was to apply the lessons learned from the
57 development of the SARS-CoV vaccine candidate and accelerate the COVID-19 vaccine

58 development efforts using microbial fermentation in the yeast *Pichia pastoris*; a technology that is
59 widely available and used to produce recombinant hepatitis B vaccines in several middle-income
60 countries (LMICs)¹⁸, including Bangladesh, Brazil, Cuba, India, and Indonesia. As COVID-19
61 spreads across the globe, especially among urban populations living in extreme poverty¹⁹, there will
62 be greater urgency to produce safe, effective, highly scalable, and affordable COVID-19 vaccines
63 locally or regionally. Therefore, the development of a yeast-expressed recombinant protein-based
64 COVID-19 vaccine allows developing it for global health and populations vulnerable to poverty-
65 related diseases²⁰.

66 Here, we present the first preclinical data of a COVID-19 recombinant protein-based vaccine
67 candidate, SARS-CoV2 RBD219-N1C1, formulated with Alhydrogel[®]. We demonstrate that
68 modifications made to the SARS-CoV2 RBD gene to improve production and stability preserve the
69 protein antigen functionality and its immunogenicity after Alhydrogel[®] adsorption.

70

71 2 Methods

72 2.1 Cloning and expression of the genes encoding RBD219-WT and RBD219-N1C1:

73 The RBD219-WT recombinant subunit protein contains amino acid residues 331-549 of the SARS-
74 CoV-2 spike protein (GenBank No.: QHD43416.1). It contains a hexahistidine tag at its C-terminus.
75 In the tag-free RBD219-N1C1 antigen candidate, N331 is not present, and C538 has been mutant to
76 an alanine residue to prevent aggregation due to intermolecular disulfide bonding. The DNAs for
77 both antigen candidates were individually synthesized with their codon use optimized for translation
78 in *Pichia pastoris* and ligated into pPICZ α A using the EcoRI and XbaI restriction sites (GenScript,
79 Piscataway, NJ, USA). The recombinant plasmids were electroporated into *P. pastoris* X33 following
80 the EasySelect™ *Pichia* Expression Kit Manual (Invitrogen, Carlsbad, CA, USA). Transformants
81 were selected on YPD plates containing different concentrations of Zeocin (100-2000 μ g/mL) and
82 incubated at 30°C for 72 hours. Individual colonies were screened for expression under induction
83 with methanol (0.5-2%) at the 10 mL culture level (MMGY medium) as described¹⁰. The expression
84 level of select colonies was identified by SDS-PAGE and Western Blotting using anti-SARS-CoV-2
85 antibodies (anti-SARS-CoV-2 spike rabbit monoclonal antibody, Sino Biological, Wayne, PA, USA,
86 Cat # 40150-R007), and research seed stocks of the highest expressing clones were frozen at -80 °C.

87 RBD219-WT and RBD219-N1C1 were expressed at the 5 L scale using a Celligen 310
88 benchtop fermentation system (Eppendorf, Enfield, CT, USA). For the RBD-WT, 2.5 L of basal salt
89 medium were inoculated with a seed culture to an initial OD₆₀₀ of 0.05 and grown at 30 °C, pH 5.0
90 with 30% dissolved oxygen until glycerol depletion. During the first hour of methanol induction, the
91 temperature was reduced from 30 °C to 26 °C and the pH was increased from 5.0 to 6.0. After
92 approximately 70 hours of induction (methanol feed at 1-11 mL/L/hr), the culture was harvested
93 from the fermenter, and cells were removed by centrifugation for 30 min at 12,227 x g at 4 °C. For
94 RBD219-N1C1, the fermentation process was slightly different in that low salt medium was used, the

95 induction temperature was set to 25 °C and the pH to 6.5 and, the methanol feed rate was between 1-
96 15 ml/L/hr. The fermentation supernatant (FS) was filtered (0.45 µm PES filter) and stored at -80 °C
97 before purification.

98 A hexahistidine-tagged SARS-CoV-2 RBD219-WT was purified from fermentation
99 supernatant (FS) by immobilized metal affinity chromatography followed by size exclusion
100 chromatography (SEC). The FS was concentrated and buffer exchanged to buffer A (20 mM Tris-
101 HCl pH 7.5 and 0.5 M NaCl) using a Pellicon 2 cassette with a 10 kDa MWCO membrane
102 (MilliporeSigma, Burlington, MA, USA) before being applied to a Ni-Sepharose column (Cytiva,
103 Marlborough, MA, USA). The column was washed with buffer A plus 30 mM imidazole and elution
104 was undertaken in buffer A containing 250 mM imidazole. The RBD219-WT protein was further
105 purified using a Superdex 75 prep grade column (Cytiva, Marlborough, MA, USA) pre-equilibrated
106 in buffer B (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) after concentrating eluates from the Ni
107 column using an Amicon Ultra-15 concentrator with a 10 kDa MWCO membrane (MilliporeSigma,
108 Burlington, MA, USA). Monomeric RBD219-WT was pooled, aseptically filtered using a 0.22 µm
109 filter, and stored at -80 °C.

110 For the purification of the tag-less RBD219-N1C1, ammonium sulfate salt was added to the
111 FS to a final concentration of 1 M (w/v) before the sample was applied to a Butyl Sepharose HP
112 column (Cytiva, Marlborough, MA, USA). The column was washed with buffer C (30 mM Tris-HCl
113 pH 8.0) with 1 M ammonium sulfate and protein was eluted with buffer C containing 0.4 M
114 ammonium sulfate. A polishing step using a Superdex 75 prep grade column (Cytiva, Marlborough,
115 MA, USA) pre-equilibrated in buffer B followed.

116

117 **2.2 SDS-PAGE**

118 To evaluate the size of RBD219-WT and RBD219-N1C1, 2 µg of these two proteins were loaded
119 onto a 4-20% tris-glycine gel under non-reduced and reduced conditions. These two proteins were
120 also treated with PNGase-F (NEB, Ipswich, MA, USA) under the reduced condition to remove N-
121 glycans and loaded on the gel to assess the impact of the glycans on the protein size. Gels were
122 stained using Coomassie Blue and analyzed using a Bio-Rad G900 densitometer with Image Lab
123 software.

124

125 **2.3 Vaccine formulation and Alhydrogel[®]-protein binding study**

126 SARS-CoV-2 RBD219-N1C1 was diluted in 20 mM Tris, 150 mM NaCl, pH 7.5 (TBS buffer) before
127 mixing with Alhydrogel[®] (aluminum oxy-hydroxide; Catalog # 250-843261 EP, Brenntag, Ballerup,
128 Denmark). To calculate the Langmuir binding isotherm of RBD219-N1C1 to Alhydrogel[®], RBD219-
129 N1C1 and Alhydrogel[®] were mixed at different ratios (from 1:2 to 1:20). The RBD219-
130 N1C1/Alhydrogel[®] mixture was stored for one hour at RT, to reach an equilibrium state. The
131 Alhydrogel[®] formulations were centrifuged at 13,000 x g for 5 min, and the supernatant was
132 removed. The protein in the supernatant fraction and the pellet fraction were quantified using a micro
133 BCA assay (ThermoFisher, Waltham, MA, USA).

134

135 **2.4 ACE-2 binding assay**

136 For the ACE-2 binding study, the Alhydrogel[®]-RBD vaccine formulations were blocked overnight
137 with 0.1% BSA. After hACE-2-Fc (LakePharma, San Carlos, CA, USA) was added, the samples
138 were incubated for 2 hours at RT. After incubation, the Alhydrogel[®] was spun down at 13,000 x g for
139 5 min. The hACE-2-Fc which did not bind to the RBD on the Alhydrogel[®] remained in the
140 supernatant. The hACE-2-Fc content in the supernatant was quantified by ELISA using 96-Well

141 MaxiSorp Immuno plates (ThermoFisher, Waltham, MA, USA) coated overnight with 200 ng/well of
142 RBD219-WT protein. After blocking with 0.1% BSA, 100 μ L supernatant sample were added to
143 each well. Plates were washed 4 times with an automated plate washer using PBS with Tween
144 (PBST). A secondary antibody against human Fc was used to detect hACE-2-Fc bound the proteins
145 on the plate. Plates were washed 5 times with an automated plate washer using PBST before 100 μ L
146 TMB solution were added. The enzymatic reaction was stopped with HCl and absorption readings
147 were made at 450 nm. The final concentration of the hACE-2 bound on the Alhydrogel[®] was
148 determined as [hACE-2-Fc on Alhydrogel[®]] = [Total hACE-2-Fc] – [hACE-2-Fc in supernatant].

149

150 **2.5 Immunogenicity testing**

151 To examine RBD-specific antibodies in mouse sera, indirect ELISAs were conducted. 96-well
152 NUNC ELISA plates were coated with 2 μ g/mL RBD219-WT in 100 μ L 1x coating buffer per well
153 and incubated overnight at 4 °C. The next day the coating buffer was discarded, and plates were
154 blocked with 200 μ L/well 0.1% BSA in PBST for 2 hours at room temperature. Mouse serum
155 samples were diluted from 1:200 to 1: 437,400 in 0.1% BSA in PBST. Blocked ELISA plates were
156 washed once with 300 μ L PBST using a Biotek 405TS plate washer and diluted mouse serum
157 samples were added to the plate in duplicate, 100 μ L/well. As negative controls, pooled naïve mouse
158 serum (1:200 diluted) and blanks (0.1% BSA PBST) were added as well. Plates were incubated for 2
159 hours at room temperature, before being washed four times with PBST. Subsequently, 1:6,000
160 diluted goat anti-mouse IgG HRP antibody (100 μ L/well) was added in 0.1% BSA in PBST. Plates
161 were incubated 1 hour at room temperature, before washing five times with PBST, followed by the
162 addition of 100 μ L/well TMB substrate. Plates were incubated for 15 min at room temperature while
163 protected from light. After incubation, the reaction was stopped by adding 100 μ L/well 1 M HCl. The

164 absorbance at a wavelength of 450 nm was measured using a BioTek Epoch 2 spectrophotometer.
165 Duplicate values of raw data from the OD₄₅₀ were averaged. The titer cutoff value was calculated
166 using the following formula: Titer cutoff = 3 x average of negative control + 3 x standard deviation
167 of the negative control. For each sample, the titer was determined as the lowest dilution of each
168 mouse sample with an average OD₄₅₀ value above the titer cutoff. When a serum sample did not
169 show any signal at all and a titer could not be calculated, an arbitrary baseline titer value of 67 was
170 assigned to that sample (baseline).

171

172 **2.6 Pseudovirus assay:**

173 Pseudovirus was prepared in HEK-293T cells by previously reported methods²¹. Cells were
174 transfected with 2.5 µg of the plasmid encoding the SARS-CoV-2 spike protein (p278-1) and 3.7 µg
175 of luciferase-encoding reporter plasmid (pNL4-3.lucR-E) and Gag/Pol-encoding packaging construct
176 (pΔ8.9). Pseudovirus containing supernatant was recovered after 48 h and passed through a 0.45 µm
177 filter before use.

178 For each serum sample, 30 µL pseudovirus were incubated with serial dilutions of heat-
179 inactivated serum (eight dilutions in a 4-fold step-wise manner) for 1 h at 37 °C. Next, 100 µL of
180 these sera-pseudovirus mixtures were added to 293T-hACE2 cells in 96-well poly-D-lysine coated
181 culture plates. Following 48 h incubation in a 5% CO₂ environment at 37 °C, the cells were lysed
182 with 100 µL Promega Glo Lysis buffer, 15 min RT. Finally, 20 µL lysate was added to 100 µL luc
183 substrate (Promega Luciferase Assay System, Madison, WI, USA). The amount of luciferase was
184 quantified by luminescence (relative luminescence units (RLU)), using a Promega GloMax
185 luminometer (Steady-Glo program). The percent virus inhibition was calculated as (1- RLU of
186 sample/ RLU of negative control) x 100. Serum from vaccinated mice was also characterized by the
187 IC₅₀-value, defined as the serum dilution at which the virus infection was reduced to 50% compared

188 with the negative control (virus + cells). When a serum sample did not neutralize 50% of the virus
189 when added at a 1:10 dilution, the IC₅₀ titer could not be calculated and an arbitrary baseline titer
190 value of 10 was assigned to that sample (baseline). As a control, human convalescent sera for SARS-
191 CoV-2 (NIBSC 20/130) was used (National Institute for Biological Standards and Control, South
192 Mimms, UK).

193

194 **2.7 Statistical analysis**

195 To test for significant differences between groups in ELISA, Luminex, and flow cytometry results,
196 Kruskal-Wallis tests in combination with Dunn's multiple comparison tests were performed. ns (not
197 significant): $p > 0.05$, *: $p < 0.05$ and **: $p < 0.01$.

198

199 **2.8 Cytokine analysis**

200 **2.8.1 Preparation of splenocytes for restimulation**

201 Single-cell suspensions from mouse splenocytes were prepared using a cell dissociator
202 (GentleMACS Octo Dissociator, Miltenyi Biotec, Waltham, MA, USA) based on a previously
203 optimized protocol²². The concentration and the viability of the splenocyte suspensions were
204 measured after mixing with AOPI dye and counted using the Nexcelom Cellometer Auto 2000
205 (Lawrence, MA, USA).

206 For the re-stimulation assays, splenocyte suspensions were diluted to 8×10^6 live cells/mL in a
207 2-mL deep-well dilution plate and 125 μ L of each sample was seeded in two 96-well tissue culture
208 treated culture plates. Splenocytes were re-stimulated with 10 μ g/mL RBD219-WT, 20 ng/mL PMA
209 + 1 μ g/mL Ionomycin or just media (unstimulated). For the flow cytometry plate, the PMA/I was not

210 added until the next day. 125 μ L (2x concentration) of each stimulant was mixed with the 125 μ L
211 splenocytes suspension in the designated wells. After all the wells were prepared, the plates were
212 incubated at 37 °C 5% CO₂. One plate was used for the cytokine release assay, while the other plate
213 was used for flow cytometry. For flow cytometry, another plate was prepared with splenocytes,
214 which would be later used as fluorescence minus one – controls (FMOs).

215 **2.8.2 *In vitro* cytokine release assay**

216 After 48 hours in the incubator, splenocytes were briefly mixed by pipetting. Then plates were
217 centrifuged for 5 min at 400 x g at RT. Without disturbing the pellet 50 μ L supernatant was
218 transferred to two skirted PCR plates and frozen at – 20 °C until use.

219 For the *in vitro* cytokine release assay, splenocytes were seeded in a 96-well culture plate at
220 1×10^6 live cells in 250 μ L cRPMI. Splenocytes were then (re-)stimulated with either 10 μ g/mL
221 RBD219-WT protein, 10 μ g/mL RBD219-N1C1 protein, PMA/Iomycin (positive control), or nothing
222 (negative control) for 48 hours at 37 °C 5% CO₂. After incubation, 96-well plates were centrifuged to
223 pellet the splenocytes down and supernatant was transferred to a new 96-well plate. The supernatant
224 was stored at -20°C until assayed. A Milliplex Mouse Th17 Luminex kit (MD MilliPore) with
225 analytes IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-23, IFN- γ , and TNF- α was
226 used to quantify the cytokines secreted in the supernatant by the re-stimulated splenocytes. An
227 adjusted protocol based on the manufacturers' recommendations was used with adjustments to use
228 less sample and kit materials²³. The readout was performed using a MagPix Luminex instrument.
229 Raw data was analyzed using Bio-Plex Manager software, and further analysis was done with Excel
230 and Prism.

231 **2.8.3 Cytokine production of activated CD4+ and CD8+ T cells**

232 Surface staining and intracellular cytokine staining followed by flow cytometry was performed to
233 measure the amount of activated (CD44⁺) CD4⁺ and CD8⁺ T cells producing IFN- γ , IL-2, TNF- α ,
234 and IL-4 upon re-stimulation with S2RBD219 WT.

235 Five hours before the 24-hour re-stimulation incubation, Brefeldin A was added to block
236 cytokines from secretion. PMA/I was also added to designated wells as a positive control. After the
237 incubation, splenocytes were stained for the relevant markers. A viability dye and an Fc Block were
238 also used to remove dead cells in the analysis and to minimize non-specific staining, respectively.

239 After staining, splenocytes were analyzed using an Attune NxT flow cytometer instrument at
240 the Baylor College of Medicine Cytometry and Cell Sorting Core. Raw data was analyzed in
241 VenturiOne software and gating results were copied in Excel. The %-gating values from the non-
242 stimulated controls were subtracted from the re-stimulated controls to observe the difference in %-
243 gating induced by the re-stimulation.

244 The gating strategy from the analysis of the results is shown in **Supplemental Figure 1**. From
245 all events collected the doublets are removed to obtain only single-cell events. Then events are
246 selected on size and granularity to obtain splenocytes only. Following the removal of dead
247 splenocytes, a gate is set to only select Activated (CD44⁺) T cells (CD3⁺)²⁴. T cells are then
248 separated into CD4⁺ T helper cells and CD8⁺ cytotoxic T cells. For T helper cells the events positive
249 for IFN-g, TNF-a, IL-2, and IL-4 were selected, while for cytotoxic T cells only IFN- γ , TNF- α , and
250 IL-2 positive events were gated.

251 **3 Results**

252 Here we report on the expression of a modified, recombinant RBD of the SARS-CoV-2 spike protein
253 using the yeast (*P. pastoris*) expression system. The candidate antigen selection, modifications, and
254 production processes were based on eight years of process development, manufacture, and preclinical
255 prior experience with a SARS-CoV recombinant protein-based receptor-binding domain (RBD)⁹⁻¹¹.
256 The RBDs of the SARS-CoV-2 and SARS-CoV share significant amino acid sequence similarity
257 (>75% identity, >80% homology) and both use the human angiotensin-converting enzyme 2 (ACE2)
258 receptor for cell entry^{25,26}. Process development using the same procedures and strategies used for the
259 production, scale-up, and manufacture of the SARS-CoV recombinant protein allowed for a rapid
260 acceleration in the development of a scalable and reproducible production process for the
261 SARS-CoV-2 RBD219-N1C1 protein, suitable for its technological transfer to a manufacturer.

262 We found that the modifications used to minimize yeast-derived hyperglycosylation and
263 optimize the yield, purity, and stability of the SARS-CoV RBD219-N1 protein were also relevant to
264 the SARS-CoV-2 RBD expression and production process. The modified SARS-CoV-2 antigen,
265 RBD219-N1C1, when formulated on Alhydrogel[®], was shown to induce virus-neutralizing antibodies
266 in mice, equivalent to those levels elicited by the wild-type (RBD219-WT) recombinant protein
267 counterpart.

268 3.1 **Cloning and expression of the modified SARS-CoV-2 RBD**

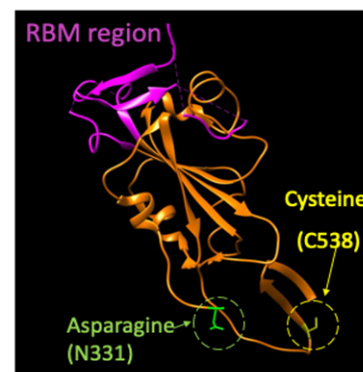
269 The wild-type SARS-CoV-2 RBD amino acid sequence comprises residues 331-549 of the spike (S)
270 protein (GenBank: QHD43416.1) of the Wuhan-Hu-1 isolate (GenBank: MN908947.3) (**Figure 1**).
271 In the RBD-219-WT construct, the gene fragment was expressed in *P. pastoris*. After fermentation at
272 the 5 L scale, the hexahistidine-tagged protein was purified by immobilized metal affinity
273 chromatography, followed by size-exclusion chromatography. We observed glycosylation and
274 aggregation during these initial expression and purification studies, and therefore, similar to our

275 previous strategy ¹⁰, we generated a modified construct, the RBD219-N1C1, by deleting the N331
276 residue and mutating the C538 residue to alanine. The additional mutation of C538 to A538 was done
277 because we observed that in the wild-type sequence nine cysteine residues likely would form four
278 disulfide bonds. Therefore, the C538 residue was likely available for intermolecular cross-linking,
279 leading to aggregation. As a result, in the RBD219-N1C1 construct, and based on the modifications,
280 the *Pichia*-derived hyperglycosylation, as well as aggregation via intermolecular disulfide bridging,
281 were greatly reduced. We note that the deleted and mutated residues are structurally far from the
282 immunogenic epitopes and specifically the receptor-binding motif (RBM) of the RBD (**Figure 1**). On
283 SDS-PAGE tris-glycine gels, the RBD219-WT protein migrated at approximately 28 kDa under non-
284 reduced conditions and 33 kDa in the reduced condition, while the RBD219-N1C1 protein migrated
285 at approximately 24 kDa under non-reduced conditions and 29 kDa under reduced conditions.
286 However, after the N-glycans were removed enzymatically, these two proteins showed a similar
287 molecular weight of approximately 25 kDa (**Supplemental Figure 2**). The purity of both proteins
288 was analyzed by densitometry resulting in levels >95%.

A)

S2-RBD	331	NITNLCPFGE	VFNATRFASV	YAWNKRKISN	CVADYSVLYN	SASFSTFKCY	380
S2-RBD-N1C1	331	ITNLCPFGE	VFNATRFASV	YAWNKRKISN	CVADYSVLYN	SASFSTFKCY	380
S2-RBD	381	GVSPTKLNDL	CFTNVYADSF	VIRGDEVRFQI	APGQTGKIAD	YNYKLPDDFT	430
S2-RBD-N1C1	381	GVSPTKLNDL	CFTNVYADSF	VIRGDEVRFQI	APGQTGKIAD	YNYKLPDDFT	430
S2-RBD	431	GCVIAWNSNN	LDSKVGGNYN	YLRLFRKSN	LKPFERDIST	EIQAGSTPC	480
S2-RBD-N1C1	431	GCVIAWNSNN	LDSKVGGNYN	YLRLFRKSN	LKPFERDIST	EIQAGSTPC	480
S2-RBD	481	NGVEGFNCYF	PLQSYGFQPT	NGVGYQPYRV	VVLSFELLHA	PATVCGPKKS	530
S2-RBD-N1C1	481	NGVEGFNCYF	PLQSYGFQPT	NGVGYQPYRV	VVLSFELLHA	PATVCGPKKS	530
S2-RBD	531	TNLVKNKCVN	FNENGLTGT				549
S2-RBD-N1C1	531	TNLVKNKAVN	FNENGLTGT				549

B)



289

290 **Figure 1 A) Amino acid sequence alignment between SARS-CoV-2 RBD219-WT (S2-RBD) and**
291 **RBD219-N1C1 (S2-RBD-N1C1). In the N1C1-mutant, the N-terminal glutamine residue (N331, green) is**
292 **removed and a C538A mutation (yellow) was introduced. Neither mutation is inside the receptor-**
293 **binding motif (RBM, purple). B) The structure model of RBD219-WT was extracted from the crystal**
294 **structure of the SARS-CoV-2 spike protein (PDB ID 6VXX). The RBM (N436-Y508) is again shown in**

295 **purple while the deleted asparagine (N331) and mutated cysteine (C538, mutated to alanine) in**
296 **RBD219-N1C1 are highlighted in green and yellow, respectively**

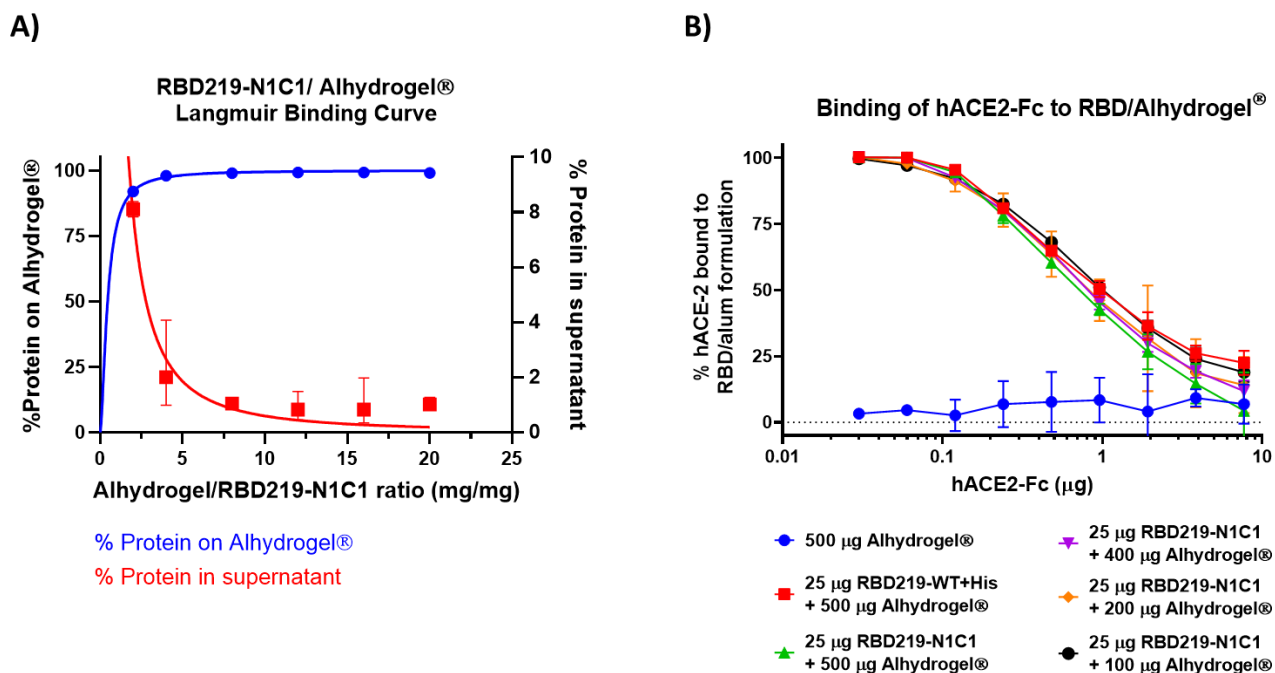
297

298 **3.2 ACE-2 binds to recombinant SARS-CoV-2 RBD219-N1C1 protein formulated on**
299 **Alhydrogel[®]**

300 When mixing 25 µg of either RBD219-WT or RBD219-N1C1 proteins to 500 µg of Alhydrogel[®], we
301 observed that >98% of the proteins bind to Alhydrogel[®] after 15 min of incubation. Only when the
302 Alhydrogel[®] was reduced to less than 100 µg (Alhydrogel[®]/RBD219 ratio <4), the Alhydrogel[®]
303 surface was saturated, and protein started to be detected in the supernatant (**Figure 2A**). It is known
304 that unbound protein may impact the immunogenicity of the vaccine formulation, therefore we
305 proceeded to only evaluate formulations with Alhydrogel[®]/RBD219 ratios higher than 4.

306 **Figure 2B** shows that hACE-2-Fc, a recombinant version of the human receptor used by the
307 virus to enter the host cells, can bind with the RBD proteins that are adsorbed on the surface of the
308 Alhydrogel[®]. This demonstrates that bound RBD proteins are structurally and possibly functionally
309 active and that after adsorption the protein does not undergo any significant conformational changes
310 that could result in the loss of possible key epitopes around the receptor-binding motif (RBM).

311 We saw no statistical differences between the binding of hACE-2-Fc to RBD219-WT (red,
312 Figure 2B) or RBD219-N1C1 (green, Figure 2B) proteins, based on an unpaired t-test (P=0.670).
313 Likewise, we saw no relation between the amount of Alhydrogel[®] to which the RBD was bound and
314 the interaction with hACE-2-Fc, indicating that the surface density of the RBD proteins on the
315 Alhydrogel[®] plays no role in the presentation of ACE binding sites.



316

317 **Figure 2 A) Langmuir binding isotherm of RBD219-N1C1 to Alhydrogel®. B) ELISA data, comparing**
318 **the binding interaction of hACE-2-Fc to RBD219-WT bound Alhydrogel® (red) and RBD219-N1C1**
319 **bound on different amounts of Alhydrogel® (green, purple, orange, and black). Five hundred µg**
320 **Alhydrogel® alone served as a negative control (blue). Data are shown as the geometric mean (n=3) with**
321 **95% confidence intervals.**

322

323 3.3 Recombinant RBD219-N1C1 protein, formulated with Alhydrogel®, elicits a strong 324 neutralizing antibody response in mice

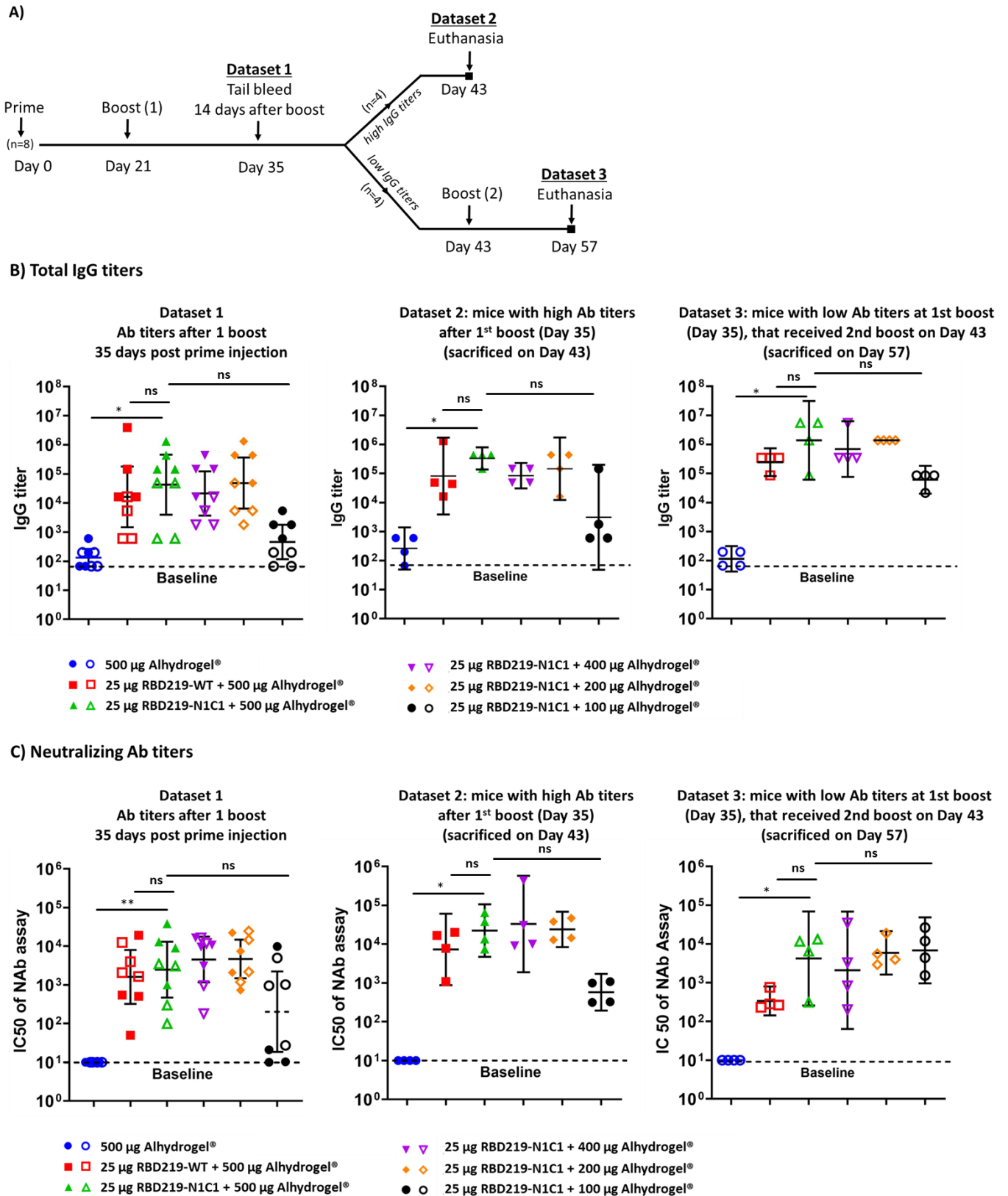
325 The recombinant RBD219-N1C1 protein (25 µg) was formulated with various amounts (100 – 500
326 µg) of Alhydrogel®. Controls included a cohort receiving only Alhydrogel® and another receiving the
327 RBD219-WT antigen, also formulated with 500 µg Alhydrogel®. Six- to eight-week-old female
328 BALB/c mice were immunized 2-3 times subcutaneously at approximately 21-day intervals (**Figure**
329 **3A**). Blood samples were taken on day 35 from all study animals to assess total IgG antibody titers,
330 as well as neutralizing antibody titers (Dataset 1). Half of the mice, those with the highest IgG titers
331 in their respective group, were euthanized on day 43 to allow the evaluation of the cellular immune
332 response after two immunizations. For this dataset (Dataset 2), we also measured IgG and

333 neutralizing antibody titers. The remaining mice received a third vaccination on day 43 and were
334 euthanized on day 57 for the assessment of humoral and cellular immune responses (Dataset 3).

335 **Humoral immune response:** On day 35 (Dataset 1), after receiving two vaccinations, all
336 groups that had received the recombinant protein formulated with at least 200 µg Alhydrogel[®]
337 produced similar and robust IgG titers. The group receiving the protein with only 100 µg
338 Alhydrogel[®], produced a lower IgG response, albeit slightly higher than the negative control that had
339 been immunized with 500 µg Alhydrogel[®] alone (**Figure 3B, Supplemental Table 1**). Importantly,
340 based on a Mann-Whitney test, we determined that there was no statistical difference between the
341 groups vaccinated with the modified and the wild-type version of the RBD protein ($p=0.3497$). The
342 average neutralizing antibody titers observed on day 35 (IC_{50} range: 5.0×10^3 to 9.4×10^3 ,
343 **Supplemental Table 2**) matched with the total IgG titers, showing equally high IC_{50} values for all
344 vaccines that contained at least 200 µg Alhydrogel[®] and lower IC_{50} values for the vaccine with only
345 100 µg Alhydrogel[®] and no IC_{50} values for the adjuvant-only control (**Figure 3C**).

346 On day 43, 22 days after receiving the boost vaccination, half of the mice in each group
347 ($N=4$), those with the highest IgG titers, were sacrificed to determine the total IgG, the IgG subtypes,
348 and the neutralizing antibody titers. As we observed on day 35, all animals that had received the
349 vaccine produced strong antibody titers, with the groups receiving ≥ 200 µg Alhydrogel[®] eliciting a
350 higher titer than those that received only 100 µg of Alhydrogel[®], albeit no statistical significance was
351 detected (**Figures 3B**). For all animals, as typical for vaccine formulations containing aluminum, the
352 IgG2a:IgG1 titer ratio was <0.1 (**Supplemental Figure 3**). In the pseudovirus neutralization assay
353 for the day 43 samples (**Figure 3C**), all vaccines containing ≥ 200 µg Alhydrogel[®] elicited IC_{50} titers
354 that, on average, were several-fold higher than on day 35 (IC_{50} range: 1.1×10^4 to 1.2×10^5 ,
355 **Supplemental Table 2**). There again was no difference between the RBD219-WT and RBD-N1C1
356 vaccines.

357 On day 57, all remaining animals were sacrificed. In contrast to the animals studied on days
358 35 and 43, these animals had received a second boost vaccination. A robust immune response in all
359 vaccinated mice, including those immunized with the protein adsorbed to 100 µg Alhydrogel[®]
360 achieved high average IgG titers. The total IgG titers in the mice sacrificed on day 57, had increased
361 after the third vaccination, compared to the titers seen on day 35. Likewise, we observed a
362 corresponding increase in the average IC₅₀ values (IC₅₀ range: 3.8×10^2 to 1.1×10^4 , **Supplemental**
363 **Table 2**) for all animals, including those immunized with the protein adsorbed to 100 µg
364 Alhydrogel[®]. Interestingly, for this time point, the cohort receiving 25 µg RBD219-N1C1 with 500
365 µg Alhydrogel[®] appeared to show higher neutralizing antibody titers than the corresponding
366 RBD219-WT group, albeit that difference was not statistically significant.



367

368 **Figure 3. A) Study design. B) Total IgG titers of Datasets 1, 2, and 3 measured respectively at days 35,**
 369 **43, and 57 post the prime injection. IgG titers were determined against RBD219-WT protein. Closed**

370 **data points represent data from mice with the highest IgG titers (Dataset 2), open data points represent**
371 **data from mice with the lowest IgG titers (Dataset 3). C) IC50 values measured by a pseudovirus**
372 **neutralization assay. Datasets 1, 2, and 3 are measured respectively at day 35, 43, and 57 after the first**
373 **injection. Baselines indicate the lowest dilution measured. Lines on each group represent the geometric**
374 **mean and 95% confidence intervals.**

375

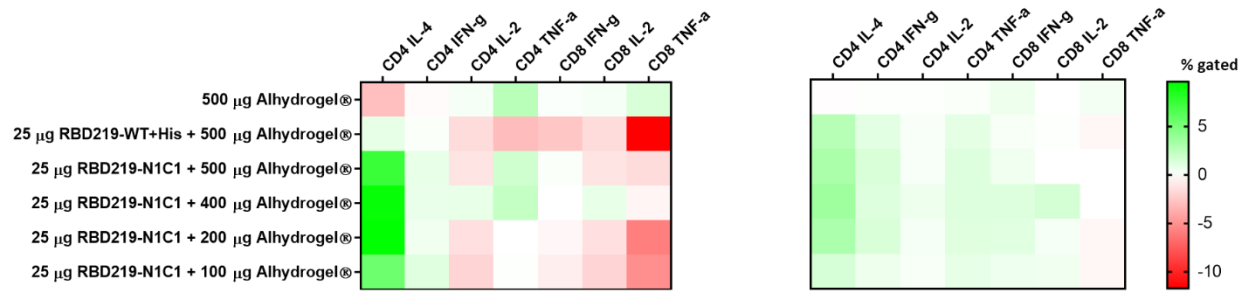
376 **Cellular immunity:** For all animals sacrificed on day 43 (having received two vaccinations)
377 and day 57 (having received three vaccinations), the cellular immune response was characterized
378 through the restimulation of isolated mouse splenocytes with the recombinant RBD219-WT protein.
379 For all samples, we employed Flow Cytometry to quantify intracellular cytokines in CD4⁺ and CD8⁺
380 cells after restimulation (**Figure 4A**). On day 43, high percentages of CD4⁺-IL-4 and, to a slightly
381 lesser extent CD4⁺-TNF α producing cells were detected. Conversely, as expected for an
382 Alhydrogel[®]-adjuvanted vaccine, low levels of IL-2 producing CD4⁺ cells were seen. In a cytokine
383 release assay, strong IFN- γ , IL-6, and IL-10 secretion was observed independent of whether the
384 animals had received two or three immunizations, whereas low amounts of secreted Th1-typical
385 cytokines such as IL-2 or IL-12 were seen (**Figure 4B**).

386

A) Cytokines production by stimulated T-cells

Dataset 2: mice with high IgG titers after 1st boost (Day 35), (sacrificed on Day 43)

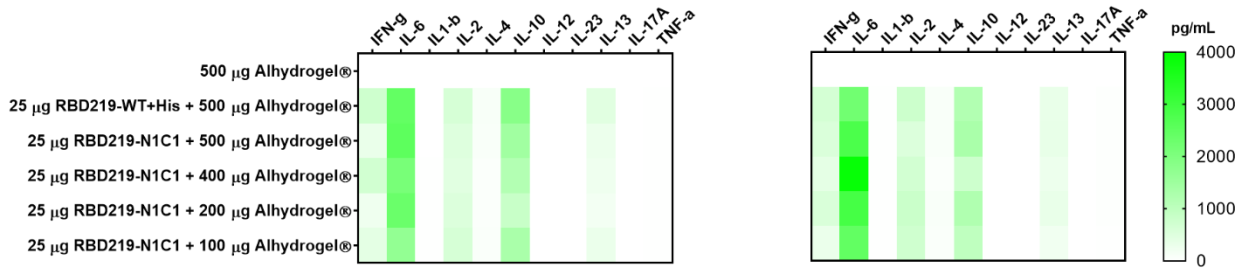
Dataset 3: mice with low IgG titers at 1st boost (Day 35), that received 2nd boost on Day 43 (sacrificed on Day 57)



B) Cytokines secreted by stimulated splenocytes

Dataset 2: mice with high IgG titers after 1st boost (Day 35), (sacrificed on Day 43)

Dataset 3: mice with low IgG titers at 1st boost (Day 35), that received 2nd boost on Day 43 (sacrificed on Day 57)



387

388 **Figure 4: A) Heatmap of the cytokine response of CD4+ and CD8+ T cells after restimulation with**
389 **SARS-CoV-2 RBD219-WT or RBD219-N1C1, re-stimulated splenocytes were surface and**
390 **intracellularly stained and subsequently analyzed by flow cytometry. Splenocytes were obtained from**
391 **mice who received two vaccinations (day 43) or three vaccinations (Day 57). Non-stimulated controls**
392 **were subtracted from re-stimulated samples. B) Heatmap of secreted cytokines in supernatant from re-**
393 **stimulated splenocytes from mice who received two vaccinations (day 43) or three vaccinations (Day 57).**
394 **Cytokine concentrations of non-stimulated controls were subtracted from re-stimulated samples.**

395

396 **4 Discussion**

397 Here we report on a yeast-expressed SARS-CoV-2 RBD219-N1C1 protein and its potential as a
398 vaccine candidate antigen for preventing COVID-19. Building on extensive prior experience
399 developing vaccines against SARS-CoV and MERS-CoV¹⁰⁻¹², we initially selected and compared the
400 SARS-CoV-2 RBD219-WT and the SARS-CoV-2 RBD219-N1C1 proteins for their potential to
401 induce high titers of virus-neutralizing antibodies, T-cell responses, and protective immunity.

402 Previously we observed that the SARS-CoV RBD219-N1 antigen, formulation with
403 Alhydrogel[®] elicited high levels of neutralizing antibodies without evidence of eosinophilic immune
404 enhancement. That RBD-based vaccine was even superior to the full-length spike protein in inducing
405 specific antibodies and fully protected mice from SARS-CoV infection while preventing eosinophilic
406 pulmonary infiltrates in the lungs upon challenge⁹.

407 In this work, using the SARS-CoV-2 RBD219 protein analog, we observed that, just like in
408 the case of the SARS-CoV RBD antigen, the deletion of the N-terminal asparagine residue reduced
409 hyperglycosylation, thus allowing for easier purification of the antigen obtained from the yeast
410 expression system. Moreover, mutagenesis of a free cysteine residue further improved protein
411 production through the reduction of aggregation. Based on the predicted structure of the RBD, no
412 impact on the functionality of the RBD219-N1C1 antigen was expected, and using an ACE-2 *in vitro*
413 binding assay we indeed showed similarity to the RBD219-WT antigen. In addition, we showed that,
414 in mice, the modified RBD219-N1C1 antigen triggered an equivalent immune response to the
415 RBD219-WT protein when both proteins were adjuvanted with Alhydrogel[®].

416 Similar to our previous findings with the SARS-CoV RBD antigen⁹, we show the RBD219-
417 N1C1 protein when formulated with Alhydrogel[®] elicits a robust neutralizing antibody response with
418 IC50 values up to 4.3×10^5 in mice, as well as an expected T-cell immunological profile. Some of the

419 titers of virus-neutralizing antibodies exceed the titer, 2.4×10^4 , measured in-house with human
420 convalescent serum research reagent for SARS-CoV-2 (NIBSC 20/130, National Institute for Biological
421 Standards and Control, UK).

422 In a mouse virus challenge model for the SARS CoV RBD recombinant protein vaccine, we
423 found that Alhydrogel[®] formulations induced high levels of protective immunity but did not
424 stimulate eosinophilic immune enhancement, suggesting that Alhydrogel[®] may even reduce immune
425 enhancement. This prior experience offers the potential for Alhydrogel[®] as a key adjuvant for
426 consideration during coronavirus vaccine development²⁷. Such findings have led to a reframing of the
427 basis for immune enhancement linked to coronavirus respiratory infections²⁸. A recent analysis and
428 review by the NIH ACTIV Vaccine Working Group confirms that aluminum or Th2 responses
429 remain viable options for vaccine development concluding that “it is not possible to clearly prioritize
430 or down-select vaccine antigens, adjuvants, biotechnology platforms, or delivery mechanisms based
431 on general immunological principles or the available preclinical data”²⁹.

432 Therefore, the RBD219-N1C1 vaccine antigen on Alhydrogel[®] merits its evaluation as a
433 COVID-19 vaccine possibly with or without other immunostimulants. Looking at the landscape of
434 recombinant protein-based COVID-19 vaccines, the WHO lists several advanced COVID-19
435 candidates that are based on recombinant proteins³⁰, and at least seven COVID-19 vaccines include
436 aluminum as part of the adjuvant component^{7,13,15,16,31-37}, often in combination with other
437 immunostimulants, such as CpG, to achieve a balanced immune response. Furthermore, these
438 recombinant protein vaccines, including RBD219-N1C1, might find an additional important use as a
439 booster if one of the newer platform vaccines, e.g., mRNA or adenovirus-based vaccines induce
440 lower than expected immunogenicity or protection. Such prime-boost approaches have been used
441 successfully with the chimp adenovirus vaccine for malaria and other systems, for example^{38,39}.

442 The selection of the *P. pastoris* expression platform for the production of the RBD antigen
443 was motivated by the intent to develop a low-cost production process that could easily be transferred
444 to manufacturers in LMICs. Currently, there are several types of COVID-19 vaccine candidates in
445 advanced clinical trials^{6,40-45}. The focus of some of the initiatives behind these vaccines is to provide
446 vaccines for the developed world that might struggle to be successful without advanced
447 infrastructure. Being able to match the existing experience in LMICs with the production of other
448 biologics in yeast increases the probability of successful technology transfer²⁰. For example,
449 currently, the recombinant hepatitis B vaccine is produced in yeast by several members of the
450 Development Country Vaccine Manufacturers Network (DCVMN), and we foresee that, given the
451 existing infrastructure and expertise, those facilities could be repurposed to produce a yeast-produced
452 COVID-19 vaccine⁴⁶. Recently, the research cell bank and production process for the RBD219-N1C1
453 antigen was technologically transferred to a vaccine manufacturer in India and produced under cGMP
454 conditions with the intent to enter into clinical development. In addition, preclinical studies using the
455 RBD219-WT and RBD219-N1C1 antigens are ongoing to further optimize and evaluate other novel
456 formulations, including a challenge study in a non-human primate model.

457

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459

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468 **Author Contributions**

469 BZ and JW planned and executed all cloning and small-scale expression studies. ZL conducted the
470 fermentations. JL executed the protein purifications. WHC and RTK conducted the characterization
471 of the purified proteins. JP and BK conducted mouse studies. LV, RA, MJVM, ACAL, JAR ran
472 ELISAs. LV and CP executed the cellular assays. JP, CP, MJVM, and JAR conducted pseudovirus
473 studies. RA and JP executed the ACE-2 binding study. MEB, JP, PJH, US, and WHC drafted the
474 manuscript. All authors were involved in the experimental design and planning and reviewed and
475 edited the final submission.

476 **Data availability**

477 All data generated or analysed during this study are included in this published article (and its
478 supplementary information files).

479

480

481 **Competing Interests Statement**

482 The authors declare that Baylor College of Medicine recently licensed the RBD219-N1C1
483 technology to an Indian manufacturer for further development. The research conducted in this paper
484 was performed in the absence of any commercial or financial relationships that could be construed as
485 a potential conflict of interest.

486

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