Yeast-Expressed SARS-CoV Recombinant Receptor-Binding Domain (RBD219-N1) Formulated with Aluminum Hydroxide Induces Protective Immunity and Reduces Immune Enhancement

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Abstract

We developed a severe acute respiratory syndrome (SARS) subunit recombinant protein vaccine candidate based on a high-yielding, yeast- engineered, receptor-binding domain (RBD219-N1) of the SARS beta-coronavirus (SARS-CoV) spike (S) protein. When formulated with Alhydrogel®, RBD219-N1 induced high-level neutralizing antibodies against both pseudotyped virus and a clinical (mouse-adapted) isolate of SARS-CoV. Here, we report that mice immunized with RBD219-N1/Alhydrogel® were fully protected from lethal SARS-CoV challenge (0% mortality), compared to ~ 30% mortality in mice when immunized with the SARS S protein formulated with Alhydrogel®, and 100% mortality in negative controls. An RBD219-N1 formulation Alhydrogel® was also superior to the S protein, unadjuvanted RBD, and

AddaVax (MF59-like adjuvant)-formulated RBD in inducing specific antibodies and preventing cellular infiltrates in the lungs upon SARS-CoV challenge. Specifically, a formulation with a 1:25 ratio of RBD219-N1 to Alhydrogel® provided high neutralizing antibody titers, 100% protection with non-detectable viral loads with minimal or no eosinophilic pulmonary infiltrates. As a result, this vaccine formulation is under consideration for further development against SARS-CoV and potentially other emerging and re-emerging beta-CoVs such as SARS-CoV-2.

Keywords: coronavirus, vaccine, eosinophil infiltration, severe acute respiratory syndrome, recombinant protein

INTRODUCTION

Coronaviruses (CoV) are the enveloped viruses with approximately 30 kb single-strand RNA genomes. CoVs belong to the family Coronaviridae and have been found in various mammals, including bats, pangolins, and civets. Previously, they were known to only cause mild diseases to humans until the pandemic of severe acute respiratory syndrome (SARS) occurred between 2002 and 2003 [1-3]. Ever since SARS, nearly every decade, a new major coronavirus outbreak occurred: The Middle East respiratory syndrome caused by MERS-CoV first emerged in 2012 and still is circulating in camels [4]; the current COVID-19 pandemic caused by SARS-CoV-2 was first discovered in December 2019, and have currently infected more than 10 million worldwide.

The disease caused by SARS coronavirus (SARS-CoV) led to almost 800 deaths and more than 8,000 infections, leading to an overall fatality rate of approximately 10 percent. Alarmingly, the fatality rate among older adults exceeded 50 percent [5]. In preparation for future outbreaks and accidental and/or

56 intentional releases of SARS-CoV, intensive efforts have been made to develop vaccines against SARS-
57 CoV.
58
59 For the past two decades, several antigens have been identified and developed as SARS-CoV vaccine
60 candidates. Initially, whole inactivated virus (WIV) or modified vaccinia virus Ankara expressing SARS
61 vaccines were developed [5], however, eosinophilic immunopathology was observed in mice and non-
62 human primates immunized with these viral-vectored vaccines [6-11]. Even though historically there
63 have been reports that alum adjuvanted vaccines could induce enhancement, such as in the 1960s with
64 the RSV vaccine or even with WIV and S proteins,[12] it was shown later that alum-adjuvanted WIV
65 elicited less immunopathology than WIV alone [12]. suggesting that alum might reduce immune
66 enhancement, a process possibly linked to mixed Th1, Th17, and Th2 responses [11, 13]. Additional
67 evidence emerged that the virus N protein had a key but not exclusive role in immune enhancement [7,
68 11]. Based on these studies, the recombinant S protein of SARS-CoV was used as a vaccine candidate [5],
69 but the full-length S-protein also induced immunopathology, with epitopes outside of the receptor-
70 binding domain (RBD) of the S protein implicated in eliciting this phenomenon [14, 15]. Therefore, the
71 RBD of the S protein was selected as a substitute for the full-length S protein [16-21].
72
73 Recombinant RBD formulated with Sigma's adjuvant system® (consisting of Monophosphoryl-lipid
74 A/Trehalose dicorynomycolate adjuvant, a skewed Th1/Th2 adjuvant) or with Freund's adjuvant
75 (Freund's complete in prime and Freund's incomplete in boost; a Th1/Th2 balanced adjuvant scheme)
76 was shown to elicit neutralizing antibodies and highly protective immunity in the vaccinated animals,
77 while eliminating or significantly reducing eosinophilic immunopathology [18, 20-23]. In our previous

78 studies, we have expressed wild-type RBD193 (residues 318–510) /RBD219 (residues 318–536) in yeast,
79 however, because of the three N glycosylation sites on these two wild-type constructs, we further
80 generated the deglycosylated forms as follows: N1: 1st glycosylation site deleted; N2: 1st glycosylation site
81 deleted and 2nd glycosylation site mutated; and N3: 1st glycosylation site deleted, 2nd and 3rd glycosylation
82 sites mutated. We developed a production process for several of these tag-free yeast-expressed
83 recombinant RBD constructs [24]. Such studies down-selected several candidates and ultimately
84 identifying one, RBD219-N1 (residues 319-536), as a promising vaccine candidate, due to its ability to
85 induce in immunized mice a stronger anti-RBD-specific antibody response and neutralizing antibodies
86 when adjuvanted with aluminum hydroxide (Alhydrogel®). The protein production process [25] was
87 transferred to the Pilot Bioproduction Facility (PBF) at Walter Reed Army Institute of Research (WRAIR),
88 and the clinical grade RBD219-N1 (drug substance) was manufactured under current Good
89 Manufacturing Practices (cGMP) and is suitable for further Phase I clinical trials.
90
91 In this work, the RBD219-N1 formulated with Alhydrogel® resulted in significantly increased antigen-
92 specific IgG titers and neutralizing antibody responses when compared to other RBD constructs. After
93 challenge with SARS-CoV, 100% of mice immunized with RBD219-N1 survived, while only 89% of mice
94 immunized with other RBD constructs and less than 70% of the mice immunized with SARS-CoV spike
95 protein survived and none survived in the control group. The aluminum formulated RBD minimized
96 immune enhancement compared with other adjuvants formulations with either the RBD or full-length S
97 protein. Finally, an Alhydrogel® dose-ranging study further indicated that by formulating RBD219-N1 with
98 Alhydrogel® at the ratio of 1:25, higher IgG titers could be elicited with no detectable viral load upon
99 challenge.

100

101 **MATERIALS AND METHODS**

102

103 **Generation of recombinant yeast-expressed RBD of SARS-CoV**

104 The yeast-expressed SARS-CoV RBD193-N1, wt-RBD219, and RBD219-N1 were expressed and purified as
105 previously described [24, 25]. Briefly, 1 mL of *P. pastoris* X33 seed stock expressing RBD193-N1, wt
106 RBD219, and RBD219-N1 was inoculated into 500 ml BMG (buffered minimal glycerol) medium and the
107 culture was incubated overnight at 30°C with constant shaking at 250 rpm until an OD600 of ~10.
108 Approximately 250 ml of overnight culture were inoculated into 5 L sterile Basal Salt Media or Low Salt
109 medium [24]. Fermentation was maintained at 30°C, pH 5.0 and 30% of dissolved oxygen concentration
110 until the exhaustion of glycerol, and the pH and the temperature were then ramped to 6.5 and 25°C,
111 respectively, over an hour followed by continuous feeding of methanol at 11 ml/L/hr for ~70 hours. The
112 fermentation supernatant (FS) was harvested for further purification. To purify RBD193-N1, wt-RBD219,
113 and RBD219-N1, ammonium sulfate was added to the FS until the molarity reached 2 M. The FS
114 containing 2 M ammonium sulfate was purified by hydrophobic interaction chromatography using Butyl
115 Sepharose HP resin followed by size exclusion chromatography using Superdex 75 resin [24, 25].

116

117 **Reagents**

118 Alhydrogel® (aluminum oxyhydroxide; Catalog # 250-843261 EP) was purchased from Brenntag
119 (Ballerup, Denmark), AddaVax (MF59-like adjuvant; squalene oil-in-water emulsion; Catalog # vac-adx-
120 10) was purchased from Invivogen (San Diego, CA, USA). The SARS S protein vaccine, produced in the
121 baculovirus/insect cell expression platform and pre-formulated with aluminum (Reagent # 50-09014,

122 50-09015, 50-09016), was obtained directly from NIH via BEI Resources, NIAID, NIH (Manassas, VA,
123 USA).
124
125 **Binding Study**
126 One ml of TBS containing 18 to 180 µg RBD219-N1 and 400 µg Alhydrogel® were prepared to study the
127 binding of RBD219-N1 to Alhydrogel® at different ratios (from 1:2 to 1:22). The prepared RBD219-N1/
128 Alhydrogel® slurry was mixed for one hour to ensure the binding of RBD219-N1 to Alhydrogel® reached
129 an equilibrium state. The slurry was then centrifuged at 13,000 x g for 5 minutes, and the supernatant
130 was collected while the Alhydrogel® pellet was resuspended with an equal volume of removed
131 supernatant. The RBD219-N1 protein content in the supernatant fraction and the pellet fraction were
132 then measured using a micro BCA assay (ThermoFisher, Waltham, MS, USA). Similarly, the presence of
133 RBD219-N1 in the pellet and supernatant fractions was also evaluated using SDS-PAGE. Briefly, after the
134 slurry was centrifuged and separated into pellet and supernatant fractions, the Alhydrogel® pellet was
135 further resuspended with desorption buffer (100 mM sodium citrate, 92 mM dibasic sodium phosphate
136 at pH 8.9) and mixed for 1 hour. The desorbed RBD was then separated from Alhydrogel® by
137 centrifugation at 13,000 x g for 5 minutes. Ten microliters of desorbed RBD from the pellet fraction and
138 free RBD in the supernatant fraction were loaded on 4-20% Tris-glycine gels and stained by Coomassie
139 blue.
140
141 **Animals**
142 Six- to eight-week-old, female Balb/c mice were purchased from Charles River (Wilmington, MA, USA),
143 and housed in an approved biosafety level 3 animal facility at the University of Texas Medical Branch

144 (UTMB) at Galveston, Texas. All of the experiments were performed according to National Institutes of
145 Health and United States Department of Agriculture guidelines using experimental protocols approved
146 by the Office of Research Project Protections, Institutional Animal Care and Use Committee (IACUC) at
147 UTMB.
148
149 **Study Design**
150 Three sets of pre-clinical studies were conducted to evaluate: (1) different yeast-expressed recombinant
151 antigens, including RBD193-N1, wt-RBD219, and RBD219-N1), and spike protein vaccine as a control; (2)
152 two of the most common adjuvants used in licensed vaccines, Alhydrogel®® and AddaVax (MF59-like
153 adjuvant); and (3) two Alhydrogel® doses (1:8 and 1:25 ratios) to formulate RBD219-N1, and spike protein
154 vaccine as a control. Mice were immunized either subcutaneously (s.c.) or intramuscularly (i.m.) followed
155 by two boosters at 21-day intervals. The same route, number, and frequency of immunization were
156 followed among all the groups within the same study. Mouse-adapted SARS-CoV (MA15 strain) was used
157 in these studies to challenge the mice. This virus was generated by serially passaged in the respiratory
158 tract of young BALB/c mice, resulting in minimal mutations in only 6 amino acids, and has been reported
159 to show dose-dependent weight loss and mortality as well as associated pulmonary histopathology in
160 BALB/c mice [26], and thus is widely used in a mouse model to evaluate SARS-CoV vaccine and
161 therapeutics. The detailed study designs are described as the following:
162
163 Antigens screening. Mice (15 per group) were immunized s.c. with 100 µL yeast-expressed recombinant
164 RBD proteins (RBD193-N1, wt-RBD219, and RBD219-N1) formulated with 10 mg/mL Alhydrogel® on days
165 0 (20 µg RBD), 21 (10 µg RBD), and 42 (10 µg RBD). TBS/Alhydrogel® buffer and 9 µg of alum pre-

166 formulated SARS S protein were used as the negative and positive controls, respectively. Sera from 5
167 mice were collected on day 50 to evaluate the pre-challenge neutralizing antibody titers. All mice were
168 then challenged intranasally (i.n.) with 10x LD50 SARS-CoV MA15 virus (~5.6 logs TCID50) on day 52.
169 Three mice in each group were further sacrificed On days 55 and 56 to determine the viral loads. The
170 remaining 9 mice in each group were used to monitor clinical disease (weight loss) and mortality daily
171 for up to three weeks.

172 Adjuvant screening. Mice (4 per group) were immunized intramuscularly (i.m.) with 100 µL RBD219-N1
173 formulated with two adjuvants on days 0 (20 µg RBD), 21 (10 µg RBD), and 42 (10 µg RBD). A total of
174 three groups were tested, including RBD219-N1 with 500 µg Alhydrogel® (sometimes referred to in the
175 manuscript as "alum") in group 1, RBD219-N1 in 50% (v/v) MF59-like adjuvant in group 2. Mice
176 immunized with RBD alone in group 3 were used as a negative control. On day 52, sera were collected
177 to evaluate IgG and neutralizing antibody titers. On day 63, mice were i.n. challenged with SARS-CoV (2x
178 LD50 (~10^5 TCID50) SARS-CoV (MA-15), and finally, on day 66 and day 69, or day 3 and day 6 post-
179 challenge, lungs from 2 mice were collected for histopathology and viral titration.

180 Alhydrogel® dose range screening. Mice (6 per group) were i.m. immunized with Alhydrogel® formulated
181 RBD219-N1 on days 0 (10 or 20 µg RBD), 21 (10 µg RBD), and 42 (10 µg RBD). In group 1, a formulation
182 of 0.2 mg/mL RBD in 5 mg/mL Alhydrogel® (2.5 mg/mL aluminum) with the dose of 20 µg/10 µg/10 µg of
183 RBD for prime/1st boost/2nd boost was used for immunization, respectively. In group 2, the formulation
184 0.2 mg/mL RBD in 1.6 mg/mL Alhydrogel® (consisted of 0.8 mg/mL aluminum) was tested, more
185 specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was used while
186 in group 3, 10 µg RBD were used for all immunizations. Alhydrogel® alone and pre-formulated SARS-S
187 protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive controls. On day 52,

188 sera were collected to evaluate IgG and neutralizing antibody titers. On day 63, mice were i.n. challenged
189 with SARS-CoV (2x LD50 (~10^5 TCID50) SARS-CoV (MA-15)), and finally, on day 66 (day 3 post-challenge)
190 and day 70 (day 6 post-challenge), lungs from 3 mice were collected for histopathology and viral titration.

191 **ELISA**

192 RBD-specific IgG titers of polyclonal sera from the immunized mice were measured by ELISA, as
193 previously described [18, 19, 24]. Briefly, 96-well ELISA plates were pre-coated with yeast-expressed RBD
194 protein (1 µg/ml) overnight at 4 °C. After blocking and then incubating with serially diluted mouse sera,
195 bound IgG antibody was detected using HRP-conjugated anti-mouse IgG (1:2000), followed by the same
196 protocol as described [18, 19, 24].

197

198 **Titration of SARS CoV-specific neutralizing antibodies**

199 Mice were anesthetized with isoflurane and then bled from the retro-orbital sinus plexus. After heat
200 inactivation at 56 °C for 30 min, sera were stored at 4 °C. The standard live virus-based
201 microneutralization (MN) assay was used as previously described [12, 27]. Briefly, serially two-fold and
202 duplicate dilutions of individual immune sera were prepared in 96-well microtiter plates with a final
203 volume of 60 µl per well before adding 120 infectious SARS-CoV (MA-15) particle in 60 µl to individual
204 wells. The plates were mixed well and cultured at room temperature for 1 h before transferring 100 µl
205 of the immune serum-virus mixtures into designated wells of confluent Vero E6 cells grown in 96-well
206 microtiter plates. Vero E6 cells cultured with medium with or without virus were included as positive
207 and negative controls, respectively. After cultivation at 37°C for 3 days, individual wells were observed
208 under the microcopy for the status of virus-induced formation of cytopathic effect. The efficacy of

209 individual sera was calculated and expressed as the lowest concentration capable of completely
210 preventing virus-induced cytopathic effect in 100% of the wells.
211
212 **Collection of lungs, histology, immunohistochemistry, and virus titration**
213 After the SARS-CoV challenge, mice were euthanized on different days depending on the study, and their
214 lungs were removed. Lung lobes were placed in 10% neutral buffered formalin for histological
215 examination using either hematoxylin and eosin (for cellular infiltrates) or immunohistochemistry (IHC),
216 specific for eosinophils, as described previously[12, 27]. For virus quantitation, the remaining tissue
217 specimen was processed as previously described [12, 27]. Evaluations for histopathology were done by
218 an experimental human pathologist masked as to the vaccine/dosage of each specimen source;
219 assessment of the extent of pathologic damage and the eosinophilic component of the inflammatory
220 infiltrates was then provided.
221
222 **Statistical analysis**
223 Neutralizing antibody titers, weight loss, lung virus titers, IgG titers, histopathologic score, and
224 eosinophilic infiltration scores were averaged for each group of mice. T-tests were routinely used to
225 evaluate the statistical variation between two groups.
226
227 **RESULTS**
228 **Vaccine integrity evaluation through binding and point-of-injection studies**
229 As a vaccine, recombinant protein antigens alone often do not induce a sufficient immune response,
230 necessitating their evaluation as proteins formulated with adjuvants. When Alhydrogel® is used as an

231 adjuvant, the antigen is typically fully adsorbed to the alum salt to maximize potency. The binding
232 efficiency of RBD219-N1 to Alhydrogel® was performed to evaluate the minimum RBD219-N1 to
233 Alhydrogel® ratio required to ensure complete protein binding. By measuring the protein content in the
234 supernatant and the pellet fraction after adsorption and centrifugation, the percentage of RBD bound
235 onto Alhydrogel® at different RBD219-N1 to Alhydrogel® ratio was determined (Figure 1A). An
236 Alhydrogel® to RBD ratio greater than 7.4 resulted in complete adsorption. SDS-PAGE analysis of a
237 formulation of 0.2 mg/mL RBD with 1.6 mg/mL Alhydrogel® (1:8 ratio) further confirmed that no protein
238 remained in the supernatant fraction after adsorption (Figure 1B).
239
240 **Antigen screening**
241
242 In this pilot study, we compared safety, immunogenicity, and efficacy of the various yeast-expressed
243 RBD- and S vaccines [12] using a lethal mouse model of SARS--CoV infection. Figure 2a shows that mice
244 immunized with RBD-219N1 had the highest titer of neutralizing antibodies compared to mice
245 immunized with alum-adjuvanted RBD193-N1, wt-RBD219, and the S protein vaccines. Importantly,
246 endpoint evaluation for mortality has shown that a 100% survival rate was found for mice immunized
247 with alum-adjuvanted RBD219-N1, while mice immunized with alum-adjuvanted wt-RBD219 and
248 RBD193-N1 vaccines showed 88% survival and those immunized with alum-adjuvanted S protein showed
249 only 67% survival. All mice in the TBS control group died within 6 days post-challenge (Figure 2B).
250 Furthermore, mice immunized with RBD219-N1, similar to wt-RBD219 and RBD193-N1, consistently
251 showed less than 10% weight loss throughout the study period, while mice immunized with other alum-
252 formulated vaccines including the S protein showed a maximum of 15-20% weight loss (Figure 2C). This
253 was accompanied by more than a 3-log reduction of infectious viral loads within the lungs when
254 compared to mice vaccinated with TBS/Alhydrogel® (Figures 2D and 2E). None of the mice given

255 TBS/Alhydrogel® produced detectable neutralizing antibodies, whereas their geometric means of lung
256 virus titers were $10^{9.9}$ and $10^{8.9}$ $TCID_{50}$/g on days 1 and 2 post-challenge, respectively. With these results,
257 RBD219-N1 was chosen for further development.
258
259 **Adjuvant screening**
260 It is known that alum (generally a Th2 adjuvant) and MF59 (a Th1/Th2 balanced adjuvant in the form of
261 oil-in-water emulsion) are two of the most common adjuvants used in licensed vaccines with very well-
262 established safety records[28, 29]. In this study, we compared Alhydrogel® and AddaVax (MF59-like
263 adjuvant) for their ability to improve the efficacy of RBD219-N1 in mice. As shown in Figure 3 and
264 Supplementary Table 1, mice immunized with RBD219-N1 formulated with Alhydrogel® produced potent
265 neutralizing antibody responses, resulting in complete protection against a subsequent SARS-CoV
266 infection. In contrast, RBD219-N1 with MF59-like adjuvant-induced high IgG titers (Supplementary Table
267 1) but failed to elicit protective neutralizing antibody responses and did not protect against SARS-CoV
268 infection, as evaluated by the isolation of the infectious virus and quantitative PCR (qPCR) for viral RNA.
269 Unlike MF59-like adjuvant formulated RBD219-N1 and RBD219-N1 alone, we also noted that RBD219-
270 N1 formulated with Alhydrogel® did not induce a cellular infiltration within the lungs (Figure 4 and
271 Supplementary Table 1). Taken together, these results suggest that RBD219-N1/Alhydrogel® was
272 potentially both effective and safe, and therefore Alhydrogel® was chosen as the optimal adjuvant and
273 further studied for the dose-ranging study.
274
275
276 **Alhydrogel® dose-ranging study**
277

278 Although RBD219-N1 formulated on Alhydrogel® at a ratio of 1:25 was effective in immunized animals
279 against lethal SARS-CoV challenge without inducing apparent pulmonary immunopathology, it was of
280 further interest to compare different aluminum ratios, including the efficacy of RBD219-N1 formulated
281 on Alhydrogel® at both 1:25 and 1:8 ratios in mice. Sera were collected 10 days after the last vaccination
282 to test RBD219-N1-specific IgG antibody responses by ELISA and neutralizing antibodies against live
283 SARS-CoV infections. Results showed that mice immunized with RBD219-N1/Alhydrogel® at a 1:25 ratio
284 produced significantly higher neutralizing antibody titers and RBD219-N1-specific IgG titers than those
285 vaccinated at a 1:8 ratio (Figure 5A-B). Consistent with the antibody responses, mice immunized with an
286 RBD219-N1/Alhydrogel® ratio of 1:25 were completely protected against lethal challenge with SARS-CoV,
287 as indicated by the undetectable infectious virus within the lungs and also a lack of morbidity and
288 mortality (Figure 6). In contrast, infectious virus was recovered from the lungs of mice immunized with
289 a 1:8 ratio of RBD219-N1/Alhydrogel® (Groups 2 and 3) by day 5 post-challenge and one mouse from
290 each group died. As expected, mice immunized with Alhydrogel® alone (Group 4) were not protected
291 against a lethal viral challenge and had detectable virus in the lungs resulting in the death of 2 mice on
292 day 4 post-challenge, while SARS-CoV S protein formulated on Alhydrogel® (Group 5) was also
293 immunogenic and protective against SARS-CoV infection. Histopathologic examination of lung tissues
294 using IHC (specific for eosinophils) revealed minimal eosinophilic infiltration for mice in group 1 (1:25
295 ratio) while somewhat increased infiltration was observed in groups 2 and 3 (1:8 ratio) and the worst
296 eosinophils infiltration was found in the mice immunized with S protein among all groups
297 (Supplementary Table 2 and Figure 7). The results confirm the protective efficacy of RBD219-
298 N1/Alhydrogel®, and its superiority to the S-protein in terms of protection and reduction or prevention

299 in eosinophilic infiltration, as well as the favorable effects of Alhydrogel® for eliciting high titer
300 neutralizing antibody and maximal reductions in immune enhancement comparing to other groups.
301
302
303 **DISCUSSION**
304 The RBD of the S1 protein of SARS-CoV, which is responsible for the attachment of the angiotensin-
305 converting enzyme-2 (ACE2) receptor and initiates the process for cell binding and entry, has been
306 proven as a promising vaccine candidate [30]. It has been expressed as a recombinant protein with a
307 hexahistidine tag, a GST protein or Fc fragment in several different expression platforms including *E. coli*,
308 insect cells, and mammalian cells to simplify the purification process and these constructs were shown
309 to trigger neutralizing antibodies and immunity [16, 18-21]. However, an additional tag on a recombinant
310 protein-based vaccine should be avoided because it can potentially trigger an undesired immune
311 response. In our previous studies [24, 25], we have expressed and purified several tag-free recombinant
312 RBD constructs in yeast using a scalable process and discovered that the yeast-expressed RBD219-N1
313 induced a stronger RBD-specific antibody response and a high level of neutralizing antibodies in
314 immunized mice when formulated with Alhydrogel®, and thus, a very promising vaccine candidate. In this
315 study, we conducted for the first time an efficacy study in which we screened several different yeast-
316 expressed RBD proteins and compared them to the S protein as a positive control. RBD219-N1 formulated
317 with Alhydrogel® triggered higher neutralizing titers than the other Alhydrogel®- formulated RBD
318 constructs or the S protein; the RBD219-N1 formulated with Alhydrogel® immunized mice were fully
319 protected with 100% survival rate. The RBD219-N1 construct was further selected for adjuvant screening
320 and adjuvant dose-ranging studies.

321

322 Before testing different adjuvants, we investigated the immunization routes (s.c. and i.m) for RBD219-N1
323 adsorbed to Alhydrogel® by evaluating the IgG antibody responses (Supplementary Figure. 1A),
324 neutralizing antibody titer against SARS pseudovirus and live SARS-CoV infections (Supplementary
325 Figures. 1B and 1C). It was found that both immunization routes were able to induce high titers of specific
326 IgG and neutralizing antibodies against infections of SARS pseudovirus in ACE2/293T cells
327 (Supplementary Figure.1B) and live SARS-CoV in Vero cells (Supplementary Figure.1C). Although the
328 antibody responses induced through the s.c. route were significantly higher than those through the i.m.
329 route, the i.m. route was selected for subsequent adjuvant optimization because i.m. injection of the
330 vaccines containing adjuvants has less chance to induce adverse local effects than s.c. injection[31] and
331 the majority of the clinically used vaccines are administered via the i.m. route [32].

332

333 The superiority of the RBD219-N1 vaccine antigen to the S protein was reflected both in terms of eliciting
334 neutralizing antibodies and protective immunity, and that the alum-adjuvanted RBD219-N1 resulted in
335 little to no cellular or eosinophilic immunopathology compared to either the S-protein or an M59-like
336 adjuvant-formulated RBD219-N1 vaccine. With regards to the former observation, it was previously
337 shown that removal of immune-enhancing epitopes located outside the S-protein RBD domain may result
338 in an immunogen, which is less likely to induce immunopathology [15, 33]. This finding led to the initial
339 selection of the RBD as a vaccine antigen [34]. With regards to adjuvant selection, alum prevented or
340 reduced immune enhancement, a finding that confirms a previous observation for the doubly inactivated
341 virus, viral-like particle vaccines, and S protein[12]. For example, Tseng et al. reported that mice
342 immunized with aluminum-formulated virus-like-particle and inactivated virus vaccines showed less

343 immunopathology than the non-adjuvanted vaccines. Such findings provided the basis for suggesting
344 that eosinophilic immunopathology may occur through mechanisms other than Th2 immunity [35, 36].
345
346 To understand the mechanisms of potential immunopathology linked to SARS vaccines, it's been shown
347 that high levels of eosinophilic immunopathology were observed with modified vaccinia virus Ankara-
348 based vaccine platform vaccines,[6-11] and this vaccine platform was found to induce both Th1 (IFN-
349 gamma, IL-2) and Th2 (IL-4, IL-5) cytokines and down-regulation of anti-inflammatory cytokines (IL-10,
350 TGF-beta) upon infection, causing severe infiltration of neutrophils, eosinophils, and lymphocytes into
351 the lung. These pieces of evidence suggest that Th2 is not the sole factor but rather a mixed Th1 and Th2
352 response is responsible for the immunopathology. Additionally, it was found that IL-6 was shown to have
353 a prominent role in SARS-CoV-induced immune enhancement [11] in experimental animals, as well as in
354 lung pathology in SARS patients [37]. The prominent role of IL-6 in host Th17 immune responses suggest
355 that this pathway might also comprise a component of coronavirus vaccine immune enhancement[13].
356 The finding that Th17 lymphocytes activate eosinophils [35], and that eosinophils comprise a key element
357 of Th17 responses is consistent with these findings [35]. Of interest, monoclonal antibodies directed at
358 interfering with IL-6 binding with its receptor are now being investigated as possible immunotherapies
359 for patients with COVID19 [38].
360
361 Finally, in the Alhydrogel® dose-ranging study, we observed that a higher concentration of Alhydrogel®
362 was required to trigger a fully protective immune response with attenuated eosinophils infiltration, while
363 S protein induced a higher degree of eosinophilic cellular infiltration, which is consistent with the
364 previous finding [12]. Table 1A further summarized the comparison of eosinophilic immunopathology

365 induced by different SARS-CoV vaccines, including the S protein, virus-like particle (VLP) expressing spike
366 protein, and the inactivated whole virus vaccines. The results indicated that the vaccine using VLP
367 expressing S protein triggered worse eosinophilic infiltration than the inactivated whole virus vaccines,
368 and S protein showed the least eosinophil infiltration among them either with or without formulated
369 with alum. Our studies find that the RBD can elicit far less immunopathology than even the S protein.
370 Similar to what was seen for the whole virus vaccine against SARS, lung eosinophil infiltration was
371 observed in mice immunized with MERS gamma irradiation-inactivated whole virus vaccine after
372 challenge with MERS-CoV (Table 1B) [39]. These findings further suggested that Alhydrogel® formulated
373 RBD219-N1 was a safer SARS-CoV vaccine than the ones listed in Table 1A.
374
375 Collectively, all the preclinical data suggested that SARS-CoV RBD219-N1 formulated with alum is a
376 potentially safe and efficacious vaccine against SARS-CoV infection. We have developed the scalable
377 process and partnered with WRAIR and manufactured RBD219-N1 protein under current good
378 manufacturing practices (cGMP) in 2016. The bulk drug substance has been frozen (-70°C to -80°C) in a
379 temperature-regulated storage location and under stability testing since its manufacturing date (July
380 2016) and remains stable. This vaccine candidate is ready for formulation and can be rapidly transitioned
381 to clinical testing. The preclinical data reported here suggest that SARS-CoV RBD219-N1 formulated with
382 Alhydrogel®is a safer and more efficacious vaccine against SARS-CoV infection compared to many other
383 candidate vaccines. This vaccine is also under evaluation as part of a broader strategy to accelerate as a
384 universal CoV vaccine, possibly in combination with RBDs from other coronaviruses, including SARS-CoV-
385 2.
386

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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485 **FIGURE LEGENDS**
486
487 **Figure 1. RBD to Alhydrogel® binding analysis.** (A) A micro BCA assay was used to quantify the % RBD
488 adsorbed on Alhydrogel® at different RBD to Alhydrogel® (Al) ratios; (B) SDS-PAGE analysis: the
489 supernatant and pellet fractions for a formulation of 0.2 mg/mL RBD219-N1 with 1.6 mg/mL Alhydrogel
490 (1:8 ratio) were analyzed. 10 µL of the desorbed RBD from the pellet fraction (P) and 10 µL of supernatant
491 (S) were loaded on the gel and stained with Coomassie Blue. M: protein marker.
492
493 **Figure 2. Vaccination-induced protection against lethal MA-15 infection at different stages.** Post
494 immunization: (A) Neutralizing antibody titers after immunization. Post challenge: (B) daily survival rates,
495 (C) daily weight loss, and the viral loads in the lung on day 1 (D) and day 2 (E).Groups of mice (N=15 per
496 group) were immunized 3 times with yeast expressed RBDs (20, 10, and 10 µg respectively) or 9 µg of S
497 protein for each immunization at 3-week intervals. Mice given TBS/alum were included as controls. The
498 titers of neutralization antibodies were determined on day 50. All vaccinated mice were challenged with
499 5.6 logs (~ 10X LD_{50}) $TCID_{50}$/60 µL of MA-15 intranasally (IN). Three challenged mice in each group were
500 euthanized on days 1 and 2 post-challenge, respectively. The remaining mice in each group (N=9) were
501 monitored daily for clinical manifestations (e.g., weight loss), and mortality.
502
503 **Figure 3 Comparison of Alhydrogel (Al) and AddaVax (MF-59-like adjuvant). (**A)qPCR for the expression
504 of the SARS-CoV np gene in the lungs of mice and (B) neutralizing antibody-100 (NT100) titers of mice
505 differentially vaccinated with RBD219-N1 (log_2). The mice (N=4) were vaccinated with RBD219-N1
506 formulated in Alhydrogel®, AddaVax (MF59-like adjuvant), and no adjuvant. On day 52, sera were
507 collected to evaluate IgG and neutralizing antibody titers. On day 63, mice were i.n. challenged with
508 SARS-CoV. Each bar represented an individual mice, two mice per group were sacrificed on day 3 and
509 day 6 posted infection (dpi) to evaluate viral load.
510
511 **Figure 4 Lung histopathology of infiltrates from mice immunized with different formulations.**
512 Photomicrographs of lung tissue from Balb/c mice to evaluate eosinophil infiltration after challenge with
513 SARS-CoV that had previously been immunized with RBD219-N1 formulated with (A) Alhydrogel® (Al), (B)
514 AddaVax (MF59-like adjuvant), and (C) no adjuvant. (A) Alhydrogel® (Al): Perivascular mononuclear
515 infiltrations (lymphocytes and monocytes/macrophages) along with very few eosinophils, only one seen
516 in this field, indicated by a red arrow. (B) AddaVax (MF59-like adjuvant): Severe inflammatory
517 infiltrations with more than 50% eosinophils, red arrows highlight some of those, and (C) no adjuvant:
518 Inflammatory infiltrations with less than 50% eosinophils. Scale bar = 100 µm.
519
520 **Figure 5. Neutralizing (A) and RBD-specific IgG antibody (B) responses of mice immunized with**
521 **RBD219-N1/Alhydrogel® (Al) at 1:25 or 1:8 ratios.** In group 1, a formulation of 0.2 mg/mL RBD in 5
522 mg/mL Alhydrogel® with the dose of 20 µg/10 µg/10 µg of RBD for prime/1st boost/2nd boost was used
523 for immunization, respectively. In group 2, the formulation 0.2 mg/mL RBD in 1.6mg/mL Alhydrogel® was
524 tested, more specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was
525 used while in group 3, 10 µg RBD were used for all immunizations. Alhydrogel® alone and pre-formulated
526 SARS-S protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive controls
527

528 **Figure 6**. **Lung viral loads of differentially immunized mice challenged intranasally with SARS-CoV on**
529 **(A) day 3 post-challenge and (B) day 6 post-challenge.** In group 1, a formulation of 0.2 mg/mL RBD in 5
530 mg/mL Alhydrogel® with the dose of 20 µg/10 µg/10 µg of RBD for prime/1st boost/2nd boost was used
531 for immunization, respectively. In group 2, the formulation 0.2 mg/mL RBD in 1.6mg/mL Alhydrogel® was
532 tested, more specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was
533 used while in group 3, 10 µg RBD were used for all three immunizations. Alhydrogel® alone and pre-
534 formulated SARS-S protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive
535 controls
536
537 **Figure 7 Eosinophilic infiltration in mice immunized with different doses of Alhydrogel®.**
538 Photomicrographs of lung tissue from Balb/c mice after challenge with SARS-CoV. (A) In group 1, a
539 formulation of 0.2 mg/mL RBD in 5 mg/mL Alhydrogel® with the dose of 20 µg/10 µg/10 µg of RBD for
540 prime/1st boost/2nd boost was used for immunization, respectively. (B) In group 2, the formulation 0.2
541 mg/mL RBD in 1.6mg/mL Alhydrogel® was tested with a dosing regimen of 20 µg/10 µg/10 µg RBD for
542 prime/1st boost/2nd boost (C) In group 3, 10 µg RBD were used for all three immunizations. (D
543 Alhydrogel® alone in group 4 was used as a negative control while (E) alum-preformulated SARS-S protein
544 (3 µg) in group 5 was used as a positive control. Scale bar= 200 µm.
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572 **TABLE**

573

574 **Table 1.** Historical lung histopathology data in mouse preclinical studies for SARS and MERS vaccines.
575 Comparison of eosinophil infiltration induced by (A) different SARS vaccines and (B) different adjuvants
576 for the whole virus MERS vaccine. DIV: double-inactivated whole virus vaccine, BPV: Beta propiolactone-
577 inactivated whole virus vaccine.

578

Vaccine type	Average eosinophil infiltration score	reference
(A) SARS Vaccine		
Study 1 **(Score scale: 0-4)** Score based on percent eosinophils on histologic evaluation. 7-8 mice per group. 5 microscopy fields for each mouse lung were evaluated.		
Whole virus (DIV)	~3	Tseng *et al.*, 2012[12]
Spike protein	~2	
Spike protein virus-like particle	>3	
Whole virus (DIV) with alum	~2	
Spike protein with alum	~1.5	
Spike protein virus-like particle with alum	>3	
PBS control	~0	
Study 2 **(Score scale: 0-3)** Score for eosinophils as percent of infiltrating cells for each vaccine dosage group. N= 7-8 per group. 10 to 20 microscopy fields for each mouse lung were scored. Scoring: 0= ,5% of cells, 1= 5–10% of cells, 2= 10–20% of cells, 3= 20% of cells.		
Spike protein	~1.5	Tseng *et al.*, 2012[12]
Whole virus (DIV)	~2.5	
Whole virus (BPV)	Not available	
Spike protein with alum	~1.5	
Whole virus (DIV) with alum	~2.5	
Whole virus (BPV) with alum	~2.5	
PBS control	0	
(B) MERS Vaccine		
Study 1 **(Score scale: 0-3)** 0- no pathology, 1- mild, 2- moderate, and 3-severe.		
Alum alone	0	Agrawal *et al.*, 2016[39]
MF59-like adjuvant alone	0	
Whole virus with alum	2	
Whole virus with MF59-like adjuvant	2	
Whole virus	2	

579

580

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583 **SUPPLEMENTAL DATA**

584 **Supplementary Table 1.** Immunogenicity and efficacy results for adjuvant screening. n/s: not
585 significant, M: moderate, S: severe. ND: Not detected. N/A: not available

586

587 **Supplementary Table 2.** Immunogenicity and efficacy results for the Alhydrogel dose-ranging study.

588 * Sacrifice; ** Not applicable; # Not detected; § Death due to over anesthetization

589

590

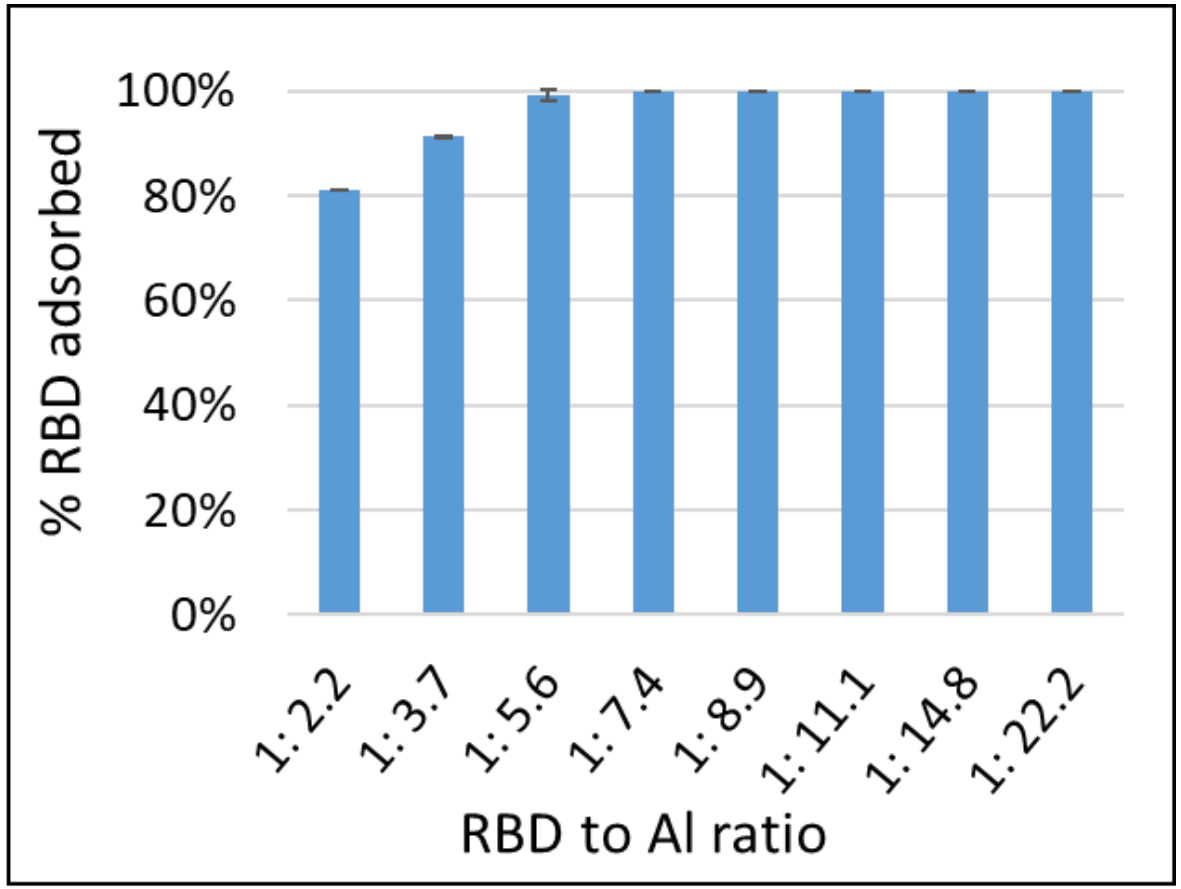
591 **Supplementary Figure 1. Optimization of immunization routes.** Mice were immunized with RBD219-
592 N1 formulated with or without Alhydrogel® (1:25 ratio) subcutaneously (s.c.) or intramuscularly (i.m.),
593 three times, at 3-week intervals. Sera were collected 10 days after the last immunization and tested for
594 IgG antibody responses and for neutralizing antibodies against SARS pseudovirus and live SARS- CoV
595 infections. (A) Detection of IgG antibody response by ELISA in mouse sera. Neutralization antibody
596 titers against SARS pseudovirus

597 (B) and live SARS-CoV (C) in mouse sera. [Alhydrogel® abbreviated as Alum.]. Psudovirus was prepared
598 as previously described in Chen et al., 2014 [20].

599

Fig. 1

(A)


% RBD adsorbed
100%
80%
60%
40%
20%
0%
1:2.2
1:3.7
1:5.6
1:7.4
1:8.9
1:11.1
1:14.8
1:22.2
RBD to Al ratio

(B)

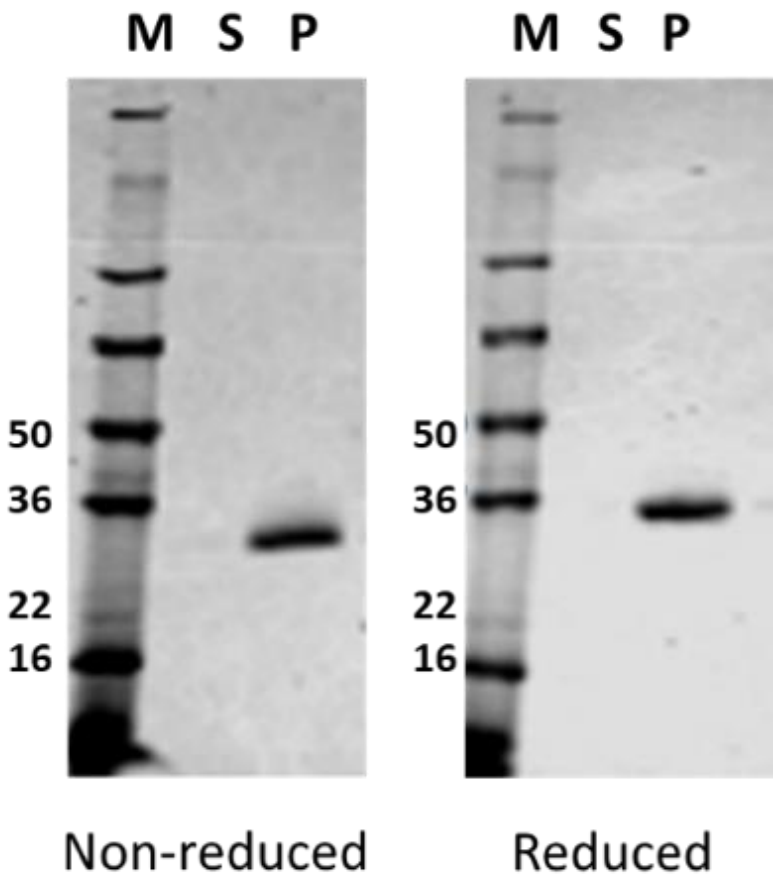
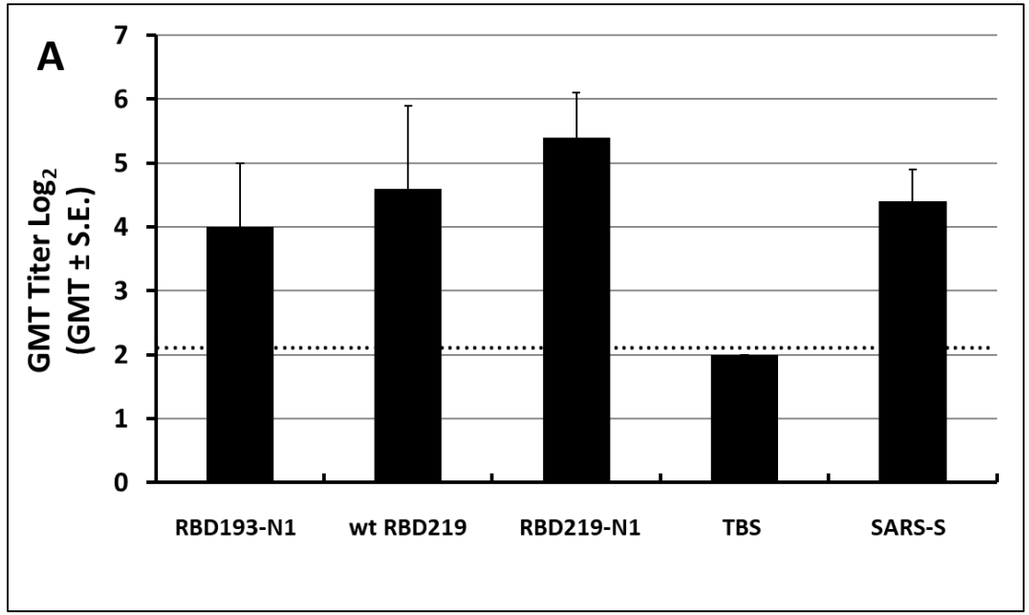
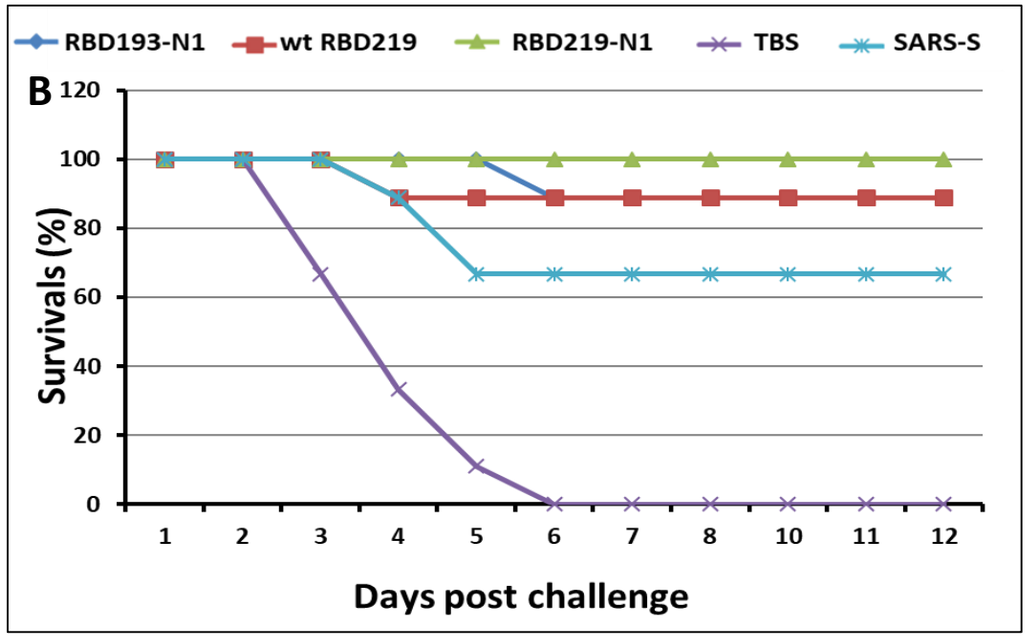
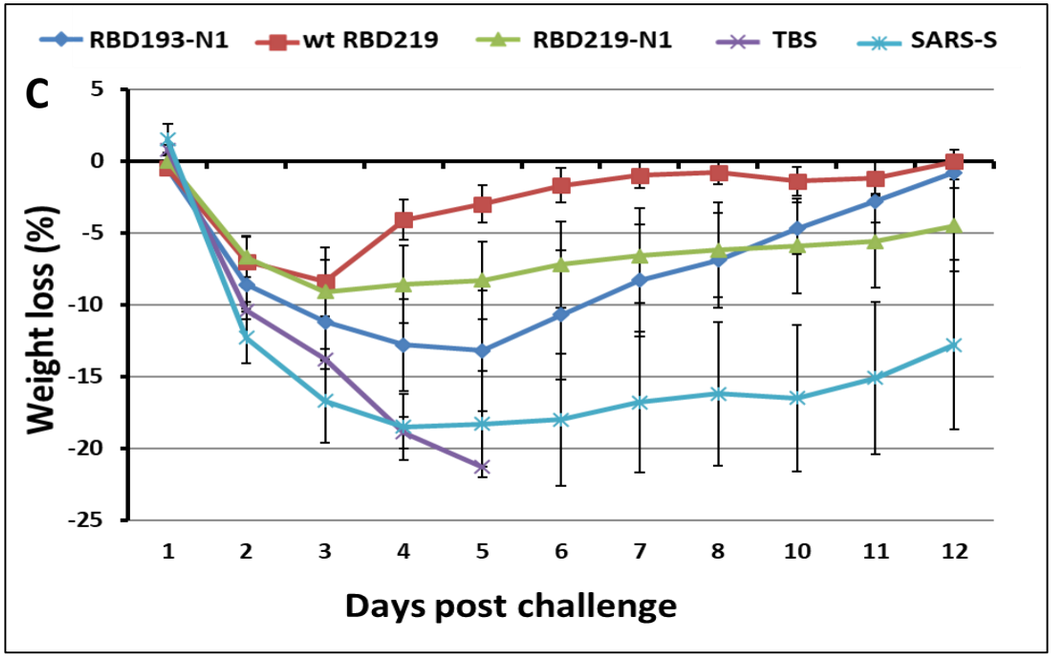
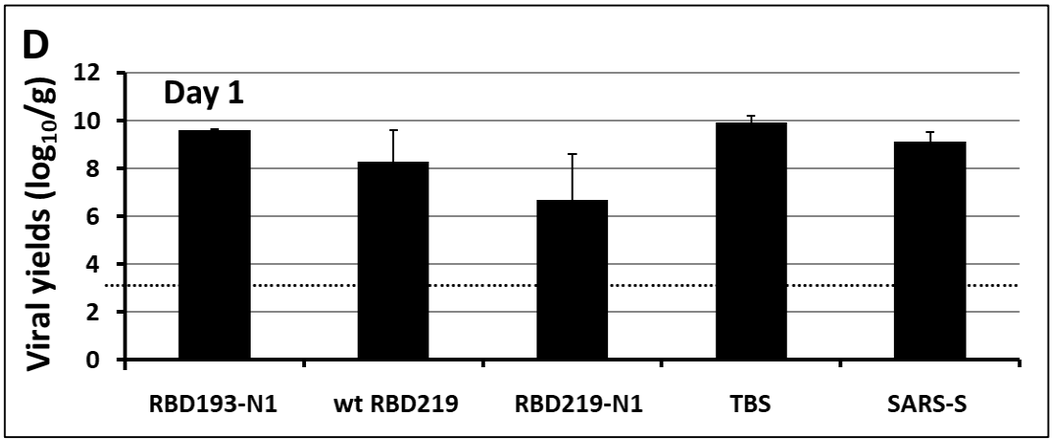

M S P
M S P
50
36
22
16
50
36
22
16
Non-reduced
Reduced

Fig. 2


A
GMT Titer Log2
(GMT ± S.E.)
0
1
2
3
4
5
6
7
RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S


RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S
B
Survivals (%)
0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
10
11
12
Days post challenge


RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S
C
Weight loss (%)
5
0
-5
-10
-15
-20
-25
1
2
3
4
5
6
7
8
10
11
12
Days post challenge


D
Day 1
Viral yields (log10/g)
0
2
4
6
8
10
12
RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S

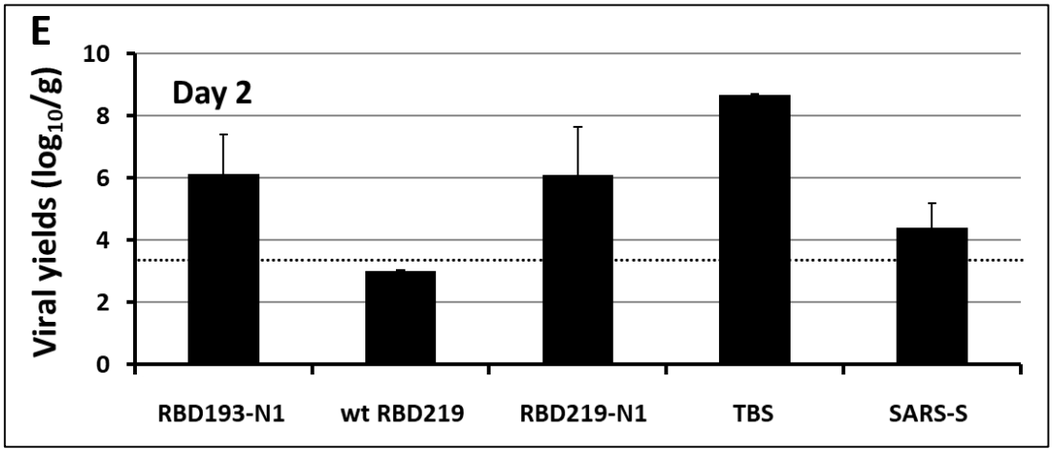

E
Day 2
Viral yields (log10/g)
0
2
4
6
8
10
RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S

Fig. 3

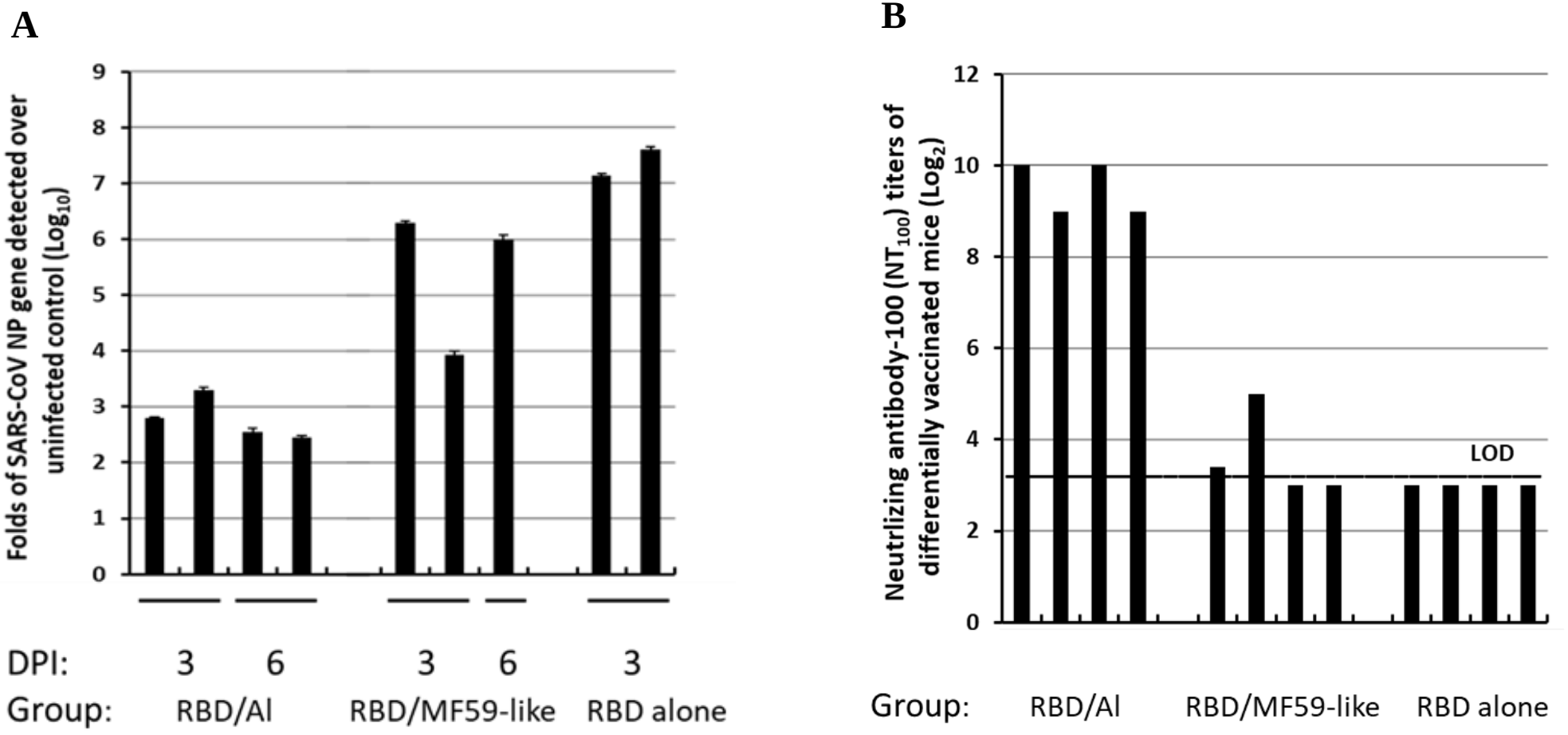
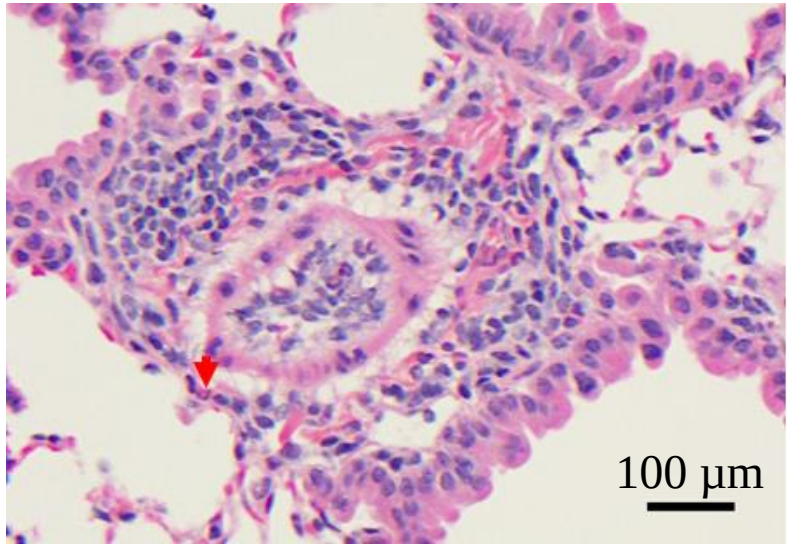
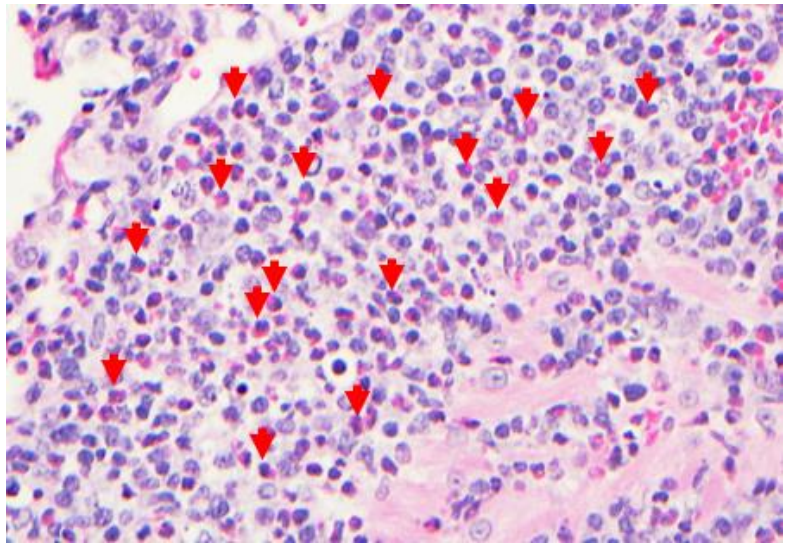

A
Folds of SARS-CoV NP gene detected over uninfected control (Log10)
0
1
2
3
4
5
6
7
8
9
DPI: 3 6 3 6 3
Group: RBD/Al RBD/MF59-like RBD alone
B
Neutrlizing antibody-100 (NT100) titers of differentially vaccinated mice (Log2)
0
2
4
6
8
10
12
LOD
Group: RBD/Al RBD/MF59-like RBD alone

Fig 4

A. RBD/Al



B. RBD/MF59-like



C. RBD alone

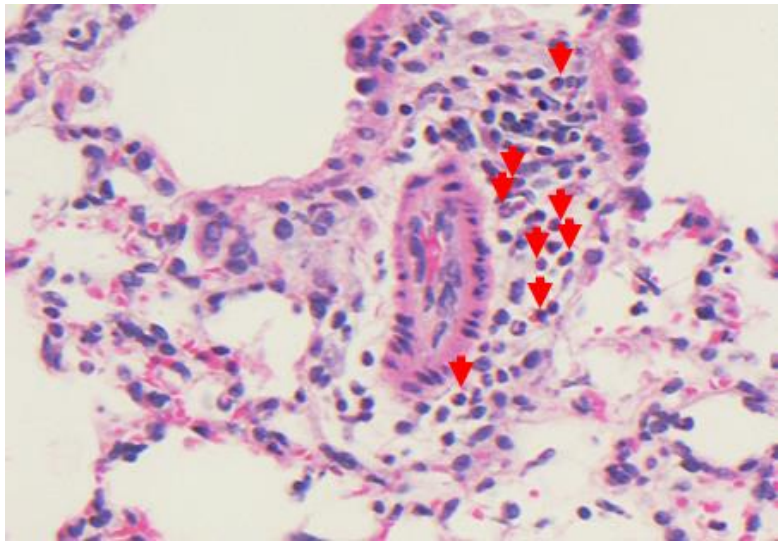
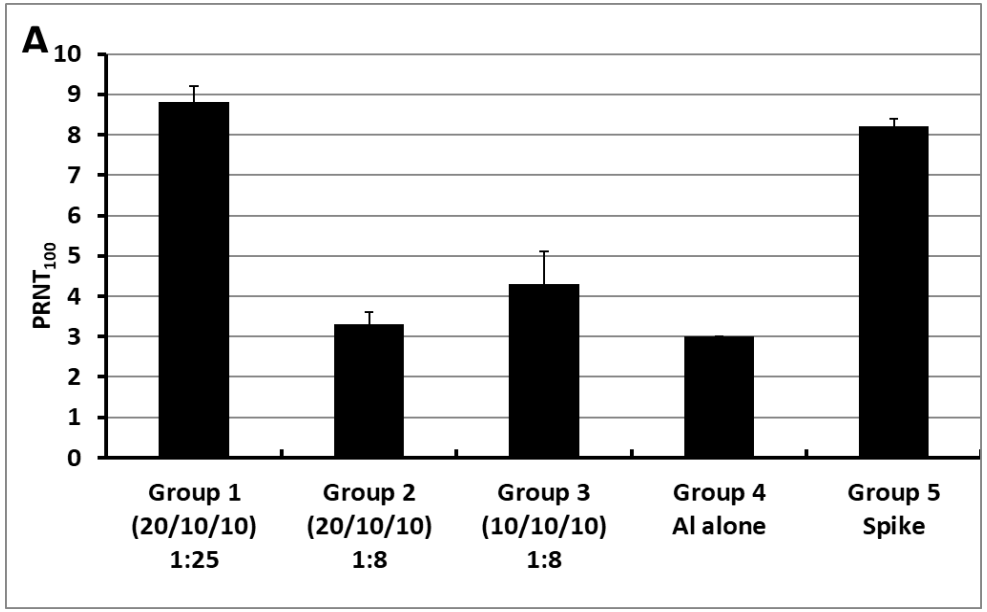


Fig. 5


A
PRNT100
10
9
8
7
6
5
4
3
2
1
0
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

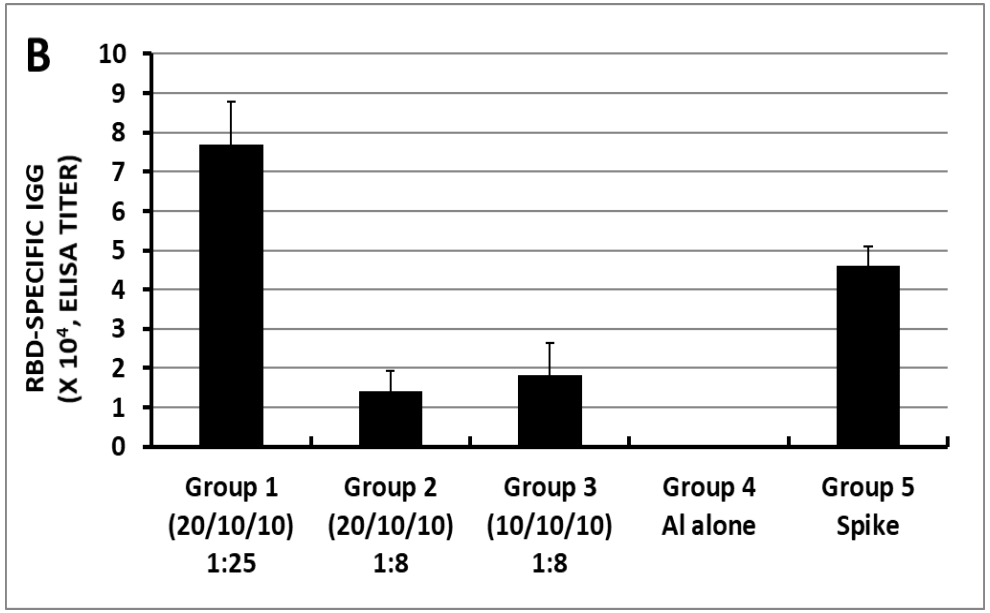
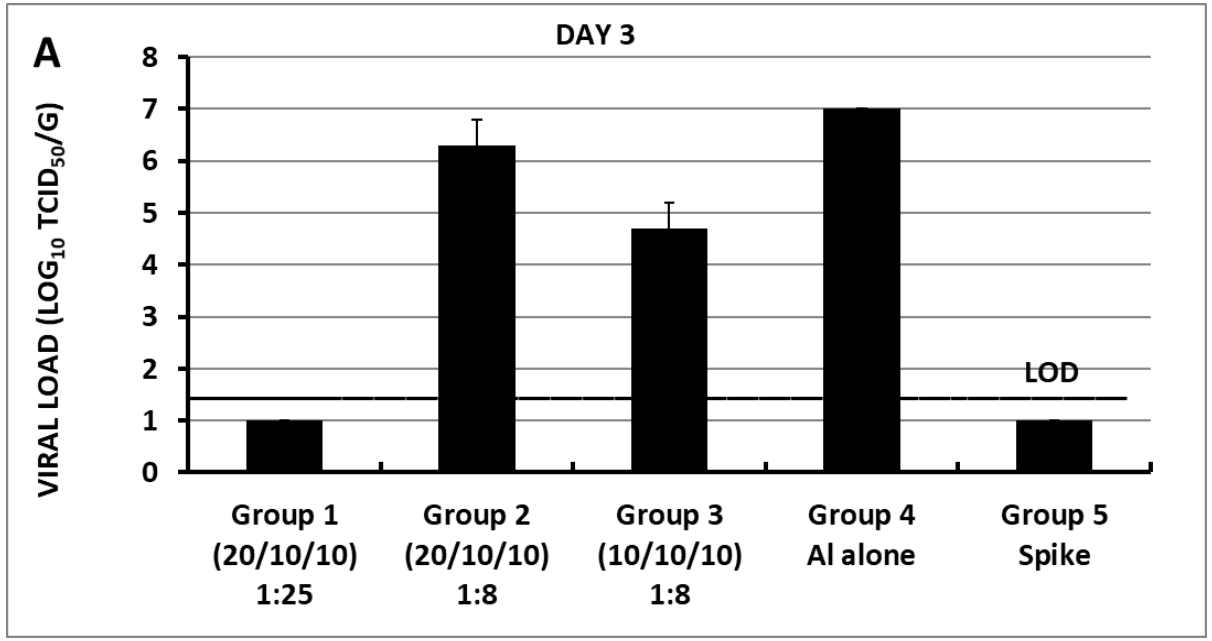

B
RBD-SPECIFIC IGG (X 10^4, ELISA TITER)
10
9
8
7
6
5
4
3
2
1
0
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

Fig. 6


A
DAY 3
VIRAL LOAD (LOG10 TCID50/G)
8
7
6
5
4
3
2
1
0
LOD
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

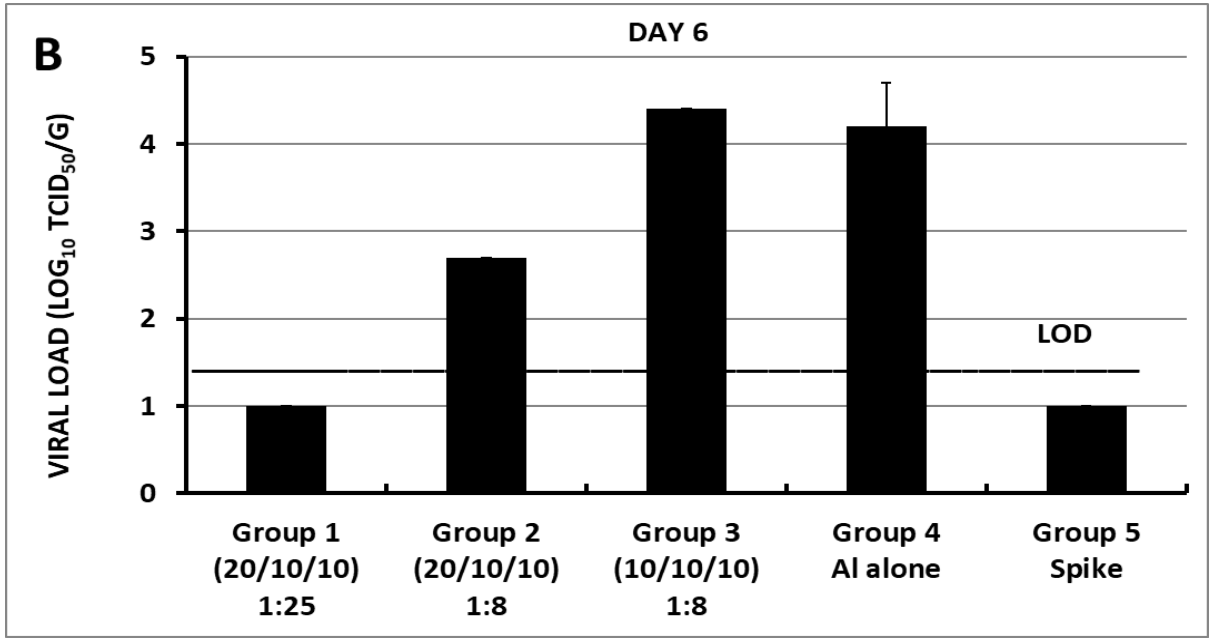
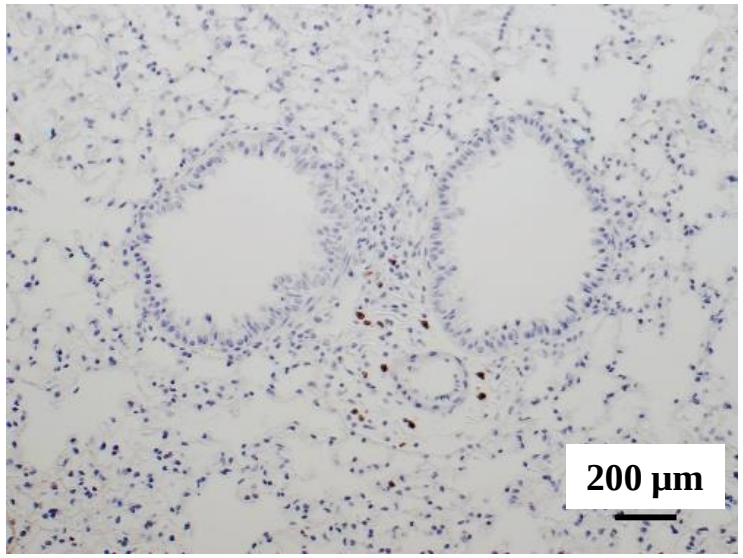
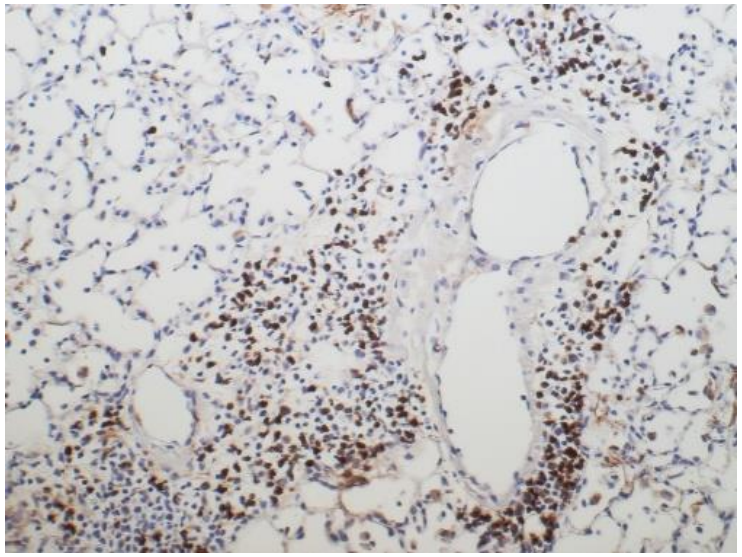

B
DAY 6
VIRAL LOAD (LOG10 TCID50/G)
5
4
3
2
1
0
LOD
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

Fig. 7

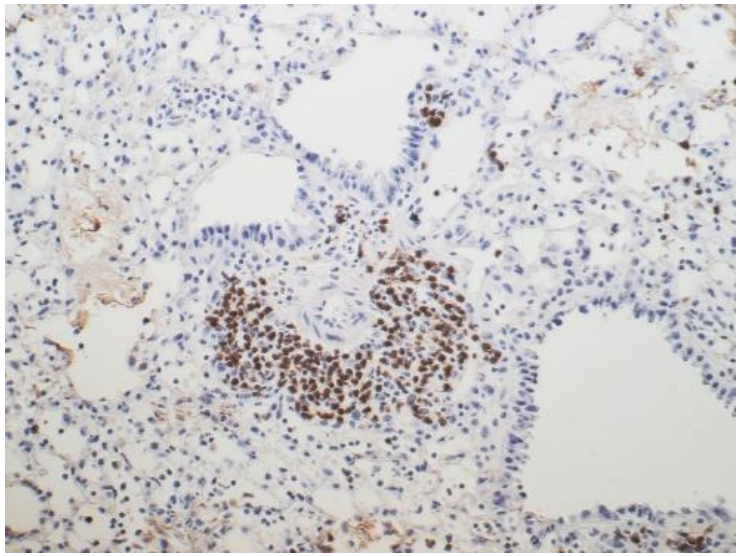
A. Group 1 RBD (20,10,10); (RBD: Alhydrogel® = 1:25)



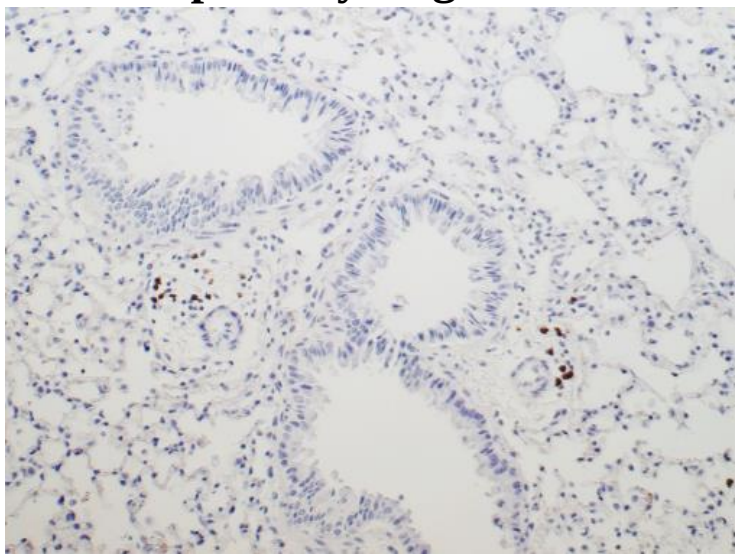
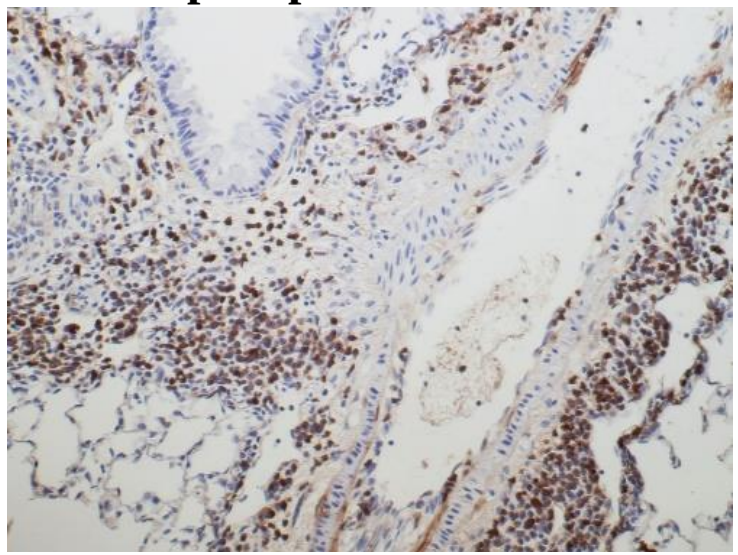
B. Group 2 RBD (20,10,10); (RBD: Alhydrogel® = 1:8)



C. Group 3 RBD (10,10,10); (RBD: Alhydrogel® = 1:8)



D. Group 4 Alhydrogel® alone



E. Group 5 Spike