Yeast-Expressed SARS-CoV Recombinant Receptor-Binding Domain (RBD219-N1) Formulated with Alum 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 other emerging and re-emerging beta-CoVs such as SARS-CoV-2.

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

INTRODUCTION

The pandemic of severe acute respiratory syndrome (SARS) between 2002 and 2003. This disease caused by SARS coronavirus (SARS-CoV) led to close to 800 deaths and more than 8,000 infections, which concluded approximately 10 percent of overall fatality rate. Alarmingly, the fatality rate among older adults exceeded 50 percent [1]. In preparation for future outbreaks and accidental and/or intentional releases of SARS-CoV, intensive efforts have been made to develop vaccines against SARS-CoV.

For the past two decades, several antigens have been identified and developed as SARS-CoV vaccine candidates. Initially, whole inactivated virus (WIV) or modified vaccinia virus Ankara expressing SARS vaccines were developed [1], however, eosinophilic immunopathology was observed in mice and non-human primates immunized with these viral-vectored vaccines [2-7]. Even though historically there have been reports that alum adjuvanted vaccines could induces enhancement, such as in the 1960's with the RSV vaccine or even with WIV and S proteins,[8] later, it was shown that alum-adjuvanted WIV elicited

55 less immunopathology than WIV alone [8]. suggesting that alum might reduce immune enhancement, a
56 process possibly linked to mixed Th1, Th17, and Th2 responses [7, 9]. Additional evidence emerged that
57 the virus N protein had a key but not exclusive role in immune enhancement [3, 7]. Based on these
58 studies, the recombinant S protein of SARS-CoV was used as a vaccine candidate [1], but the full-length
59 S-protein also induced immunopathology, with epitopes outside of the receptor-binding domain (RBD)
60 of the S protein implicated in eliciting this phenomenon [10, 11]. Therefore, the RBD of the S protein
61 was selected as a substitute for the full-length S protein [12-17].
62
63 Recombinant RBD formulated with Sigma's adjuvant system® (consisting of Monophosphoryl-lipid
64 A/Trehalose dicorynomycolate adjuvant, a skewet Th1/Th2 adjuvant) or with Freund's adjuvant
65 (Freund's complete in prime and Freund's incomplete in boost; a Th1/Th2 balanced adjuvant scheme)
66 was shown to elicit neutralizing antibodies and highly protective immunity in the vaccinated animals,
67 while eliminating or significantly reducing eosinophilic immunopathology [14, 16-19]. In our previous
68 studies [20]. we developed a production process for several tag-free yeast-expressed recombinant RBD
69 constructs. Such studies down-selected several candidates and ultimately identifying one, RBD219-N1
70 (residues 319-536), as a promising vaccine candidate, due to its ability to induce in immunized mice a
71 stronger anti-RBD-specific antibody response and neutralizing antibodies when adjuvanted with
72 aluminum hydroxide (Alhydrogel®). The protein production process was transferred to the Pilot
73 Bioproduction Facility (PBF) at Walter Reed Army Institute of Research (WRAIR), and the clinical grade
74 RBD219-N1 (drug substance) was manufactured under current Good Manufacturing Practices (cGMP)
75 and is suitable for further Phase I clinical trials.
76

77 In this work, the RBD219-N1 formulated with Alhydrogel® resulted in significantly increased antigen-
78 specific IgG titers and neutralizing antibody responses when compared to other RBD constructs. After
79 challenge with SARS-CoV, 100% of mice immunized with RBD219-N1 survived, while only 89% of mice
80 immunized with other RBD constructs and less than 70% of the mice immunized with SARS-CoV spike
81 protein survived and none survived in the control group. The alum formulated RBD minimized immune
82 enhancement compared with other adjuvants formulations with either the RBD or full-length S protein.
83 Finally, an Alhydrogel® dose-ranging study further indicated that by formulating RBD219-N1 with
84 Alhydrogel® at the ratio of 1:25, higher IgG titers could be elicited with no detectable viral load upon
85 challenge.
86
87 **MATERIALS AND METHODS**
88
89 **Generation of recombinant yeast-expressed RBD of SARS-CoV**
90 The yeast-expressed SARS-CoV RBD193-N1, wt-RBD219 and RBD219-N1 were expressed and purified as
91 previously described[20, 21]. Briefly, 1 mL of *P. pastoris* X33 seed stock expressing RBD193-N1, wt
92 RBD219, and RBD219-N1 was inoculated into 500 ml BMG (buffered minimal glycerol) medium and the
93 culture was incubated overnight at 30°C with constant shaking at 250 rpm until an OD600 of ~10.
94 Approximately 250 ml of overnight culture were inoculated into 5 L sterile Basal Salt Media or Low Salt
95 medium [20]. Fermentation was maintained at 30°C, pH 5.0 and 30% of dissolved oxygen concentration
96 until the exhaustion of glycerol, and the pH and the temperature were then ramped to 6.5 and 25°C,
97 respectively, over an hour followed by continuous feeding of methanol at 11 ml/L/hr for ~70 hours. The
98 fermentation supernatant (FS) was harvested for further purification. To purify RBD193-N1, wt-RBD219,

99 and RBD219-N1, ammonium sulfate was added to the FS until the molarity reached 2 M. The FS
100 containing 2 M ammonium sulfate was purified by hydrophobic interaction chromatography using Butyl
101 Sepharose HP resin followed by size exclusion chromatography using Superdex 75 resin [20, 21].
102
103 **Reagents**
104 Alhydrogel® (aluminum oxy hydroxide) was purchased from Brenntag (Ballerup, Denmark), AddaVax
105 (MF59-like adjuvant; squalene oil-in water emulsion) was purchased from Invivogen (San Diego, CA, USA).
106 The SARS S protein vaccine, produced in the baculovirus/insect cell expression platform and pre-
107 formulated with aluminum, was obtained from BEI Resources, NIAID, NIH (Manassas, VA, USA).
108
109 **Binding Study**
110 One ml of TBS containing 18 to 180 µg RBD219-N1 and 400 µg Alhydrogel® were prepared to study the
111 binding of RBD219-N1 to Alhydrogel® at different ratios (from 1:2 to 1:22). The prepared RBD219-N1/
112 Alhydrogel® slurry was mixed for one hour to ensure the binding of RBD219-N1 to Alhydrogel® reached
113 an equilibrium state. The slurry was then centrifuged at 13,000 x g for 5 minutes, and the supernatant
114 was collected while the Alhydrogel® pellet was resuspended with an equal volume of removed
115 supernatant. The RBD219-N1 protein content in the supernatant fraction and the pellet fraction were
116 then measured using a micro BCA assay (ThermoFisher, Waltham, MS, USA). Similarly, the presence of
117 RBD219-N1 in the pellet and supernatant fractions was also evaluated using SDS-PAGE. Briefly, after the
118 slurry was centrifuged and separated into pellet and supernatant fractions, the Alhydrogel® pellet was
119 further resuspended with desorption buffer (100 mM sodium citrate, 92 mM dibasic sodium phosphate
120 at pH 8.9) and mixed for 1 hour. The desorbed RBD was then separated from Alhydrogel® by

121 centrifugation at 13,000 x g for 5 minutes. Ten microliters of desorbed RBD from the pellet fraction and
122 free RBD in the supernatant fraction were loaded on 4-20% Tris glycine gels and stained by Coomassie
123 blue.
124
125 **Animals**
126 Six- to eight-week-old, female Balb/c mice were purchased from Charles River (Wilmington, MA, USA),
127 and housed in an approved biosafety level 3 animal facility at the University of Texas Medical Branch
128 (UTMB) at Galveston, Texas. All of the experiments were performed according to National Institutes of
129 Health and United States Department of Agriculture guidelines using experimental protocols approved
130 by the Office of Research Project Protections, Institutional Animal Care and Use Committee (IACUC) at
131 UTMB.
132
133 **Study Design**
134 Three sets of pre-clinical studies were conducted to evaluate: (1) different yeast-expressed recombinant
135 antigens, including RBD193-N1, wt-RBD219, and RBD219-N1); (2) two of the most commonly adjuvants
136 used in licensed vaccines, Alhydrogel®® and AddaVax (MF59-like adjuvant); and (3) two Alhydrogel® doses
137 (1:8 and 1:25 ratios). Mice were immunized either subcutaneously (s.c.) or intramuscularly (i.m.)
138 followed by two boosters at 21-day intervals. The detailed study designs are described as the following:
139
140 <u>Antigens screening.</u> Mice (15 per group) were immunized s.c. with 100 µL yeast-expressed recombinant
141 RBD proteins (RBD193-N1, wt-RBD219, and RBD219-N1) formulated with 10 mg/mL Alhydrogel® at days
142 0 (20 µg RBD), 21 (10 µg RBD), and 42 (10 µg RBD). TBS/Alhydrogel® buffer and 9 µg of alum pre-

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

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

157 Alhydrogel® dose range screening. Mice (6 per group) were i.m. immunized with Alhydrogel® formulated
158 RBD219-N1 at days 0 (10 or 20 µg RBD), 21 (10 µg RBD), and 42 (10 µg RBD). In group 1, a formulation of
159 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
160 RBD for prime/1st boost/2nd boost was used for immunization, respectively. In group 2, the formulation
161 0.2 mg/mL RBD in 1.6 mg/mL Alhydrogel® (consisted of 0.8 mg/mL aluminum) was tested, more
162 specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was used while
163 in group 3, 10 µg RBD were for all three immunizations. Alhydrogel® alone and pre-formulated SARS-S
164 protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive controls. At day 52,

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

168 **ELISA**

169 RBD-specific IgG titers of polyclonal sera from the immunized mice were measured by ELISA, as
170 previously described [14, 15, 20]. Briefly, 96-well ELISA plates were pre-coated with yeast-expressed RBD
171 protein (1 µg/ml) overnight at 4 °C. After blocking and then incubating with serially diluted mouse sera,
172 bound IgG antibody was detected using HRP-conjugated anti-mouse IgG (1:2000), followed by the same
173 protocol as described [14, 15, 20].

174

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

176 Mice were anesthetized with isoflurane and then bled from the retro-orbital sinus plexus. After heat
177 inactivation at 56 °C for 30 min, sera were stored at 4 °C. The assay for virus-specific neutralizing
178 antibodies was performed on serial 2-fold diluted samples of each serum as described previously [8, 22].

179

180 **Collection of lungs, histology, immunohistochemistry, and virus titration**

181 After SARS-CoV challenge, mice were euthanized on different days depending on the study and their
182 lungs were removed. Lung lobes were placed in 10% neutral buffered formalin for histological
183 examination using either hematoxylin and eosin (for cellular infiltrates) or immunohistochemistry (IHC),
184 specific for eosinophils, as described previously[8, 22]. For virus quantitation, the remaining tissue
185 specimen was processed as previously described [8, 22]. Evaluations for histopathology were done by

186 an experimental human pathologist masked as to the vaccine/dosage of each specimen source;
187 assessment of the extent of pathologic damage and the eosinophilic component of the inflammatory
188 infiltrates was then provided.
189
190 **Statistical analysis**
191 Neutralizing antibody titers, weight loss, lung virus titers, IgG titers, histopathologic score and
192 eosinophilic infiltration scores were averaged for each group of mice.
193
194 **RESULTS**
195 **Vaccine integrity evaluation through binding and point-of-injection studies**
196 As a vaccine, recombinant protein antigens alone often do not induce a sufficient immune response,
197 necessitating their evaluation as proteins formulated with adjuvants. When Alhydrogel® is used as an
198 adjuvant, the antigen is typically fully adsorbed to alum to maximize potency. The binding efficiency of
199 RBD219-N1 to Alhydrogel® was performed to evaluate the minimum RBD219-N1 to Alhydrogel® ratio
200 required to ensure complete protein binding. By measuring the protein content in the supernatant and
201 the pellet fraction after adsorption and centrifugation, the percentage of RBD bound onto Alhydrogel®
202 at different RBD219-N1 to Alhydrogel® ratio was determined (Figure 1A). An Alhydrogel® to RBD ratio
203 greater than 7.4 resulted in complete adsorption. SDS-PAGE analysis of a formulation of 0.2 mg/mL RBD
204 with 1.6 mg/mL Alhydrogel® (1:8 ratio) further confirmed that no protein remained in the supernatant
205 fraction after adsorption (Figure 1B).
206
207 **Antigen screening**
208

209 In this pilot study, we compared safety, immunogenicity, and efficacy of the various yeast-expressed
210 RBD- and S vaccines [8] using a lethal mouse model of SARS--CoV infection. Figure 2a shows that mice
211 immunized with RBD-219N1 had the highest titer of neutralizing antibodies compared to mice
212 immunized with alum-adjuvanted RBD193-N1, wt-RBD219 and the S protein vaccines. Importantly,
213 endpoint evaluation for mortality has shown that a 100% survival rate was found for mice immunized
214 with alum-adjuvanted RBD219-N1, while mice immunized with alum-adjuvanted wt-RBD219 and
215 RBD193-N1 vaccines showed 88% survival and those immunized with alum-adjuvanted S protein showed
216 only 67% survival. All mice in the TBS control group died within 6 days post-challenge (Figure 2B).
217 Furthermore, mice immunized with RBD219-N1, similar to wt-RBD219 and RBD193-N1, consistently
218 showed less than 10% weight loss throughout the study period, while mice immunized with other alum-
219 formulated vaccines including the S protein showed a maximum of 15-20% weight loss (Figure 2C). This
220 was accompanied by more than a 3-log reduction of infectious viral loads within the lungs when
221 compared to mice vaccinated with TBS/Alhydrogel® (Figures 2D and 2E). None of the mice given
222 TBS/Alhydrogel® produced detectable neutralizing antibodies, whereas their geometric means of lung
223 virus titers were $10^{9.9}$ and $10^{8.9}$ $TCID_{50}$/g on days 1 and 2 post-challenge, respectively. With these results,
224 RBD219-N1 was chosen for further development.
225
226 **Adjuvant screening**
227 It is known that alum (generally a Th2 adjuvant) and MF59 (a Th1/Th2 balanced adjuvant in the form of
228 oil-in-water emulsion) are two of the most common adjuvants used in licensed vaccines with very well-
229 established safety records[23, 24]. In this study, we compared Alhydrogel® and AddaVax (MF59-like
230 adjuvant) for their ability to improve the efficacy of RBD219-N1 in mice. As shown in Figure 3 and

231 Supplementary Table 1, mice immunized with RBD219-N1 formulated with Alhydrogel® produced potent
232 neutralizing antibody responses, resulting in complete protection against a subsequent SARS-CoV
233 infection. In contrast, RBD219-N1 with MF59-like adjuvant induced high IgG titers (Supplementary Table
234 1) but failed to elicit protective neutralizing antibody responses and did not protect against SARS-CoV
235 infection, as evaluated by the isolation of the infectious virus and quantitative PCR (qPCR) for viral RNA.
236 Unlike MF59-like adjuvant formulated RBD219-N1 and RBD219-N1 alone, we also noted that RBD219-
237 N1 formulated with Alhydrogel® did not induce a cellular infiltration within the lungs (Figure 4 and
238 Supplementary Table 1). Taken together, these results suggest that RBD219-N1/Alhydrogel® was
239 potentially both effective and safe, and therefore alum was chosen as the optimal adjuvant and further
240 studied for the dose-ranging study.

241
242
243 **Alhydrogel® dose-ranging study**
244
245 Although RBD219-N1 formulated on Alhydrogel® at a ratio of 1:25 was effective in immunized animals
246 against lethal SARS-CoV challenge without inducing apparent pulmonary immunopathology, it was of
247 further interest to compare different aluminum ratios, including the efficacy of RBD219-N1 formulated
248 on Alhydrogel® at both 1:25 and 1:8 ratios in mice. Sera were collected 10 days after the last vaccination
249 to test RBD219-N1-specific IgG antibody responses by ELISA and neutralizing antibodies against live
250 SARS-CoV infections. Results showed that mice immunized with RBD219-N1/Alhydrogel® at a 1:25 ratio
251 produced significantly higher neutralizing antibody titers and RBD219-N1-specific IgG titers than those
252 vaccinated at a 1:8 ratio (Figure 5A-B). Consistent with the antibody responses, mice immunized with an
253 RBD219-N1/Alhydrogel® ratio of 1:25 were completely protected against lethal challenge with SARS-CoV,
254 as indicated by the undetectable infectious virus within the lungs and also a lack of morbidity and

255 mortality (Figure 6). In contrast, infectious virus was recovered from the lungs of mice immunized with
256 a 1:8 ratio of RBD219-N1/Alhydrogel® (Groups 2 and 3) by day 5 post-challenge and one mouse from
257 each group died. As expected, mice immunized with Alhydrogel® alone (Group 4) were not protected
258 against a lethal viral challenge and had detectable virus in the lungs resulting in the death of 2 mice on
259 day 4 post-challenge, while SARS-CoV S protein formulated on Alhydrogel® (Group 5) was also
260 immunogenic and protective against SARS-CoV infection. Histopathologic examination of lung tissues
261 using IHC (specific for eosinophils) revealed minimal eosinophilic infiltration for mice in group 1 (1:25
262 ratio) while somewhat increased infiltration was observed in groups 2 and 3 (1:8 ratio) and the worst
263 eosinophils infiltration was found in the mice immunized with S protein among all groups
264 (Supplementary Table 2 and Figure 7). The results confirm the protective efficacy of RBD219-
265 N1/Alhydrogel®, and its superiority to the S-protein in terms of protection and reduction or prevention
266 in eosinophilic infiltration, as well as the favorable effects of Alhydrogel® for eliciting high titer
267 neutralizing antibody and maximal reductions in immune enhancement comparing to other groups.
268
269
270 **DISCUSSION**
271 The RBD of the S1 protein of SARS-CoV, which is responsible for the attachment of the receptor ACE2
272 and initiates the process for cell binding and entry, has been proven as a promising vaccine candidate
273 [25]. It has been expressed as a recombinant protein with a hexahistidine tag, a GST protein or Fc
274 fragment in several different expression platforms including *E. coli*, insect cells, and mammalian cells to
275 simplify the purification process and these constructs were shown to trigger neutralizing antibodies and
276 immunity [12, 14-17]. However, an additional tag on a recombinant protein-based vaccine should be

277 avoided because it can potentially trigger an undesired immune response. In our previous studies [20,
278 21], we have expressed and purified several tag-free recombinant RBD constructs in yeast using a
279 scalable process and discovered that the yeast-expressed RBD219-N1 induced a stronger RBD-specific
280 antibody response and a high level of neutralizing antibodies in immunized mice when formulated with
281 Alhydrogel®, and thus, a very promising vaccine candidate. In this study, we conducted for the first time
282 an efficacy study in which we screened several different yeast-expressed RBD proteins and compared
283 them to the S protein as a positive control. RBD219-N1 formulated with Alhydrogel® triggered higher
284 neutralizing titers than the other Alhydrogel®- formulated RBD constructs or the S protein; the RBD219-
285 N1 formulated with Alhydrogel® immunized mice were fully protected with 100% survival rate. The
286 RBD219-N1 construct was further selected for adjuvant screening and adjuvant dose-ranging studies.
287
288 Before testing different adjuvants, we investigated the immunization routes (s.c. and i.m) for RBD219-N1
289 adsorbed to Alhydrogel® by evaluating the IgG antibody responses (Supplementary Figure. 1A),
290 neutralizing antibody titer against SARS pseudovirus and live SARS-CoV infections (Supplementary
291 Figures. 1B and 1C). It was found that both immunization routes were able to induce high titers of specific
292 IgG and neutralizing antibodies against infections of SARS pseudovirus in ACE2/293T cells
293 (Supplementary Figure.1B) and live SARS-CoV in Vero cells (Supplementary Figure.1C). Although the
294 antibody responses induced through the s.c. route were significantly higher than those through the i.m.
295 route, the i.m. route was selected for subsequent adjuvant optimization because i.m. injection of the
296 vaccines containing adjuvants has less chance to induce adverse local effects than s.c. injection[26] and
297 the majority of the clinically used vaccines are administered via the i.m. route [27].
298

299 The superiority of the RBD219-N1 vaccine antigen to the S protein was reflected both in terms of eliciting
300 neutralizing antibodies and protective immunity, and that the alum-adjuvanted RBD219-N1 resulted in
301 little to no cellular or eosinophilic immunopathology compared to either the S-protein or an M59-like
302 adjuvant-formulated RBD219-N1 vaccine. With regards to the former observation, it was previously
303 shown that removal of immune-enhancing epitopes located outside the S-protein RBD domain may result
304 in an immunogen, which is less likely to induce immunopathology [11, 28]. This finding led to the initial
305 selection of the RBD as a vaccine antigen [29]. With regards to adjuvant selection, alum prevented or
306 reduced immune enhancement, a finding that confirms a previous observation for doubly inactivated
307 virus, viral-like particle vaccines and S protein[8]. For example, Tseng et al. reported that mice immunized
308 with alum-formulated virus-like-particle and inactivated virus vaccines showed less immunopathology
309 than the non-adjuvanted vaccines. Such findings provided the basis for suggesting that eosinophilic
310 immunopathology may occur through mechanisms other than Th2 immunity [30, 31].
311
312 To understand the mechanisms of potential immunopathology linked to SARS vaccines, it's been shown
313 that high levels of eosinophilic immunopathology were observed with modified vaccinia virus Ankara-
314 based vaccine platform vaccines,[2-7] and this vaccine platform was found to induce both Th1 (IFN-
315 gamma, IL-2) and Th2 (IL-4, IL-5) cytokines and down-regulation of anti-inflammatory cytokines (IL-10,
316 TGF-beta) upon infection, causing severe infiltration of neutrophils, eosinophils, and lymphocytes into
317 the lung. These pieces of evidence suggest that Th2 is not the sole factor but rather a mixed Th1 and Th2
318 response is responsible for the immunopathology. Additionally, it was found that IL-6 was shown to have
319 a prominent role in SARS-CoV-induced immune enhancement [7] in experimental animals, as well as in
320 lung pathology in SARS patients [32]. The prominent role of IL-6 in host Th17 immune responses suggest

321 that this pathway might also comprise a component of coronavirus vaccine immune enhancement[9].
322 The finding that Th17 lymphocytes activate eosinophils [30], and that eosinophils comprise a key element
323 of Th17 responses is consistent with these findings [30]. Of interest, monoclonal antibodies directed at
324 interfering with IL-6 binding with its receptor are now being investigated as possible immunotherapies
325 for patients with COVID19 [33].
326
327 Finally, in the Alhydrogel® dose-ranging study, we observed that a higher concentration of Alhydrogel®
328 was required to trigger a fully protective immune response with attenuated eosinophils infiltration, while
329 S protein induced a higher degree of eosinophilic cellular infiltration, which is consistent with the
330 previous finding [8]. Table 1A further summarized the comparison of eosinophilic immunopathology
331 induced by different SARS-CoV vaccines, including the S protein, virus-like particle (VLP) expressing spike
332 protein and the inactivated whole virus vaccines. The results indicated that the vaccine using VLP
333 expressing S protein triggered worse eosinophilic infiltration than the inactivated whole virus vaccines,
334 and S protein showed the least eosinophil infiltration among them either with or without formulated
335 with alum. Our studies find that the RBD can elicit far less immunopathology than even the S protein.
336 Similar to what was seen for the whole virus vaccine against SARS, lung eosinophil infiltration was
337 observed in mice immunized with MERS gamma irradiation-inactivated whole virus vaccine after
338 challenge with MERS-CoV (Table 1B) [34]. These findings further suggested that Alhydrogel® formulated
339 RBD219-N1 was a safer SARS-CoV vaccine than the ones listed in Table 1A.
340 Collectively, all the preclinical data suggested that SARS-CoV RBD219-N1 formulated with alum is a
341 potentially safe and efficacious vaccine against SARS-CoV infection. We have developed the scalable
342 process and partnered with WRAIR and manufactured RBD219-N1 protein under current good

343 manufacturing practices (cGMP) in 2016. The bulk drug substance has been frozen (-70°C to -80°C) in a
344 temperature-regulated storage location and under stability testing since its manufacturing date (July
345 2016) and remains stable. This vaccine candidate is ready for formulation and can be rapidly transitioned
346 to clinical testing. The preclinical data reported here suggest that SARS-CoV RBD219-N1 formulated with
347 alum is a safer and more efficacious vaccine against SARS-CoV infection compared to many other
348 candidate vaccines.

349

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352

353 **CONFLICT OF INTEREST**

354 Some of the authors have developed subunit vaccines against SARS and MERS coronavirus infection.
355 They are involved in the process of developing a vaccine against SARS-CoV-2.

356

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FIGURE LEGENDS

Figure 1. RBD to Alhydrogel® binding analysis. (A) A micro BCA assay was used to quantify the % RBD adsorbed on Alhydrogel® at different RBD to Alhydrogel® (Al) ratios; (B) SDS-PAGE analysis: the supernatant and pellet fractions for a formulation of 0.2 mg/mL RBD219-N1 with 1.6 mg/mL Alhydrogel (1:8 ratio) were analyzed. 10 µL of the desorbed RBD from the pellet fraction (P) and 10 µL of supernatant (S) were loaded on the gel and stained with Coomassie Blue. M: protein marker.

Figure 2. Vaccination-induced protection against lethal MA-15 infection at different stages. Post immunization: (A) Neutralizing antibody titers after immunization. Post challenge: (B) daily survival rates, (C) daily weight loss and the viral loads in lung on day 1 (D) and day 2 (E).Groups of mice (N=15 per group) were immunized 3 times with yeast expressed RBDs (20, 10 and 10 µg respectively) or 9 µg of S protein for each immunization at 3-week intervals. Mice given TBS/alum were included as controls. The titers of neutralization antibodies were determined on day 50. All vaccinated mice were challenged with 5.6 log (~ 10X LD_{50}) $TCID_{50}$/60 µL of MA-15 intranasally (IN). Three challenged mice in each group were euthanized on days 1 and 2 post-challenge, respectively. The remaining mice in each group (N=9) were monitored daily for clinical manifestations (e.g., weight loss), and mortality.

Figure 3 Comparison of Alhydrogel (Al) and AddaVax (MF-59-like adjuvant). (A)qPCR for the expression of the SARS-CoV np gene in the lungs of mice and (B) neutralizing antibody-100 (NT100) titers of mice differentially vaccinated with RBD219-N1 (log_2). The mice (N=4) were vaccinated with RBD219-N1 formulated in Alhydrogel®, AddaVax (MF59-like adjuvant) and no adjuvant. At day 52, sera were collected to evaluate IgG and neutralizing antibody titers. At day 63, mice were i.n. challenged with SARS-CoV. Each bar represented an individual mice, two mice per group were sacrificed on day 3 and day 6 posted infection (dpi) to evaluate viral load.

Figure 4 Lung histopathology of infiltrates from mice immunized with different formulations. Photomicrographs of lung tissue from Balb/c mice to evaluate eosinophil infiltration after challenge with SARS-CoV that had previously been immunized with RBD219-N1 formulated with (A) Alhydrogel® (Al), (B) AddaVax (MF59-like adjuvant), and (C) no adjuvant. (A) Alhydrogel® (Al): Perivascular mononuclear infiltrations (lymphocytes and monocytes/macrophages) along with very few eosinophils, only one seen in this field, indicated by a red arrow. (B) AddaVax (MF59-like adjuvant): Severe inflammatory infiltrations with more than 50% eosinophils, red arrows highlight some of those, and (C) no adjuvant: Inflammatory infiltrations with less than 50% eosinophils. Scale bar = 100 µm.

Figure 5. Neutralizing (A) and RBD-specific IgG antibody (B) responses of mice immunized with RBD219-N1/Alhydrogel® (Al) at 1:25 or 1:8 ratios. In group 1, a 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 prime/1st boost/2nd boost was used for immunization, respectively. In group 2, the formulation 0.2 mg/mL RBD in 1.6mg/mL Alhydrogel® was tested, more specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was used while in group 3, 10 µg RBD were for all three immunizations. Alhydrogel® alone and pre-formulated SARS-S protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive controls

Figure 6. **Lung viral loads of differentially immunized mice challenged intranasally with SARS-CoV on (A) day 3 post-challenge and (B) day 6 post-challenge.** In group 1, a 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 prime/1st boost/2nd boost was used for immunization, respectively. In group 2, the formulation 0.2 mg/mL RBD in 1.6mg/mL Alhydrogel® was tested, more specifically, a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost was used while in group 3, 10 µg RBD were for all three immunizations. Alhydrogel® alone and pre-formulated SARS-S protein (3 µg) were used in groups 4 and 5, respectively, as negative and positive controls

Figure 7 Eosinophilic infiltration in mice immunized with different doses of Alhydrogel®. Photomicrographs of lung tissue from Balb/c mice after challenge with SARS-CoV. (A) In group 1, a 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 prime/1st boost/2nd boost was used for immunization, respectively. (B) In group 2, the formulation 0.2 mg/mL RBD in 1.6mg/mL Alhydrogel® was tested with a dosing regimen of 20 µg/10 µg/10 µg RBD for prime/1st boost/2nd boost (C) In group 3, 10 µg RBD were for all three immunizations. (D Alhydrogel® alone in group 4 was used as a negative control while (E) alum-preformulated SARS-S protein (3 µg) in group 5 was used as a positive control. Scale bar= 200 µm.

531 **TABLE**

532

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

537

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[8]
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[8]
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[34]
MF59-like adjuvant alone	0	
Whole virus with alum	2	
Whole virus with MF59-like adjuvant	2	
Whole virus	2	

538

539

540

541

542 **SUPPLEMENTAL DATA**

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

545

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

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

548

549

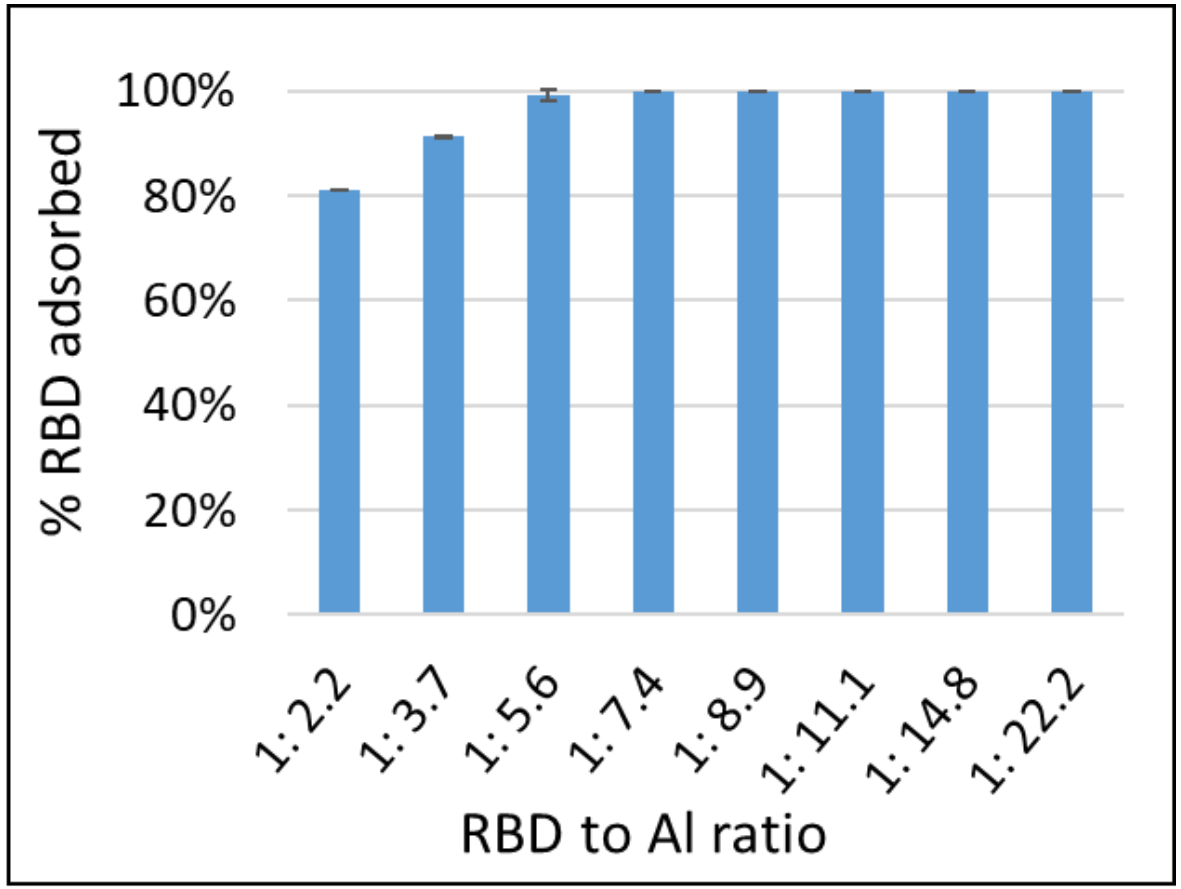
550 **Supplementary Figure 1. Optimization of immunization routes.** Mice were immunized with RBD219-
551 N1 formulated with or without Alhydrogel® (1:25 ratio) subcutaneously (s.c.) or intramuscularly (i.m.),
552 three times, at 3-week intervals. Sera were collected 10 days after the last immunization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

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

558

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)

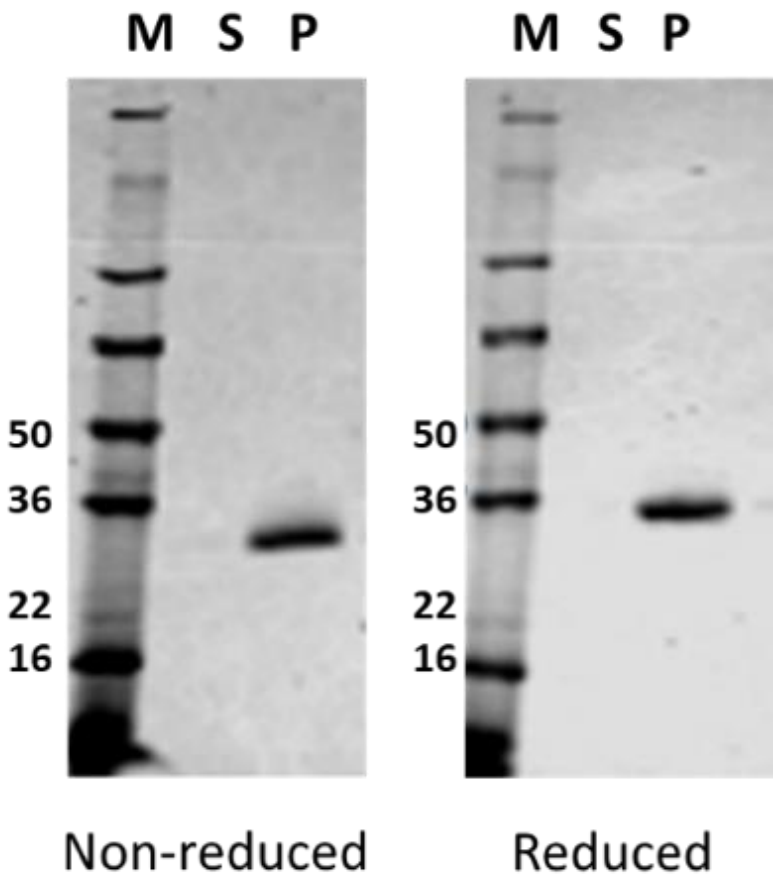
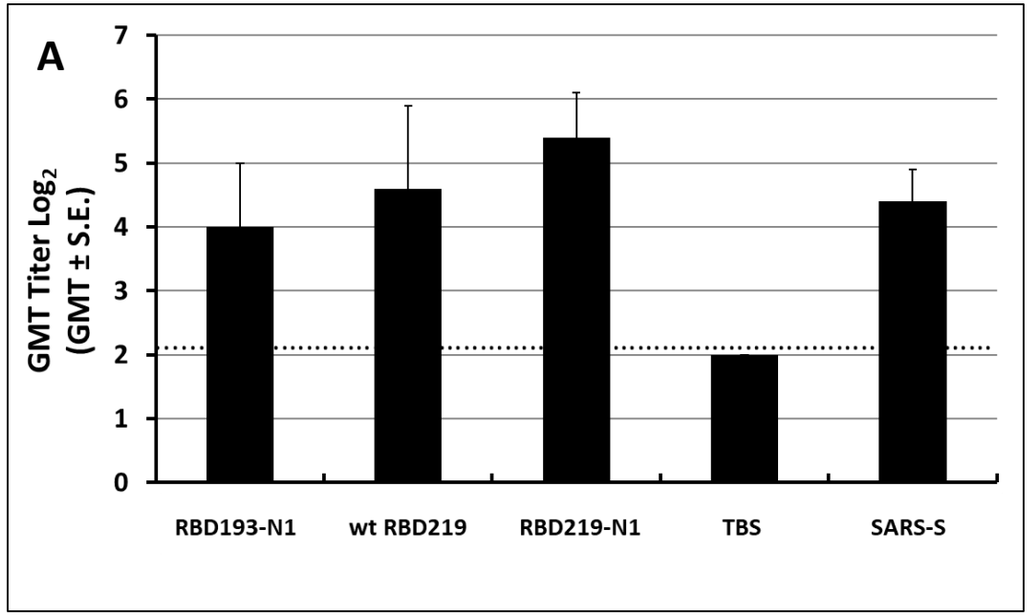
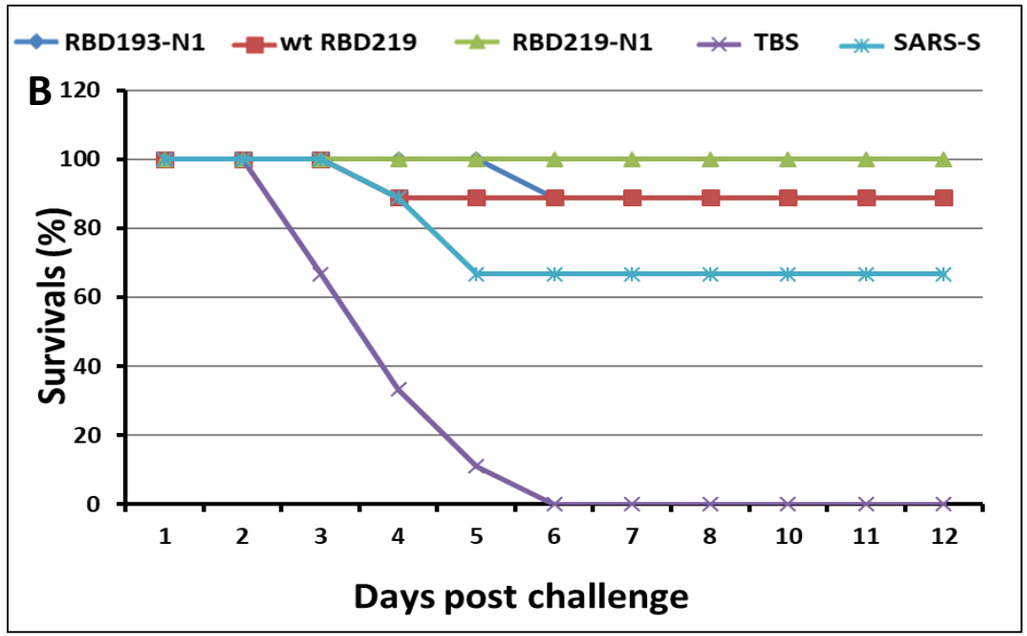
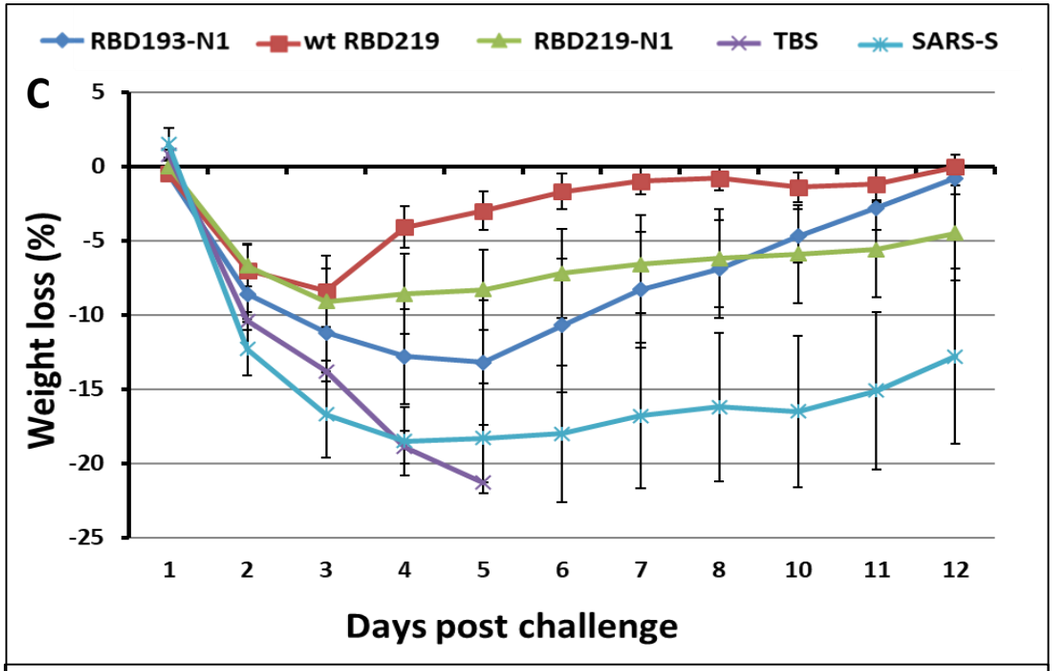
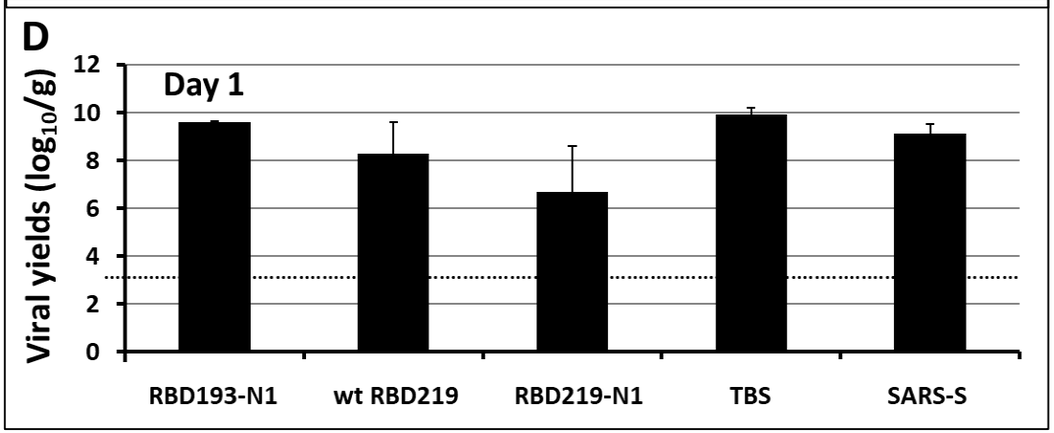

M S P
M S P
50
36
22
16
50
36
22
16
Non-reduced
Reduced

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

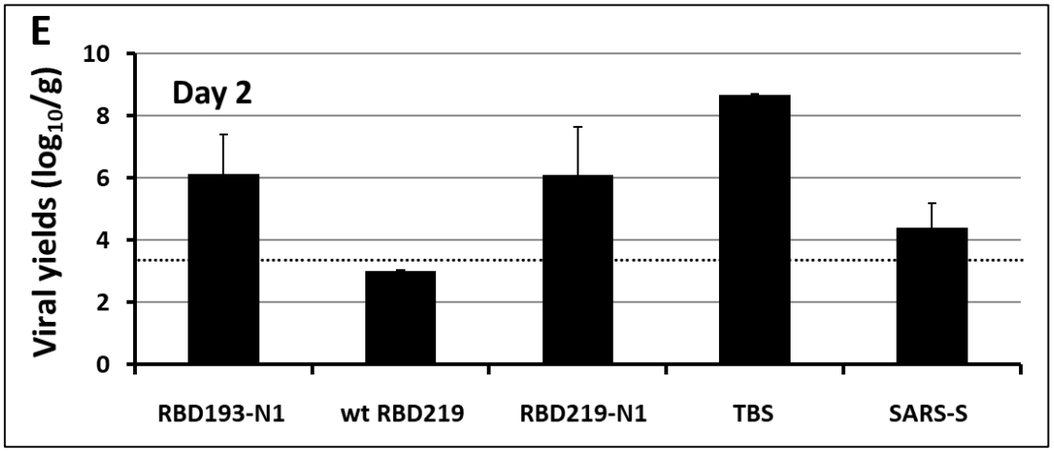
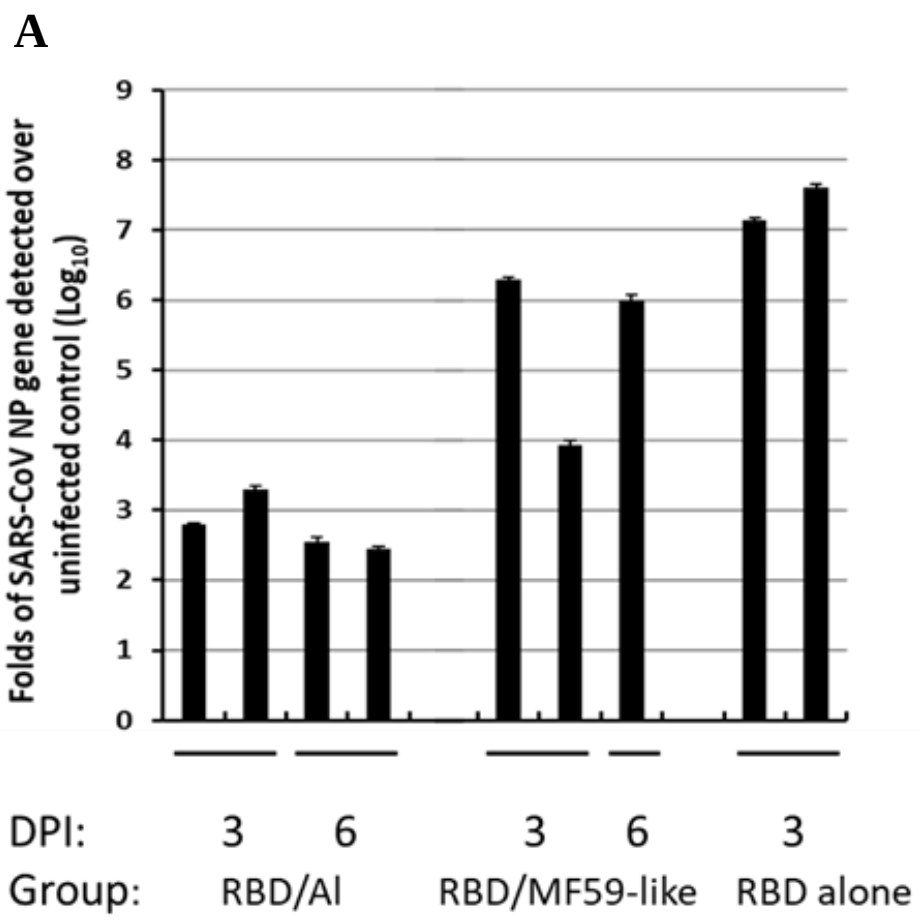

E
Day 2
Viral yields (log10/g)
0
2
4
6
8
10
RBD193-N1
wt RBD219
RBD219-N1
TBS
SARS-S

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

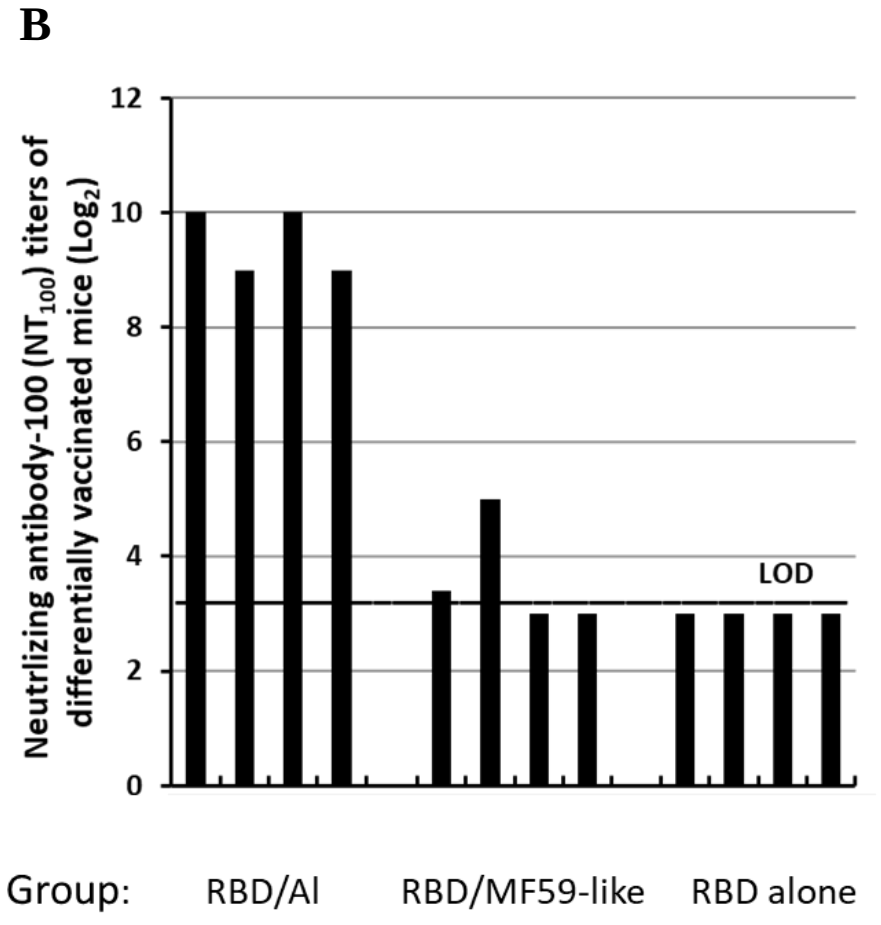
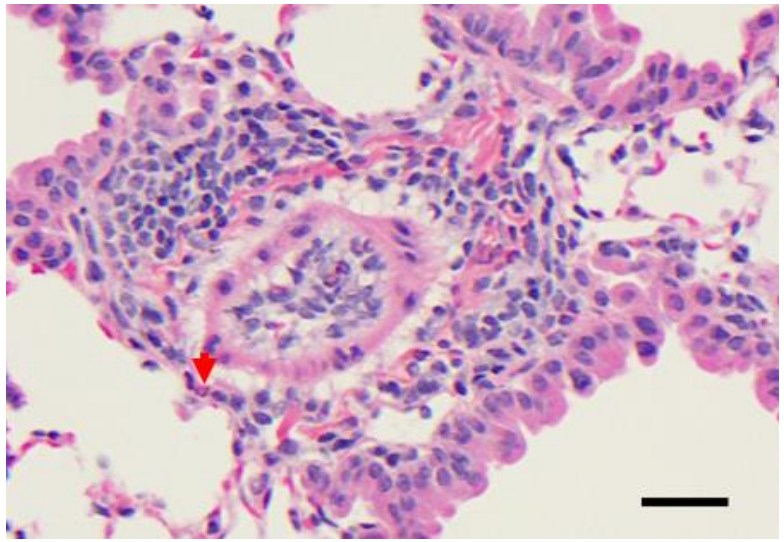
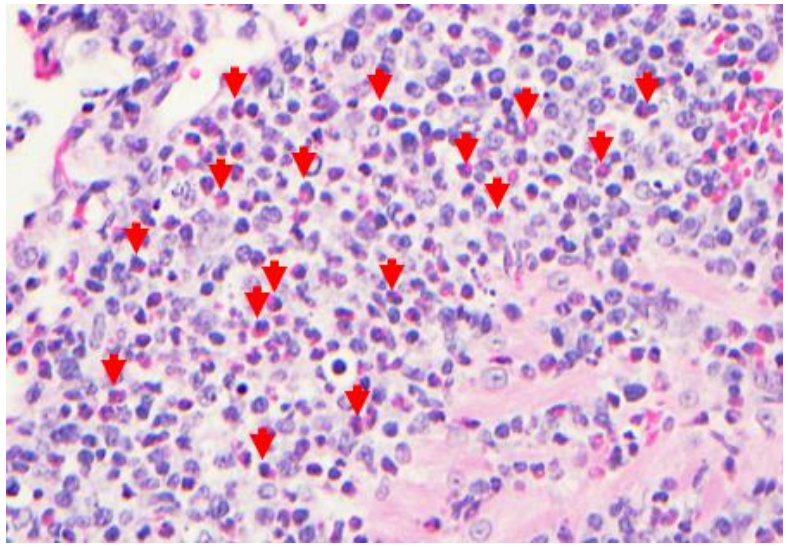

B
Neutrlizing antibody-100 (NT100) titers of differentially vaccinated mice (Log2)
0
2
4
6
8
10
12
LOD
Group: RBD/Al RBD/MF59-like RBD alone

Fig. 4

A. RBD/Al



B. RBD/MF59-like



C. RBD alone

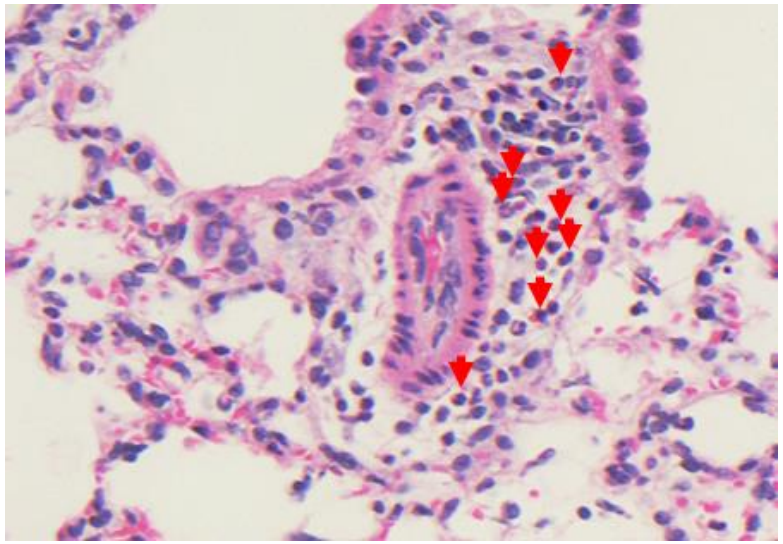
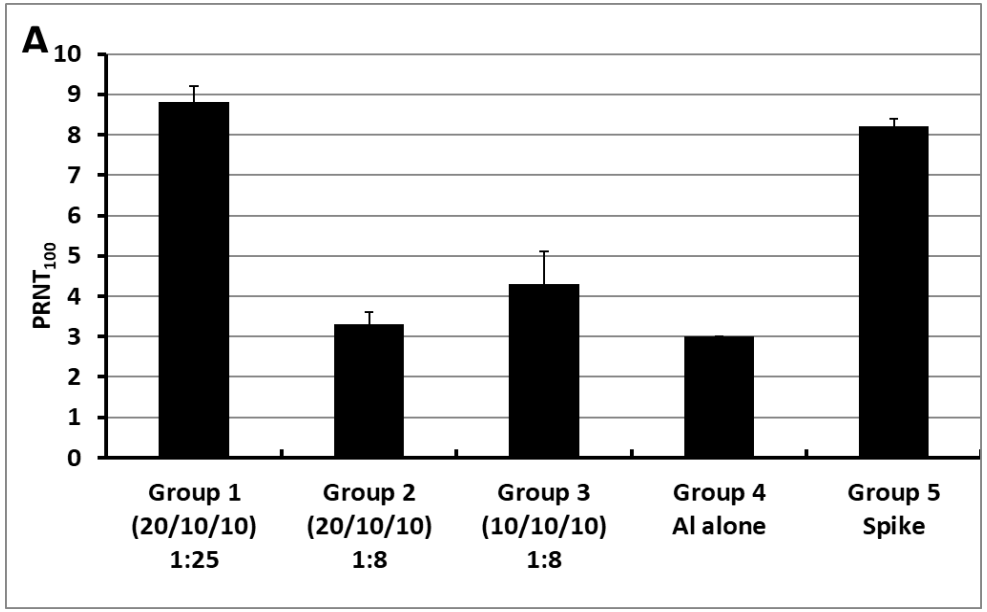


Fig. 5


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

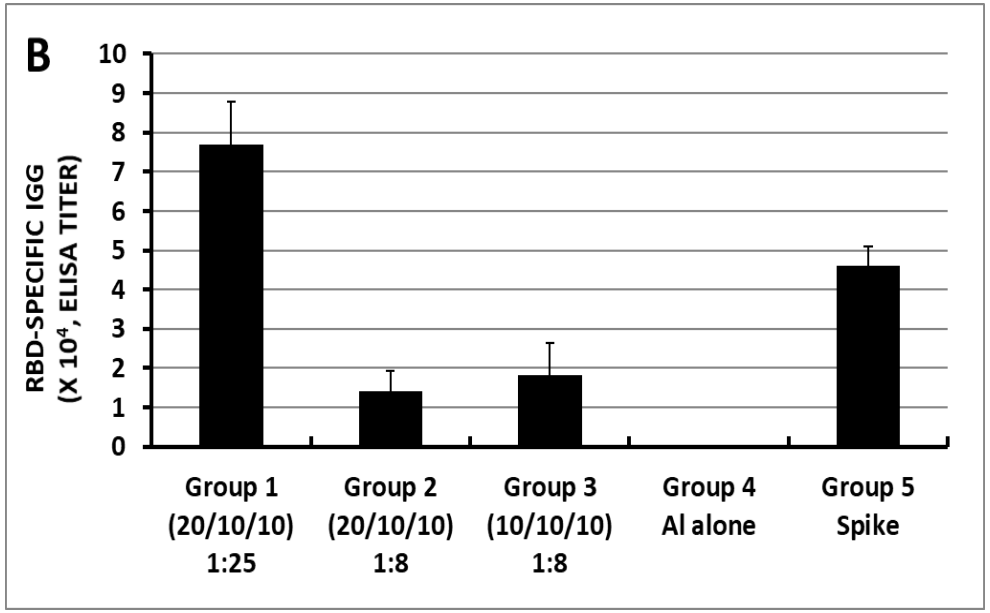
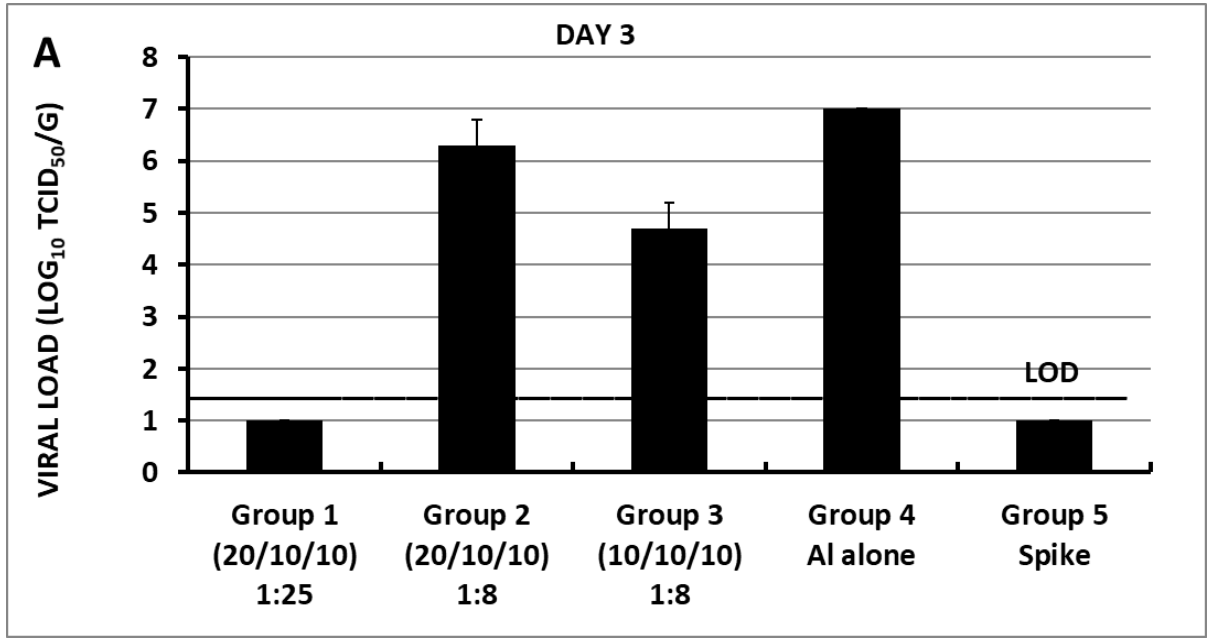

B
RBD-SPECIFIC IGG (X 10^4, ELISA TITER)
10
9
8
7
6
5
4
3
2
1
0
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

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

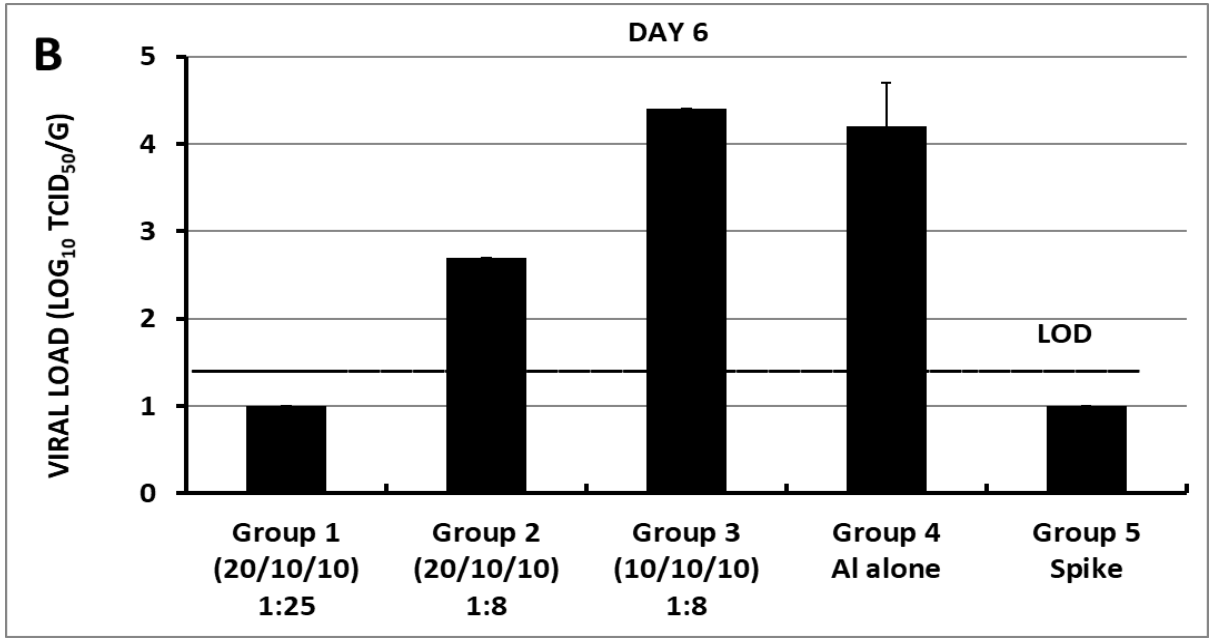
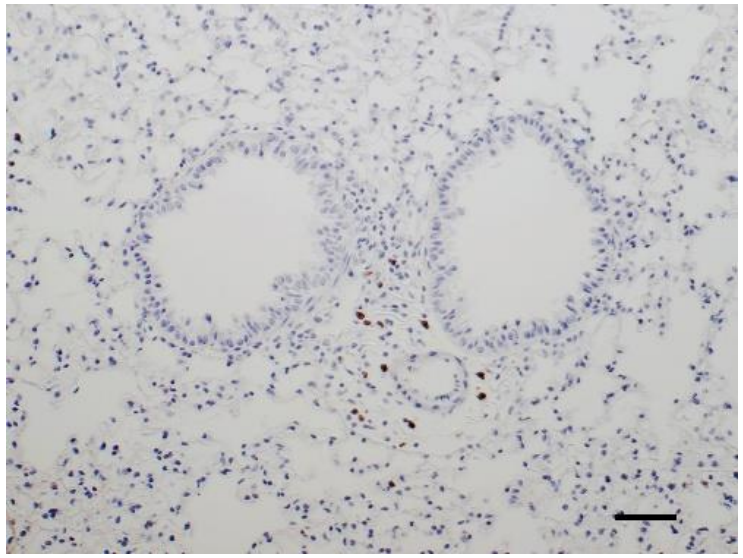
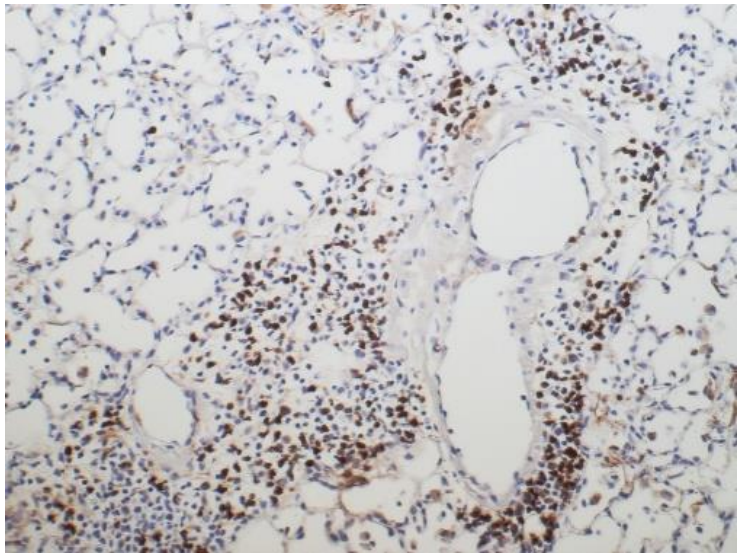

B
DAY 6
VIRAL LOAD (LOG10 TCID50/G)
5
4
3
2
1
0
LOD
Group 1 (20/10/10) 1:25
Group 2 (20/10/10) 1:8
Group 3 (10/10/10) 1:8
Group 4 Al alone
Group 5 Spike

Fig. 7

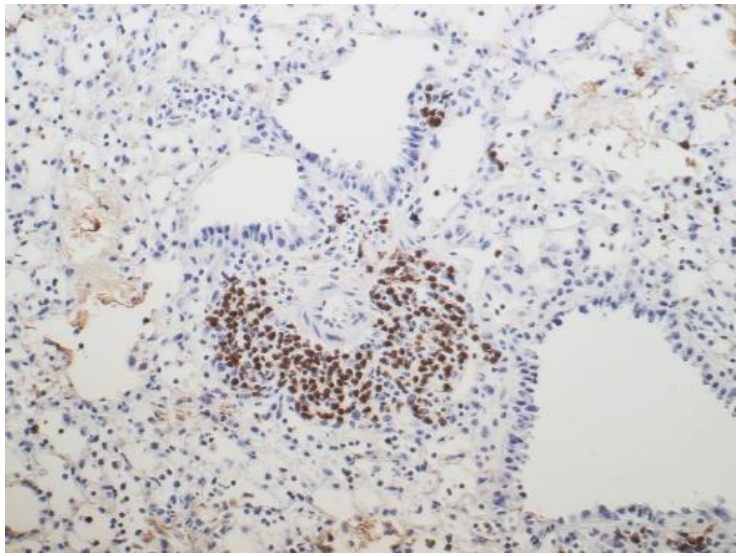
A. Group 1 RBD (20,10,10); (RBD: Alhydrogel® = 1:25)



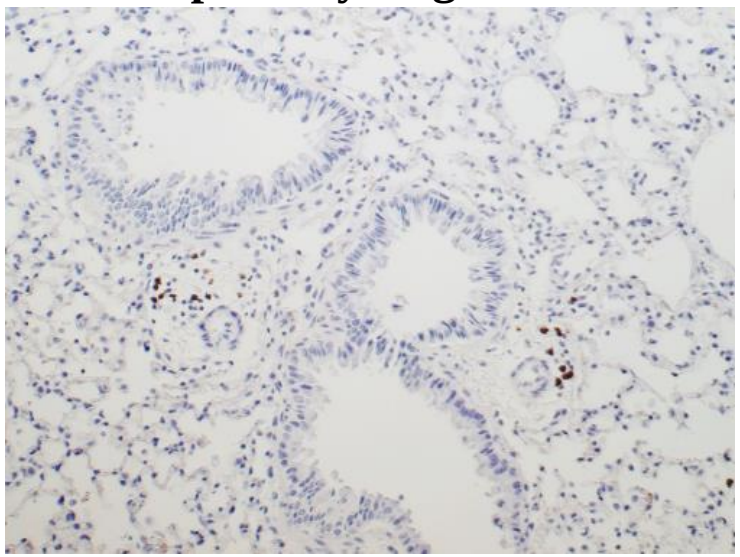
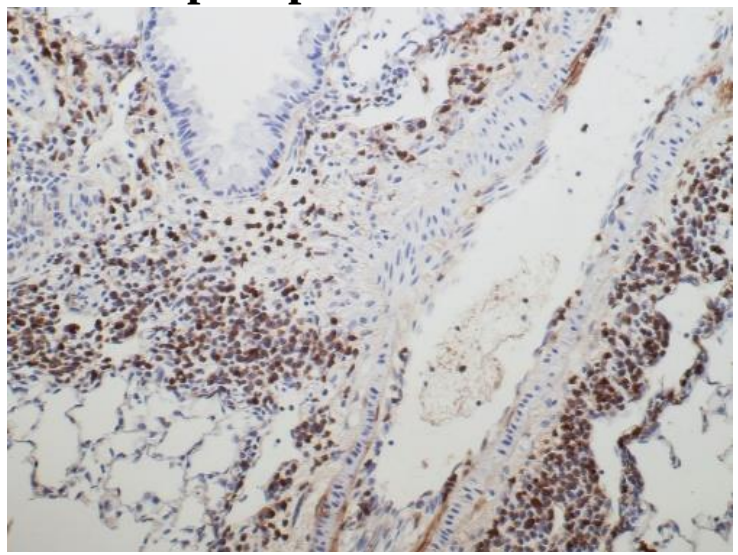
B. Group 2 RBD (20,10,10); (RBD: Alhydrogel® = 1:8)



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



D. Group 4 Alhydrogel® alone



E. Group 5 Spike