

1 **An enveloped virus-like particle vaccine expressing a stabilized prefusion form of the**
2 **SARS-CoV-2 spike protein elicits potent immunity after a single dose.**

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20 **Highlights**

21 ● VBI-2902a is a VLP-based vaccine candidate against SARS-CoV-2
22 ● VBI-2902a contains VLPs pseudotyped with a modified prefusion SARS-CoV-2 S in Alum.
23 ● VBI-2902a induces robust neutralization antibody response against SARS-CoV-2 S
24 ● VBI-2902a protects hamsters from SARS-CoV-2 induced lung inflammation
25 ● A single dose of VBI-2902a provides protective benefit in hamsters

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28

29 **Abstract**

30 Development of efficacious single dose vaccines would substantially aid efforts to stop the
31 uncontrolled spread of the COVID-19 pandemic. We evaluated enveloped virus-like particles
32 (eVLPs) expressing various forms of the SARS-CoV-2 spike protein and several adjuvants in an
33 effort to identify a COVID-19 vaccine candidate efficacious after a single dose. The eVLPs
34 expressing a modified prefusion form of SARS-CoV-2 spike protein were selected as they induced
35 the highest antibody binding titers and neutralizing activity after a single injection in mice.
36 Formulation of SARS-CoV-2 S eVLPs with aluminum phosphate resulted in balanced induction of
37 IgG2 and IgG1 isotypes and antibody binding and neutralization titers were undiminished for more
38 than 3 months after a single immunization. A single dose of this candidate, VBI-2902a (prefusion S
39 eVLPs formulated with aluminum phosphate), protected Syrian golden hamsters from challenge
40 with SARS-CoV-2 and supports the on-going clinical evaluation of VBI-2902a as a potential single
41 dose vaccine against COVID-19.

42

43 **Keywords**

44 SARS-COV-2; Vaccine; Virus-like-particles; Immunogenicity; Neutralizing antibodies

45

46 **Abbreviations**

47 eVLP, enveloped virus-like particules; CoV, coronavirus; RBD, receptor binding domain; TMCTD,
48 transmembrane cytoplasmic terminal domain; Ab, antibody; nAb, neutralizing antibody; MLV,
49 murine leukemia virus; ELISA, enzyme-linked-immuno-sorbent-assay; PRNT, plaque reduction
50 neutralization test; EPT, end-point titer; Alum, aluminum; ELISPOT, Enzyme Linked ImmunoSpot
51 Test; IP, IntraPeritoneal; IM, IntraMuscular; NRC, National Research Council Canada; VIDO,
52 Vaccine and Infectious Disease Organization

53

54 **1. Introduction**

55 SARS-CoV-2 has been circulating worldwide for more than a year with no significant sign of natural
56 exhaustion, in contrast to the previous SARS and MERS epidemics in 2003 and 2012 respectively,
57 which faded despite absence of a vaccine or specific antiviral treatments. In contrast to the SARS
58 epidemic, the COVID-19 pandemic is associated with increasing morbidity, mortality, and
59 mutagenic potential as more people are infected at an increasing rate [1]. The possibility of waning
60 immunity and isolated cases of re-infection after a period of convalescence have been reported
61 that have prompted questions about correlates of protection and the efficacy of natural immunity
62 [2-4]. Unprecedented efforts and measures have been undertaken to rapidly provide prophylactic
63 vaccines that could decrease the rate of infection and prevent severe health complications.

64 The SARS-CoV-2 spike (S) protein was identified as a major target for neutralizing antibodies
65 (nAb) due to its crucial role in mediating virus entry and its homology to S proteins from SARS,
66 MERS and other coronaviruses (CoV) for which nAb had similarly been demonstrated [5,6]. CoV S
67 proteins resemble typical of class I viral proteins. They are constituted of 2 functional subunits, S1,
68 containing the receptor binding domain (RBD) and S2, containing the fusion entry domain. Binding
69 of the RBD to the host cell receptor induces conformational changes resulting in activation of the
70 protease cleavage site upstream of the fusion domain followed by release and activation of the S2
71 fusogenic domain [7]. Unlike SARS-CoV and other CoV from the same clade, SARS-CoV-2 S
72 contains a furin cleavage site located at the boundary of S1 and S2 [6,8](Walls, Cell 2020:
73 Coutard, Antiviral Res. 2020) enabling rapid processing of the S protein during biosynthesis in
74 host cells.

75 The CoV S proteins are expressed at the viral surface as metastable prefusion trimers that
76 undergo conformational changes [6-7]. Studies of class I viral fusion proteins resulted in the design
77 of stabilized prefusion forms resistant to protease cleavage that could increase expression yield
78 and elicit potent neutralization responses in mice [9-10]. Wrapp et al. [11] described a similar
79 strategy whereby 2 consecutive prolines in the S2 subdomain between heptad repeat 1 and the
80 central helix are substituted with the addition of a C-terminus foldon trimerization domain. The
81 result was a SARS-CoV-2 stabilized S-2P antigen. Vaccine candidates containing SARS-CoV-2 S-

82 2P have demonstrated potent induction of nAb responses in laboratory animals [12-13] and
83 humans [14-15].

84 Virus-like particles (VLPs) are attractive vaccine candidates to generate nAb responses.
85 Structurally, they resemble the wild-type virus from which they are derived, but are much safer
86 because they lack genetic material and therefore the ability to replicate [16]. VLPs enable
87 repeating, array-like presentation of antigens which is a preferred means of activating B cells and
88 eliciting high affinity antibodies [17]. Indeed, VLP expression of a B cell antigen improved
89 neutralizing titers over 10-fold relative to immunization with the same amount of recombinant
90 protein [18]. Accordingly, the use of VLPs as a vaccine modality may expand higher affinity B cell
91 repertoires relative to recombinant protein or DNA/mRNA-based modalities.

92 In the present study murine leukemia virus (MLV)-based enveloped virus-like particles (eVLPs)
93 [18,19] were used to produce vaccine candidates expressing various forms of SARS-CoV-2 S. We
94 demonstrate that a modified prefusion form of S containing the ectodomain of SARS-CoV-2 S
95 fused with the transmembrane cytoplasmic terminal domain of VSV-G enabled high yields and
96 density of S expression on MLV-Gag eVLPs and induced robust nAb responses exceeding those
97 observed with SARS-CoV-2 convalescent sera after a single dose when adjuvanted with Alum
98 phosphate (VBI-2902a). This candidate, VBI-2902a, was safe and highly efficacious in a hamster
99 challenge model and offers the potential for use as a single dose vaccine. More generally, these
100 data demonstrate the high potency of antigen expression by eVLPs.

101

102 **2. Materials and Methods**

103

104 **2.1. COVID-19 human sera**

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106 Plasma samples were purchased from Biomex GmbH (Heidelberg, Germany). Samples were
107 collected under consent at donation centers in Heidelberg or Munich, from 30 individual who
108 recovered from moderate SARS-CoV-2 infection with no need for hospitalization or heavy
109 treatment. Subjects were aged 26 to 61 years old. Sera were collected at 26 to 72 days post time

110 of infection. One 61 years old woman was asymptomatic but all others experienced multiples
111 symptoms including fever, headache, anosmia, coughing, difficulty to breath, tiredness and muscle
112 pain.

113

114 *2.2. Plasmids, eVLPs production and adjuvant formulation*

115

116 All sequences coding for the full length and modified S proteins from SARS-CoV-2 were codon
117 optimized prior to synthesis and subcloned into a proprietary modified phCMV plasmid at Genscript
118 (Piscataway, NJ). The proprietary HEK293SF-3F6 GMP compliant cells were provided by the
119 National Research Council (NRC, Montreal, Canada) and grown in serum-free chemically defined
120 medium [20]. eVLPs were produced using transient polyethylenimine transfection in 293SF-3F6 by
121 co-transfection of one plasmid coding for the spike protein with a phCMV plasmid encoding
122 minimal cDNA sequence of murine leukemia virus (MLV) Gag corresponding to the full length Gag
123 deprived of its C-terminal Polymerase sequence as described elsewhere [18]. Control called
124 “empty” eVLPs or Gag eVLPs were produced by exclusive transfection of the Gag plasmid. Cell
125 culture harvests containing eVLPs were processed using a proprietary purification steps that
126 consists of clarification, tangential flow filtration, benzonase® treatment, diafiltration and
127 ultracentrifugation using sucrose cushion. The final product was sterile filtered using 0.2 µm
128 membrane prior to preparation of vaccine. Depending on the different pre-clinical mouse studies,
129 SARS-CoV-2 eVLPs vaccines were formulated with different adjuvant system including Alum
130 (Adjuphos®), AS03, MF59, and AS04. Adjuvants AS03, AS04 and its VBI modified version,
131 AddavaxTM (MF59), 2% AdjuPhos® (aluminium phosphate referred as Alum) were purchased from
132 Invivogen.

133

134 *2.3. Western blot analysis of eVLPs content*

135

136 The expression of SARS-CoV-2 S protein in eVLP preparations was analyzed by western blotting
137 as described previously [18] using rabbit polyclonal Ab (pAb) anti-spike protein of SARS-CoV-2

143 (Immune Technology Corp) followed by detection with goat anti-rabbit IgG-Fc horseradish
144 peroxidase-conjugated (Bethyl). Alternatively, human sera from COVID-19 convalescent subjects
145 was used as primary antibody followed by detection with goat anti-human IgG heavy and light
146 chain HRP-conjugated (Bethyl). Precision Protein Streptactin HRP conjugate (Bio-Rad) was used
147 as molecular weight ladder standard. Recombinant SARS-CoV-2 S(S1+S2) unmodified protein
148 (Mybiosource) or SARS-CoV-2 stabilized prefusion S protein (National Research Council of
149 Canada - NRC) were used as controls.

150

151 *2.4. Mouse immunization study*

152

153 Six- to 8-week-old female C57BL/6 mice were purchased from Jackson Laboratory (ME, USA) .
154 The animals were allowed to acclimatize for a period of at least 7 days before any procedures were
155 performed. The animal studies were conducted under ethics protocols approved by the National
156 Research Council of Canada Animal Care Committee. The animals were maintained in a controlled
157 environment in accordance with the “Guide for the Care and Use of Laboratory Animals” at the
158 NRC Animal Research facility (Institute for Biological Sciences, Ottawa, Canada). Mice were
159 randomly assigned to experimental groups and received intraperitoneal (IP) injections with 0.5 mL
160 of different adjuvanted SARS-CoV-2 immunogens. Blood was collected on day -1 (pretreatment)
161 and day 14 after each injection. All mice from each group were sacrificed 14 days after the last
162 immunization for humoral immunity assessment and or 6 days after the second immunity, where
163 spleens were collected for cellular immunity assessment.

164

165 *2.5. Hamster challenge study*

166

167 Syrian golden hamsters (males, 5-6 weeks old) were purchased from Charles River Laboratories
168 (Saint-Constant, Quebec, Canada). The study was conducted under approval of the CCAC
169 committee at the Vaccine and Infectious Disease Organization (VIDO) International Vaccine Centre
170 (Saskatchewan, Canada). Animals were randomly assigned to each experimental groups (A, B)

171 (n=12/group) in two independent experiments (Regimen II and Regimen I). Groups A placebo
172 received 0.9%-saline buffer, Groups B received VBI-2902a. Each dose of VBI-2902a contained
173 1 μ g of SPG and 125 μ g of Alum. Injection was performed by intramuscular (IM) route at one side of
174 the thighs in a 100 μ L volume. The schedule for immunization, challenge and sample collection
175 was depicted on Fig. 6a. All animals were challenged intranasally via both nares with 50 μ L/nare
176 containing 1 \times 10⁵ TCID50 of SARS-CoV-2/Canada/ON/VIDO-01/2020/Vero'76 (Seq. available at
177 GISAID EPI_ISL_413015) strain per animal. Body weights and body temperature were measured
178 at immunization for 3 days and daily from the challenge day. General health conditions were
179 observed daily through the entire study period. Blood samples and nasal washes were collected as
180 indicated on Fig. 6a. Half of the animals (6/group) were euthanized at 3 days post-infection (dpi),
181 and the remaining animals were euthanized at 14 dpi. The challenge experiments were performed
182 in the animal biosafety level 3 (ABSL3) laboratory at VIDO (Saskatchewan, Canada).

183

184 *2.6. Antibody binding titers*

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186 Anti-SARS-CoV-2 specific IgG binding titers in mouse sera were measured by standard ELISA
187 procedure described elsewhere [18], using recombinant SARS-CoV-2 S (S1+S2) protein
188 (Sinobiological). For total IgG binding titers, detection was performed using a goat anti-mouse IgG-
189 Fc HRP (Bethyl) for mouse serum, or goat anti-human IgG heavy and light chain HRP-conjugated
190 (Bethyl) for human serum. HRP-conjugated Goat anti-mouse IgG1 and HRP-conjugated goat anti-
191 mouse IgG2b HRP (Bethyl) were used for the detection of isotype subtype. Determination of Ab
192 binding titers to the RBD was performed using SARS-CoV-2 RDB recombinant protein
193 (Sinobiological). Detection was completed by adding 3,3',5,5'-tetramethylbenzidine (TMB)
194 substrate solution, and the reaction stopped by adding liquid stop solution for TMB substrate.
195 Absorbance was read at 450 nm in an ELISA microwell plate reader. Data fitting and analysis were
196 performed with SoftMaxPro 5, using a four-parameter fitting algorithm.

197 Ab binding titers in hamster sera were determined with ELISA method. Plates were coated with
198 spike S1+S2 Ag (Sinobiological). The coating concentration was 0.1 ug/mL. Plates were blocked

199 with 5% non-fat skim milk powder in PBS containing 0.05% Tween 20. Fourfold dilutions of serum
200 were used. Goat anti-Hamster IgG HRP from ThermoFisher (PA1-29626) was used as the
201 secondary antibody at 1:7000. Plates were developed with OPD peroxidase substrate (0.5 mg/ml)
202 (Thermo Scientific Pierce). The reaction was stopped with 2.5 M sulfuric acid and absorbance was
203 measured at 490 nm. Throughout the assay, plates were washed with PBS containing 0.05%
204 Tween 20. The assay was performed in duplicate. The titres were reported as the end point of the
205 dilutions.

206

207 *2.7. Virus neutralization assays*

208

209 Neutralizing activity in mouse serum samples was measured by standard plaque reduction
210 neutralization test (PRNT) on Vero cells at the NRC (Ottawa, Canada) using 100 PFU of SARS-
211 CoV-2/Canada/ON/VIDO-01/2020. Results were represented as PRNT90, PRNT80, or PRNT50
212 end point titer, corresponding to the lowest dilution inhibiting respectively 90% or 80% or 50% of
213 plaque formation in Vero cell culture.

214 Virus neutralization assays against the challenge SARS-CoV-2 virus were performed at VIDO,
215 Saskatchewan on the hamster serum samples collected at pre-challenge and at the end day; 3
216 days post-challenge or 14 days post-challenge. The study was conducted using the cell line Vero
217 E6. The serum samples were heat-inactivated for 30 min at 56°C. The serum samples were initially
218 diluted 1:10 and then serially diluted (2-fold serial dilutions). The virus was diluted in medium for a
219 final concentration of 3×10^2 TCID50/mL. Initially 60 μ L of the virus solution was mixed with 60 μ L
220 serially diluted serum samples. The mixture was incubated for 1hr at 37°C, with 5% CO₂. The pre-
221 incubated virus-serum mixtures (100 μ L/well) were transferred to the wells of the 96-well flat-
222 bottom plates containing 90% confluent pre-seeded VeroE6 cells. The plates were incubated at
223 37°C, with 5% CO₂ for 5 days. The plates were observed using a microscope on day 1 post-
224 infection for contamination and on days 3 and 5 post-infection for cytopathic effect (CPE). The
225 serum dilution factor for the last well with no CPE at 5 dpi was defined as the serum neutralization
226 titer. The initial serum dilution factor was 1:20.

227

228 *2.8. RNA extraction and purification*

229

230 RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen). Briefly, 140 μ L of hamster nasal
231 wash was added into 560 μ L viral lysis buffer (Buffer AVL). The mixture was incubated at room
232 temperature for 10 min. After brief centrifugation, the solution was transferred to a fresh tube
233 containing 600 μ L of 100% ethanol, and the tube was incubated at room temperature for 10 min.
234 RNA was then purified using QiaAmp Viral RNA Mini Kit and eluted with 60 μ L of RNase Free
235 water containing 0.04% sodium azide (elution buffer AVE). Extraction of RNA from lung lobes and
236 nasal turbinates was completed using approximately 100 μ g of tissue. The tissues were
237 homogenized in 600 μ L of lysis buffer (RLT Qiagen) with a sterile stainless steel bead in the
238 TissueLyserII (Qiagen) for 6 min, at 30 Hz. The solution was centrifuged at 5000 \times g for 5 min.
239 Supernatant was transferred to a fresh tube containing 600 μ L of 70% ethanol, and the tube was
240 incubated at room temperature for 10 min. Viral RNA was then purified using Qiagen Rneasy Mini
241 Kit (Cat No /ID: 74106) and eluted with 50 μ L elution buffer.

242

243 *2.9. Viral qRT-PCR reaction*

244

245 The qRT-PCR assays were performed on RNA from samples of nasal washes, lung tissues and
246 nasal turbinates using SARS-CoV-2 specific primers targeting the E gene (Fwd, ACAGGTACGT-
247 TAATAGTTAATAGCGT; Rev, ATATTGCAGCAGTACGCACACA) and labelled probe, ACACTA-
248 GCCATCCTTACTGCGCTTCG. The primers have an annealing temperature of approximately
249 60°C. Qiagen Quantifast RT-PCR Probe kits were used for qRT-PCR. The qRT-PCR results were
250 expressed in RNA copy number per reaction. This was done by producing a standard curve with
251 RNA extracted from a sample of SARS-CoV-2 which was cloned to determine exact copy number
252 of the gene of interest. The Ct values for individual samples were used with the standard curve to
253 determine the copy number in each sample. The qRT-PCR reactions were performed using the
254 OneStepPlus (Applied Biosystems) machine. The program was set at: Reverse transcription (RT)

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255 10 min at 50°C; Inactivation 5 min at 95°C; and then 40 cycles of denaturation for 10 sec at 95°C
256 and annealing/extension for 30 sec at 60°C.

257

258 *2.10. IFN-γ Ex-vivo ELISPOT*

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260 IFN-γ ELISPOT analyses to measure Th1 T cell responses were performed as follows. One day
261 before the spleens were removed, ELISpot plates (Millipore) were coated with IFN-γ capture
262 antibody at a concentration of 15 µg/mL (Mabtech). The following day, mice were sacrificed and
263 spleens were removed. Spleens from individual mice were processed to produce single cell
264 suspensions. Erythrocytes were lysed using a commercially available RBC lysis buffer
265 (BioLegend). Fifty microliters containing 2×10^6 splenocytes were then to each well of a pre-blocked
266 ELISPOT plate. Then, fifty microliters of stimulant pepmixes (JPT peptides) resuspended in
267 RPMI+10%FBS (R10) with recombinant mouse IL-2 (rmIL-2) (R&D Systems) were added to each
268 well. The final concentration of each peptide in the assay was 1µg/mL/peptide, and the final
269 concentration of rmIL-2 was 0.1 ng/mL. R10 alone was used as a negative control and
270 PMA+Ionomycin as a positive control. The ELISPOT plates were then placed into a humid 37°C
271 with 5% CO₂ incubator for 40-48 hours. After incubation, the plates were washed and IFN-γ
272 capture antibody was added, followed by streptomycin horseradish peroxidase (strep-HRP). The
273 plates were developed with commercially available 3-Amino-9-ethylcarbazole (AEC) substrate
274 (Sigma-Aldrich). The observed spots were counted using an ELISPOT plate reader by ZellNet and
275 the final data was reported as spot forming cells (SFC) per one million splenocytes.

276

277 *2.11. Histopathology*

278

279 At necropsy the left lung of hamsters was perfused with neutral-buffered formalin immediately after
280 collection. Tissues were fixed in neutral-buffered formalin for a week, then placed into fresh
281 neutral-buffered formalin before being transferred from containment level 3 to containment level 2

282 laboratory. Tissues were embedded, sectioned and stained with hematoxylin and eosin. Slides were
283 examined by a board-certified pathologist.

284

285 **2.12. Statistics**

286

287 All statistical analyses were performed using GraphPad Prism 9 software (La Jolla, CA). Unless
288 indicated, multiple comparison was done with Kruskall-Wallis test. The data were considered
289 significant if $p < 0.05$. Geometric means with standard deviation are represented on graphs. No
290 samples or animals were excluded from the analysis. Randomization was performed for the animal
291 studies.

292

293 **3. Results**

294

295 **3.1. Impact of SARS-CoV-2 S antigen design on expression and yield**

296

297 Four constructs were designed based on the spike protein sequence of the SARS-CoV-2 Wuhan-
298 Hu1 isolate and subcloned into expression plasmids for the production of eVLPs as described in
299 Methods (Fig. 1a). To obtain a stabilized prefusion form of S (SP), the furin cleavage site of S,
300 RRAR, was inhibited by mutation of the 3 arginines into a glycine and 2 serine (GSAS) and 2
301 proline substitutions were introduced at successive residues K986 and V987. Our previous work
302 has demonstrated that the swap of the transmembrane cytoplasmic terminal domain (TMCTD) of
303 CMV gB resulted in enhanced yields and immunogenicity of the gB glycoprotein presented on
304 eVLPs [18]. Based on this data, two additional constructs, Native-VSVg (SG) and Stabilized
305 Prefusion-VSVg (SPG) were designed by swapping the TMCTD of S with that of VSV-G.
306 Western blot analysis of eVLPs using a polyclonal Ab directed against the SARS-CoV-2 S receptor
307 binding domain confirmed the processing of SARS-CoV-2 S during biosynthesis in HEK-293 cells
308 as expected by the presence of the furin cleavage site in S1/S2 [6]

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309 (Fig. 1b, lane 2-3). Expression of S was slightly improved by the VSV-G swap in SG, and more
310 dramatically enhanced by the inhibition of the cleavage sites in SP and SPG (Fig. 1b, lane 4-5).
311 Overexpression of S in the prefusion forms showed a major band at 180 kDa, the size commonly
312 described for uncleaved S180 kDa and an additional band around 150 kDa. The additional band
313 around 150 kDa is reproducibly seen upon overexpression of uncleaved S, and most likely
314 represents the S protein deprived of N-Glycosylation [21] that would occur because of overloading
315 of the host cell machinery. Similar results were obtained after blotting with human convalescent
316 sera.

317 Quantitative analysis of protein content in eVLP preparations showed that for a similar number of
318 particles and comparable amounts of Gag protein, the amount of SARS-CoV-2 S protein was
319 increased substantially with replacement of the TMCTD and by use of the stabilized prefusion
320 construct, suggesting that the density of the S protein was enhanced using the VSV-G constructs
321 (Table 1). The best yield was reproducibly obtained when producing the eVLPs expressing the
322 prefusion-VSV-G form of S, with up to a 40-fold increase relative to eVLPs expressing native S.

323
324 *3.2. Impact of SARS-CoV-2 S antigen design on neutralizing antibody responses*
325

326 Comparison to convalescent serum is commonly used as a benchmark to help evaluate
327 immunogenicity and potential efficacy of Covid-19 candidate vaccines. However, a wide spectrum
328 of Ab responses can be observed in recovering patients, ranging from barely detectable to very
329 high levels, likely influenced by time since infection and severity of disease. To enable comparison
330 across experiments, we obtained a cohort of 20 sera from COVID-19 confirmed convalescent
331 patients with moderate COVID-19 symptoms who all recovered without specific treatment
332 intervention or hospitalization. The cohort was separated into two groups of 10 samples according
333 to high or low levels of Ab binding activity to recombinant SARS-CoV-2 S (Fig. 2a). Sera from each
334 group were then pooled and tested for neutralizing activity (Fig. 2b). As expected, the pool of
335 human sera showing higher levels of IgG titers against SARS-CoV-2 S had the highest neutralizing
336 activity, which was consistent with previous observations [22]. To provide a robust benchmark with

13

337 which to assess the immunogenicity of the vaccine candidates, only the high titer pooled sera was
338 used to assess vaccine-induced responses in animals.

339 Humoral responses of the various types of SARS-CoV-2 eVLPs were evaluated in C57BL/6 mice
340 that received 2 intraperitoneal injections at 3 week intervals (Fig. 3). The first injection of
341 unmodified S presented on eVLPs induced levels of anti-SARS-CoV-2 S Ab binding titers similar to
342 those in mice that received a recombinant trimerized prefusion S protein, but they were not
343 associated with significant (90% or greater) neutralization activity as measured in a plaque
344 reduction neutralization test (PRNT) (Fig. 3a-b). In contrast, a significant nAb response was
345 induced by a single injection of eVLPs expressing prefusion SP or SPG, with PRNT90 end-point
346 titers (EPTs) of 80 and 160 respectively. These values were higher than those observed with the
347 human convalescent control pool (PRNT90 EPT of 50). All nAb responses were greatly enhanced
348 by the second injection and reflected the responses that were observed prior to the boosting dose.
349 Notably, all forms of SARS-CoV-2 S presented on eVLPs induced higher antibody titers than
350 recombinant prefusion S protein, both in the levels of total IgG and neutralization activity, after one
351 or two injections.

352 Individual mice sera obtained 14 days after the second injection of eVLPs were evaluated for the
353 specificity of the Ab responses against the whole S1+S2 protein or the RBD (Fig. 3c-d). All
354 immunized mice that received eVLPs showed robust anti-SARS-CoV-2 Ab responses either
355 against a full length S1+S2 protein (Fig. 3c) or against the RBD protein (Fig. 3d). A more
356 homogenous response was observed in mice that received the SPG eVLPs, with all Ab EPTs
357 above 400,000 against S (5.6 Log 10), and above 650,000 against RBD (5.8 Log10).

358

359 *3.3 Influence of adjuvants on antibody and T cell responses*

360

361 A Th2-type response has been suggested to contribute to the “cytokine storm” associated with
362 vaccine-induced severe lung pathologies [23,24]. In light of these results, we tested a variety of
363 adjuvants that might enhance neutralizing Ab production while also promoting a balanced Th1/Th2
364 response. For this purpose, we compared formulation of eVLPs with Alum against a panel of

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365 adjuvants including MF59 and the adjuvant systems AS03 and AS04. We used SARS-CoV-2 native
366 S eVLPs as they were less immunogenic than eVLPs expressing the prefusion form of the S
367 protein and might better enable differences in the adjuvants_to be observed. The various
368 adjuvanted formulation of S eVLPs were compared to recombinant stabilized prefusion S protein
369 (r-SP) formulated in Alum adjuvant, which was expected to induce a Th2-biased response [25].
370 Mice received two IP injections and Ab and T cell responses were measured 14 days after the
371 second injection (Fig. 4). MF59 enhanced IFN- γ T cell responses compared to Alum (Fig. 4a) but
372 induced similar Ab responses (Fig. 4b, c) and a comparable, balanced IgG2/IgG1 ratio (Fig. 4d).
373 The AS03 and AS04 adjuvants also skewed responses towards a Th1-type T cell response. Most
374 remarkably, while r-SP in Alum preferentially induced IgG1 Ab representative of a Th2 response, S-
375 eVLPs induced balanced production of IgG1 and IgG2b indicating a balanced Th1/Th2 response
376 (Fig. 4d).

377

378 3.4. *Immunogenicity in mice of vaccine candidate VBI-2902a*

379

380 Based on the results described above, we chose to evaluate the immunogenicity and potential
381 efficacy of eVLPs expressing SPG protein formulated with Alum, named VBI-2902a, after one or
382 two injections 21 days apart. Fourteen days after a single injection, sera from mouse immunized
383 with VBI-2902a contained total anti-Spike IgG EPTs reaching geometric means of (4.8 Log 10)
384 54,891 that were associated with neutralizing PRNT90 titers of 365 (2.6 Log10). A second injection
385 boosted Ab binding titers to 228,374 (5.4 Log10) with nAb titers of 1,079 (3.0 Log10) (Fig. 5a-b).
386 Levels of nAb response were higher than those observed in sera from convalescent patients. Abs
387 were preferentially directed against the RBD and S1 with only low binding to S2 (Fig. 5c).
388 Mouse splenocytes collected 2 weeks after each immunization were stimulated *ex vivo* using two
389 different peptide pools preferentially covering the S1 domain (pepmix 1) or the S2 domain (pepmix
390 2) respectively. Numbers of IFN- γ spot forming cells (Fig. 5d) suggested preferential T cell
391 responses against the S1 domain of the spike protein rather than against the S2 domain. No major
392 increases in T cell responses were observed after the second injection of VBI-2902a.

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393 Additionally, we observed that a single dose of VBI-2902a induced a sustained Ab response for at
394 least 15 weeks without any drop in neutralization titers (Fig. 5e).

395

396 *3.5. Protective efficacy of VBI-2902a in Syrian Golden Hamsters*

397

398 The protective efficacy of VBI-2902a was examined in Syrian Gold hamsters. SARS-CoV-2
399 infection in Syrian Gold hamsters resembles features found in humans with moderate COVID-19
400 and is characterized by a rapid weight loss starting 2 days post infection (dpi) [26,27]. Two
401 immunization regimens were compared. Regimen II consisted of two IM injections of VBI-2902a or
402 saline at 3 weeks interval whereas Regimen I consisted of a single dose injection of VBI-2902a or
403 saline (Fig. 6a). Three weeks after the last injection (day 42 in Regimen II and day 21 in Regimen
404 I), all animals were inoculated intranasally with 1×10^5 TCID50 of SARS-CoV-2 per animal and
405 monitored daily for weight change, general health and behavior.

406 After a single injection of VBI-2902a the levels of anti-S IgG rapidly increased in the serum of
407 immunized animals with EPTs reaching $1-2\times10^3$ (Fig. 6b). The second injection enhanced these
408 levels approximately 10-fold to reach EPTs of $2-3\times10^4$ at day 35, which translated into robust
409 neutralization titers of over 10^3 EPT as measured by PRNT90 (Fig. 6c). In the single dose
410 regimen, the neutralization activity (GeoMean 69), was increased 250-fold to 1725 within 3 days
411 after exposure to the virus.

412 Animals in all groups lost 2-4% of body weight 2 days post infection (2dpi). Animals in the saline
413 control groups continued to lost weight until an average 15% loss at 7dpi, before gradually
414 regaining weight (Fig. 7a-b). In marked contrast, none of the hamsters immunized with two doses
415 of VBI-2902a lost any further weight after 2dpi, regaining normal weight by 7dpi, demonstrating
416 robust protection against SARS-CoV-2 disease. In the single dose regimen, the majority of the
417 animals regained body weight after 3dpi instead of 2dpi, suggesting slightly delayed but significant
418 protection against disease.

419 At 3dpi, hamsters vaccinated with either one or two doses of VBI-2902a had greatly decreased
420 viral RNA copy numbers in lungs (Fig. 8a). Two doses of VBI-2902a resulted in a 5 Log decrease

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421 in viral load in the cranial lobe and a 4 Log decrease in the caudal lobe relative to non-immunized
422 animals; a single dose of vaccine induced a 2 Log decrease in the cranial lobe and a 4 Log
423 decrease in the caudal lobe. The viral load values observed in lungs were inversely correlated with
424 the neutralization measured as PRNT90 (Fig. c-d). More viral RNA was found in nasal turbinates,
425 which may have included residual viral inoculum as suggested previously [27,28]. Data from prior
426 studies also suggested an extended persistence of the virus in nasal turbinates while barely
427 detectable in the lung [26]. Both vaccine regimens protected against the development of lung
428 pathology as indicated by reductions of the lung to body weight ratio (Fig. 9a-b) and histological
429 analysis of the lungs (Fig. 9c-d).

430

431 **4. Discussion**

432

433 The unprecedented urgency for a safe COVID-19 vaccine that can confer protection as quickly as
434 possible with as few doses as possible is evident as regulatory agencies and vaccine
435 manufacturers have discussed the risks and benefits of delaying planned second doses of currently
436 available COVID-19 vaccines to enable immunization of a greater number of individuals as quickly
437 as possible [29,30]. We have previously demonstrated that expression of proteins on the surface
438 of eVLPs dramatically enriches for neutralizing antibody, the presumed correlate of protection
439 against SARS-CoV-2, relative to recombinant proteins [18]. Accordingly, we evaluated both
440 conformations of the SARS-CoV-2 S protein as well as a variety of adjuvants in an effort to identify
441 a COVID-19 vaccine candidate with the potential to confer protection after a single dose.

442 The eVLPs particles were pseudotyped with SARS-CoV-2 unmodified S protein but expressed low
443 amounts of S that were not suitable for upscaled production. We therefore designed a modified
444 prefusion form of S that resulted in both dramatic increases in yields and enhancement of the nAb
445 response compared to native S. SPG eVLPs induced high titers of RBD Ab binding titers
446 associated with robust neutralizing responses in mice at levels that were much higher than those
447 observed with a recombinant prefusion S protein. Indeed, 14 days after a single dose of SPG
448 eVLPs formulated with aluminum phosphate nAb titers exceeded those associated with high titer

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449 COVID-19 convalescent sera, persisted and were undiminished for at least 3 months. The potency
450 observed after a single dose of VBI-2902a appears superior to what has been observed after 2
451 doses in the same strain of mice with an mRNA vaccine that has received Emergency Use
452 Authorization [12], further demonstrating the strong potency of this vaccine candidate. In a hamster
453 challenge model VBI-2902a demonstrated robust efficacy against clinical disease and lung
454 inflammation. While two doses showed greater efficacy, a single dose clearly conferred protective
455 benefit.

456 The value of eVLP expression of the modified SP protein is consistent with prior reports which
457 demonstrated that an anchored version of a stabilized prefusion S antigen provided optimal
458 induction of protective nAbs in Rhesus macaques [13]. Our construct differed from the previously
459 described S-2P [13, 31] by using the VSV-G transmembrane cytoplasmic domain to replace that of
460 S, instead of a C-terminal T4 fibritin trimerization domain. Based on previous experience and
461 published data [18,19] , we hypothesized that the use of VSV-G tail and expression in the
462 phospholipid membrane of eVLPs would result in natural trimerization of the spike ectodomains
463 providing optimal presentation of neutralization epitopes. The use of the VSV-G tail has been
464 shown to enhance expression and localization of viral glycoproteins at the phospholipid envelop of
465 the particles [32,33].

466 Aluminum salt adjuvants have a long history of safety and are a component of approved VLP-
467 based vaccines such as Gardasil® against HPV [34] and Engerix B® against HBV [35].
468 Nevertheless, theoretical concerns have been raised about the use of an aluminum-based
469 adjuvant with a SARS-CoV-2 vaccine and the potential for Th2-mediated enhanced lung pathology
470 [36,37]. Subsequent studies have demonstrated that non-neutralizing antibodies against structural
471 proteins were responsible for the pathology observed in preclinical models [38]. Use of eVLP
472 presentation of an optimized form of the SARS-CoV-2 S protein resulted in a highly potent and
473 focused neutralizing antibody response which avoided any evidence of disease enhancement or
474 increased lung inflammation. In a hamster challenge model VBI-2902a demonstrated efficacy and
475 ability to suppress lung inflammation. While two doses showed better potency, the single dose also
476 conferred protective benefit indicated by comparable results in terms of lung inflammation.

18

477 Moreover, compared to a clear Th2-biased profile observed in response to recombinant prefusion
478 stabilized S protein in Alum, the similar prefusion S construct induced a balanced Th1/Th2
479 response when presented by eVLPs (Fig. 4d). The balanced production of IgG2/IgG1 antibody
480 isotypes after VBI-2902a immunization was comparable with those described in response to the
481 recently emergency use authorized vaccine Ad26.COV2.S [25]. These results emphasize an
482 important difference in the quality of the antibody response when immunizing with soluble,
483 recombinant versus particulate forms of vaccine antigens.

484 The VBI-2902a vaccine candidate addresses several issues that have thus far hindered the speed
485 and extent of vaccination with currently available COVID-19 vaccines. This includes the need to
486 administer multiple doses and the need for storage, transport, and distribution of the vaccine at
487 freezing temperatures not typically required for prophylactic vaccines. VBI-2902a received
488 approval from Health Canada to initiate its ongoing Phase I/II clinical study (NCT04773665) to
489 assess its potential for one dose immunogenicity and potential efficacy.

490

491 **Acknowledgment**

492 The authors want to thank Adam Asselin, Matthew Yorke, Teresa Daoud, Lanjian (Isabel) Yang,
493 Rebecca Wang, Gillian Lampkin (VBI vaccines) for outstanding technical support; Traian Sulea
494 (NRC) for discussions on construct design, NRC Animal Resources Group and the VIDO
495 Saskatchewan team for remarkable care with animal experiments as well as Ammon Ding and
496 Echo Wu (Genescrypt) for their dedication in plasmid preparation. All the people cited above
497 contributed to the success of the study by the excellence of their work.

498

499 **Funding** VBI-2902a study was supported by Government of Canada Innovation, Science and
500 Industry (ISED) funding through the Strategic Innovation Fund (SIF).

501

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690 **Legends to Figures**

691

692 **Fig. 1: Constructs design and production of eVLPs expressing SARS-CoV-2 Spike protein.**

693 **(a)** Schematic representation of SARS-CoV-2 S plasmid constructs. TM-CTD: Transmembrane
694 cytoplasmic terminal domain. **(b)** Expression of SARS-CoV-2 S analyzed by Western-blot of
695 SARS-CoV-2 eVLPs and recombinant proteins using a rabbit polyclonal Ab (pAb, upper panel)
696 raised against SARS-CoV-2 RBD (Sinobiological) or COVID-19 convalescent human serum
697 (HuCS, bottom panel). eVLPs produced with Gag plasmid only (Gag eVLPs) and recombinant
698 SARS-CoV-2 S proteins were used as negative and positive controls respectively. r-S: recombinant
699 native S, r-SP: recombinant prefusion S protein containing a mutated furin cleavage domain
700 (RRAR → GSAS), replacement of 2 proline (KV → PP) and a trimerization domain.

701

702 **Fig. 2: Measure of anti-SARS-CoV-2 Ab in COVID-19 convalescent human plasma. (a)** Ab
703 binding titers against SARS-CoV-2 S (S1+S2). Plasma samples were grouped according to initial
704 screening by YHLO method in two groups Low and High Ab titers prior to be tested in the in-house
705 ELISA against a recombinant S(S1+S2). **(b)** Neutralizing activity was measured by PRNT90 as
706 described in Material and Methods. Results are represented as end point titers (EPT).

707

708 **Fig. 3: Immunogenicity of the various forms of SARS-CoV-2 S eVLPs in C57BL/6 mice.** Four
709 groups of 10 C57BL/6 mice received 2 injections of various forms of SARS-CoV-2 S eVLPs or
710 recombinant SP at day 0 and 21 a indicated on legend, S: native S, SG: S with VSV-G tail, SP:
711 prefusion S, SPG: prefusion S with VSV-G tail, r-SP: recombinant SP protein. Sera were collected
712 2 weeks after each injection. **(a)** Pooled sera from each group were analyzed for specific SARS-
713 CoV-2 S(S1+S2) total IgG; results are represented as EPT corresponding to the first dilution that
714 gave an OD 3-fold above background. **(b)** Pooled sera from each group were analyzed in PRNT
715 assay with a 90% threshold (PRNT90) as described in Material and Methods. A pool of human sera
716 from COVID-19 convalescent patients with moderate disease (HuCS) was used as reference. **(c-d)**
717 Individual sera were analyzed in ELISA using recombinant SARS-CoV-2 S(S1+S2) protein (c) or
718 recombinant SARS-CoV-2 RBD protein (d). P values from Kruskall-Wallis test comparing groups
719 are indicated in c and d.

720

721 **Fig. 4: Influence of various adjuvant in the SARS-CoV-2 S eVLPs-mediated Ab and T cell**
722 **response.** At day 0 and 21, five groups of 10 C57BL/6 mice received 2 injections of S eVLPs in the
723 presence of various adjuvants as indicated in legends and described in Material and Methods.
724 Sera and splenocytes were collected 2 weeks after the second injection. **(a)** Numbers of IFN γ
725 producing cells per million splenocytes collected 2 weeks after the second injection were measured
726 by ELISpot using peptide pool covering the entire S(S1+S2) protein. **(b)** Total IgG were measured
727 in ELISA against recombinant SARS-CoV-2 S(S1+S2) protein, results are represented as EPT. **(c-**
728 **d)** Isotype usage was determined in individual sera by specific ELISA using HRP conjugate goat
729 Ab against mouse IgG1 and IgG2. **(c)** Results are expressed as the ratio of IgG2b to IgG1. Results
730 from Kruskall-Wallis comparison of groups are indicated.

731

732 **Fig. 5: Immunogenicity of VBI-2902a and VBI-2902e in C57BL/6 mice. (a-d)** Two groups of 10
733 mice were immunized twice at 3 weeks interval with VBI-2902a or VBI-2902e containing 0.2 μ g of
734 S protein. Blood was collected 2 weeks after each injection, P1d: post 1st dose, P2d: post 2nd dose.
735 **(a)** Ab binding titer against recombinant S(S1+S2) compared to human convalescent sera,
736 measured by ELISA, **(b)** neutralization end point titers measured by PRNT90, **(c)** Ab binding titers
737 against recombinant S(S1+S2), recombinant RBD or recombinant S2 measured by ELISA in sera
738 after the 2nd dose. Results from Kruskall-Wallis comparison of groups are indicated for A and B. **(d)**
739 Numbers of IFN γ producing cells per million splenocytes collected 2 weeks after each injection
740 were measured by ELISpot using Pepmix 1 or Pepmix 2 preferentially covering SARS-CoV-2 S1
741 domain or tS2 domain respectively. **(e)** Kinetic of the humoral response after single injection of
742 VBI-2902a calculated as end point titer determined in ELISA and PRNT90.

743

744 **Fig. 6: Immunogenicity of VBI-2902a or VBI-2902e in Syrian golden hamsters. (a)** Schematic
745 representation of the challenge experiments. Each challenge experiment used 2 groups of 15
746 Syrian gold hamsters. In regimen II, animals received 2 IM injections of VBI-2902a (2 μ g of S per
747 dose) or placebo saline buffer administered at 3 weeks interval. In regimen I, animals received a

28

748 single injection of VBI-2902a or Saline buffer. Blood was collected 2 weeks after each injection.
749 Three weeks after the last injection corresponding to day 42 in regimen II and day 21 in regimen I,
750 hamsters were exposed to SARS-CoV-2 at 1×10^5 TCID50 per animal via both nares. At 3 days post
751 infection (dpi), 6 animals per groups were sacrificed for viral load analysis. The remaining animals
752 were clinically evaluated daily until end of study at 14dpi. (b) Anti-SARS-CoV-2 S(S1+S2) total IgG
753 EPT measured by ELISA 2 weeks after each immunization. (c) Neutralization activity was
754 measured by PRNT90 in immunized groups; results are represented as PRNT90 EPT.

755

756 **Fig. 7: Weight change of hamsters after exposure to SARS-CoV-2.** Hamsters from experiment
757 described in fig. 6 were monitored daily for weight change. Results are represented for each
758 animal in each groups as kinetic of weight change from SARS-CoV-2 exposure to day 9 after
759 infection. (a) represents the weight change observed in the 2-dose regimen (II), (b) represent the
760 weight change observed in the single dose regimen (I).

761

762 **Fig. 8: Viral load analysis in SARS-CoV-2 infected hamsters. (a-b)** At 3dpi, qRT-PCR assays
763 were performed on RNA from samples of nasal washes, lung tissues (cranial and caudal lobes)
764 using SARS-CoV-2 specific primers. Results were expressed as copy number per gram of tissue
765 sample. (c-d) Correlation analysis of viral loads measured in lung caudal lobe and PRNT90.

766

767 **Fig. 9: Clinical evaluation of lung pathology in immunized hamsters challenged by SARS-**
768 **CoV-2 virus. (a-b)** lung to body weight ratio in hamsters at 3dpi and 14dpi. (c-d) Histopathology
769 severity analysis of hamster lungs at 3dp and 14 dpi. Scores were evaluated on a scale from 0 to 4
770 as follow: 0, no microscopic lesions; 1, slight or questionable pneumonia; 2, clearly present, but not
771 conspicuously so; 3, moderate pneumonia; 4, severe pneumonia. Statistics were performed using
772 Kruskal-Wallis non-parametric test followed by Dunn' multiple comparisons test. Adjusted p values
773 are shown.

774 **Table 1**

775
776

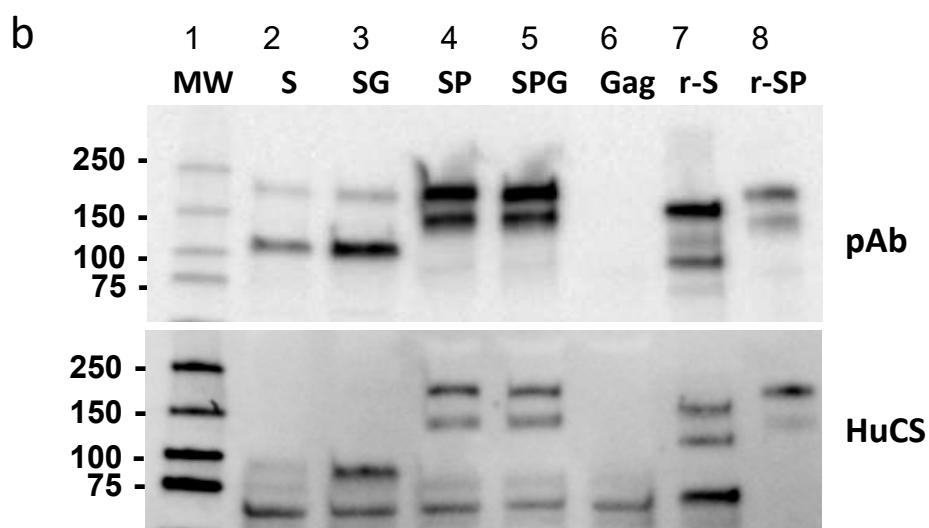
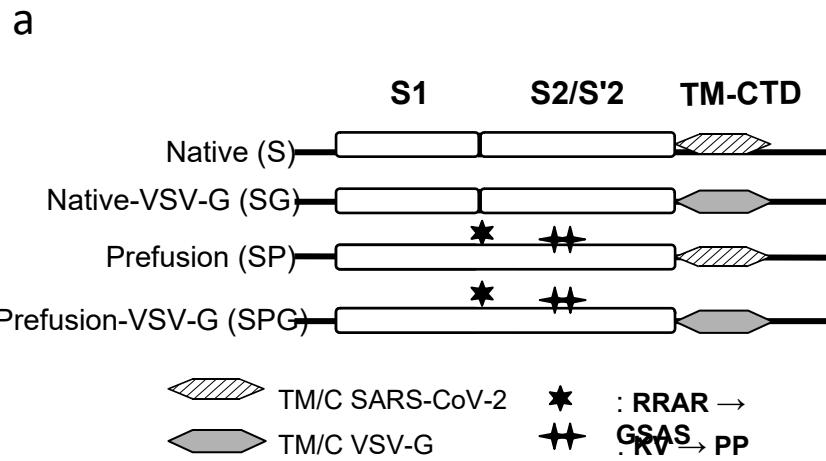
777 **Table 1: Optimisation of SARS-CoV-2 S protein yields by alteration of the sequence construct**

Spike Construct	Gag total amount (mg)	SARS-CoV-2 S total amount (mg)	S to Gag ratio (%)	Particle number /mL
Native (S)	23	0.16	0.07	4.37×10^{11}
Native-VSV-G (SG)	19	0.5	0.26	4.37×10^{11}
Prefusion (SP)	32	0.23	0.71	4.37×10^{11}
Prefusion-VSV-G (SPG)	23	0.64	2.78	4.37×10^{11}

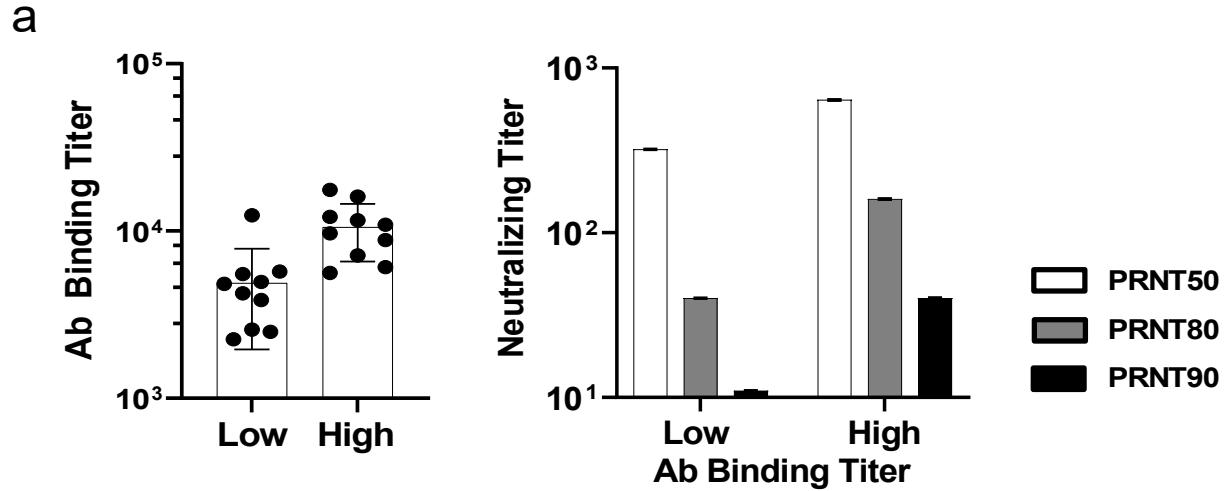
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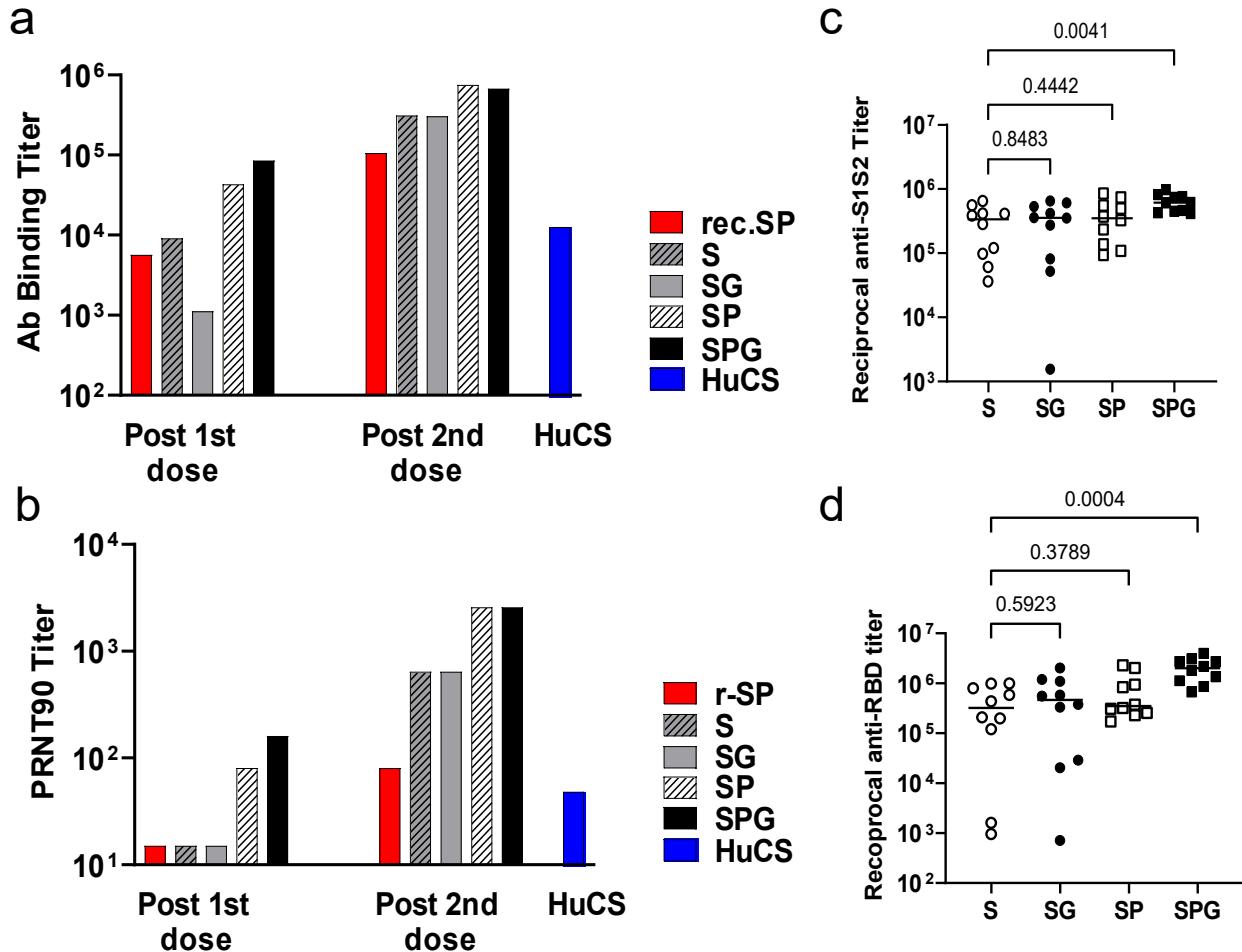
FIGURE 1



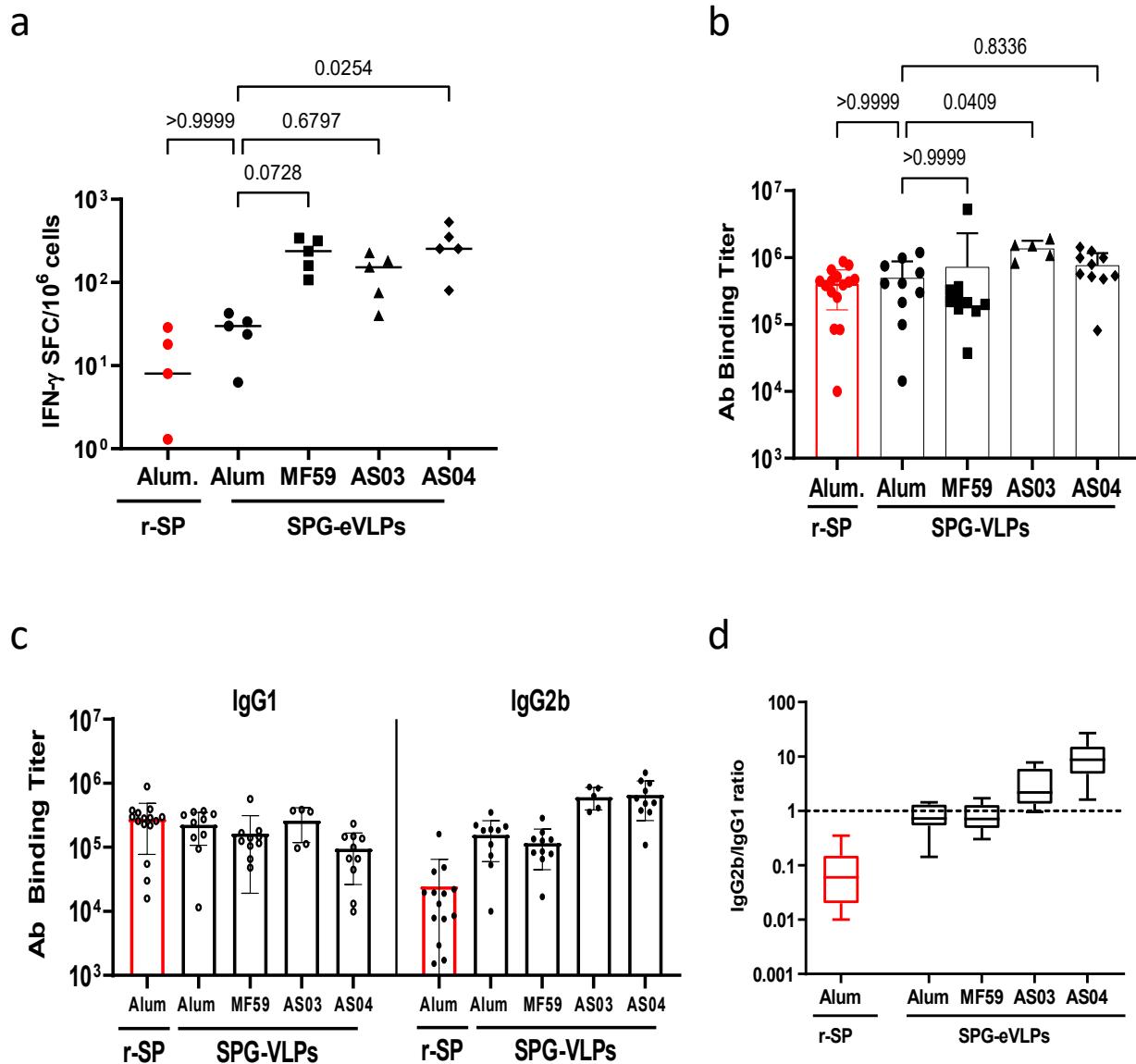
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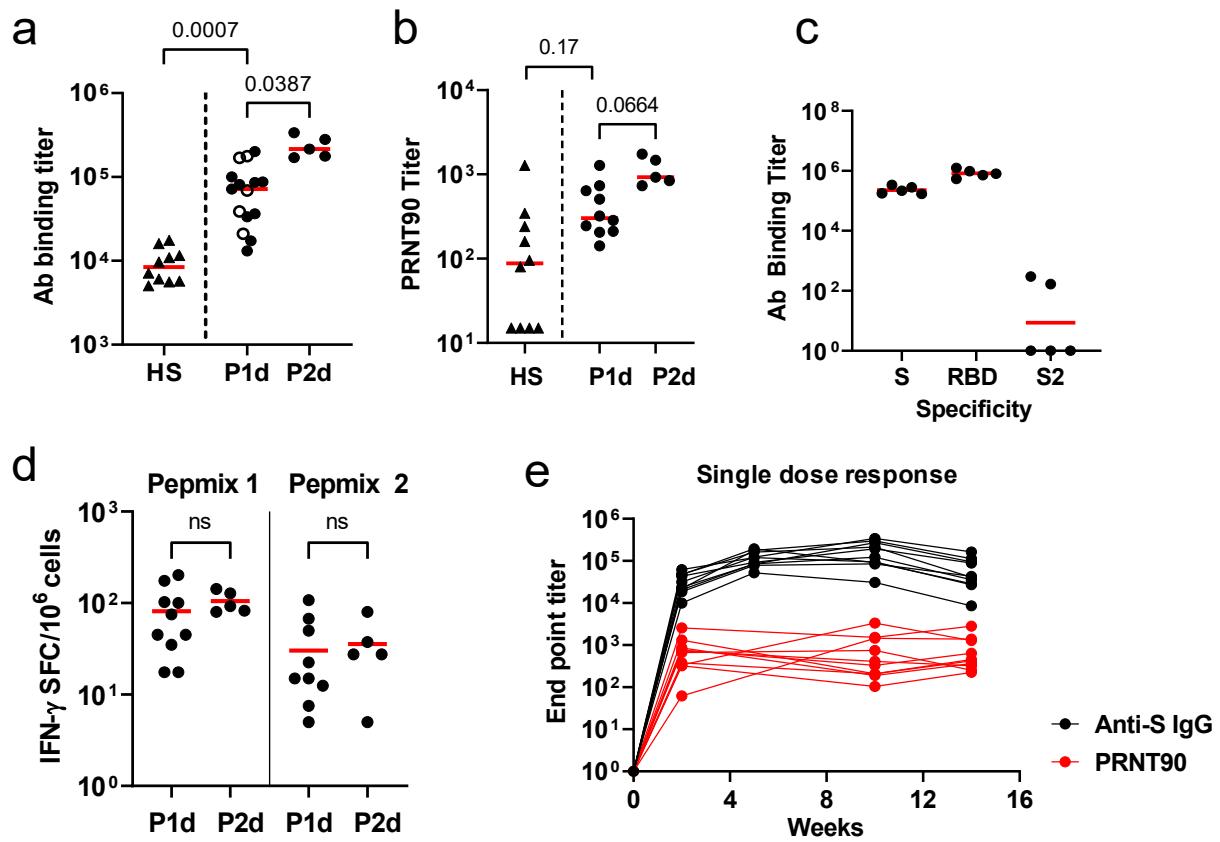
781 FIGURE 3



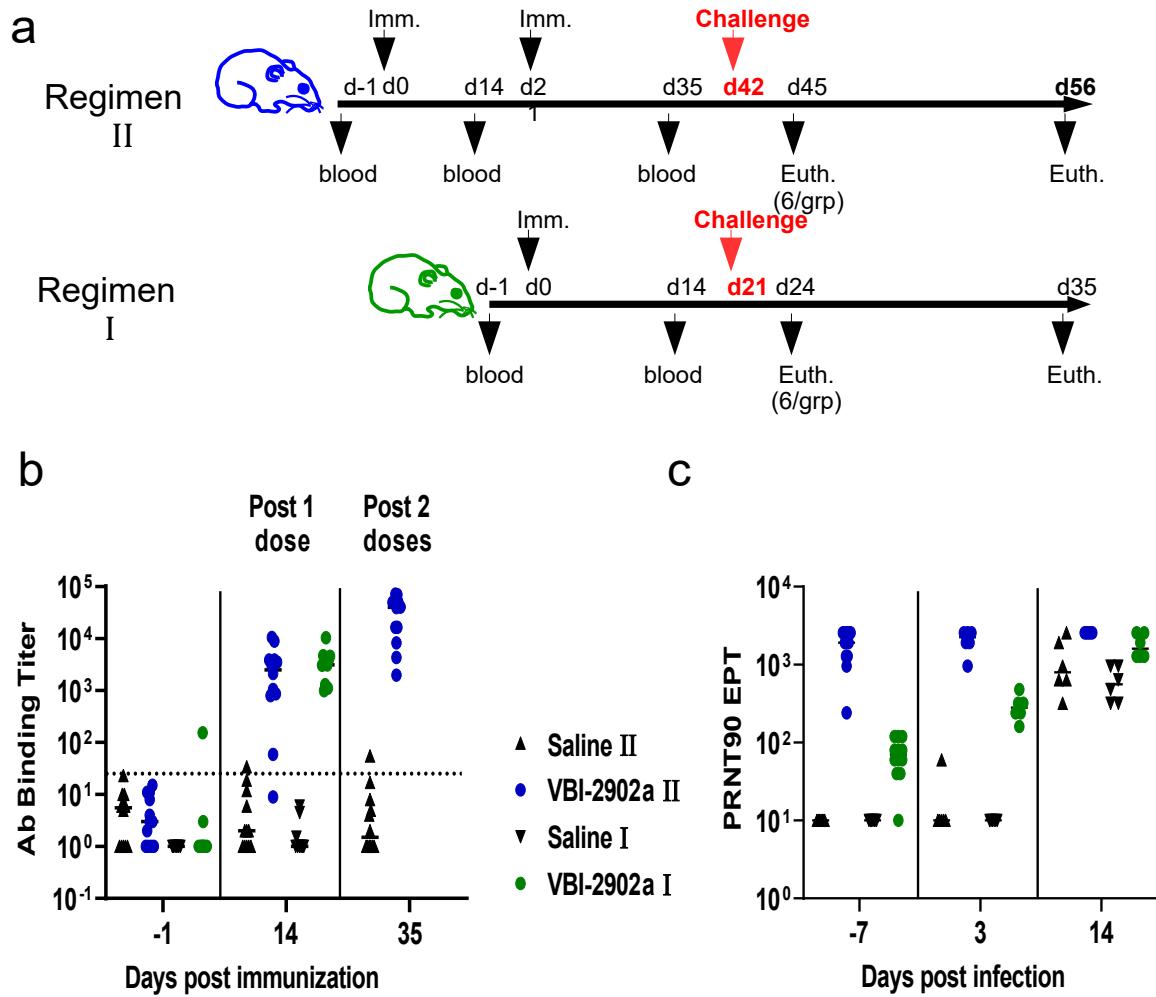
782 FIGURE 4



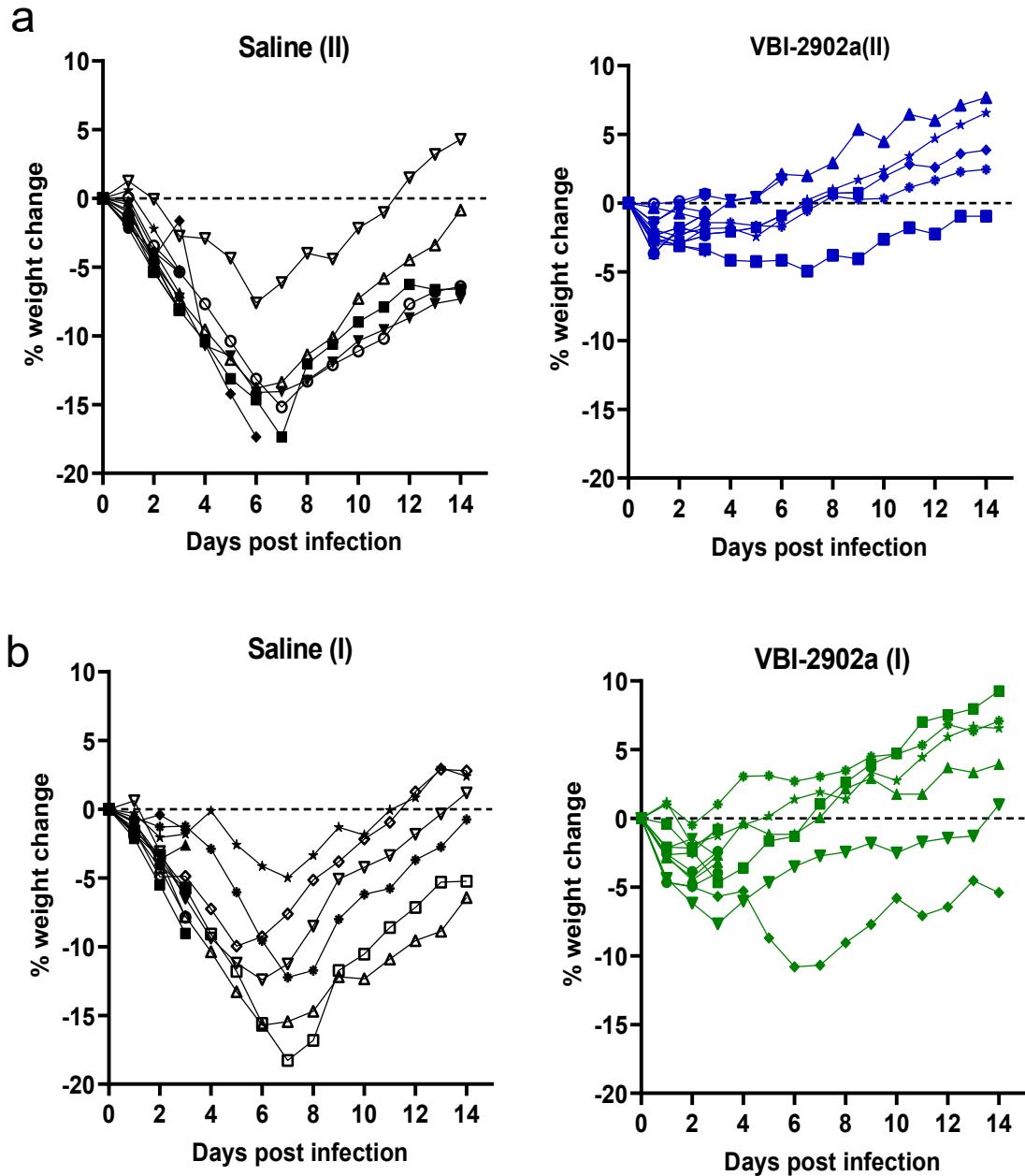
783 FIGURE 5



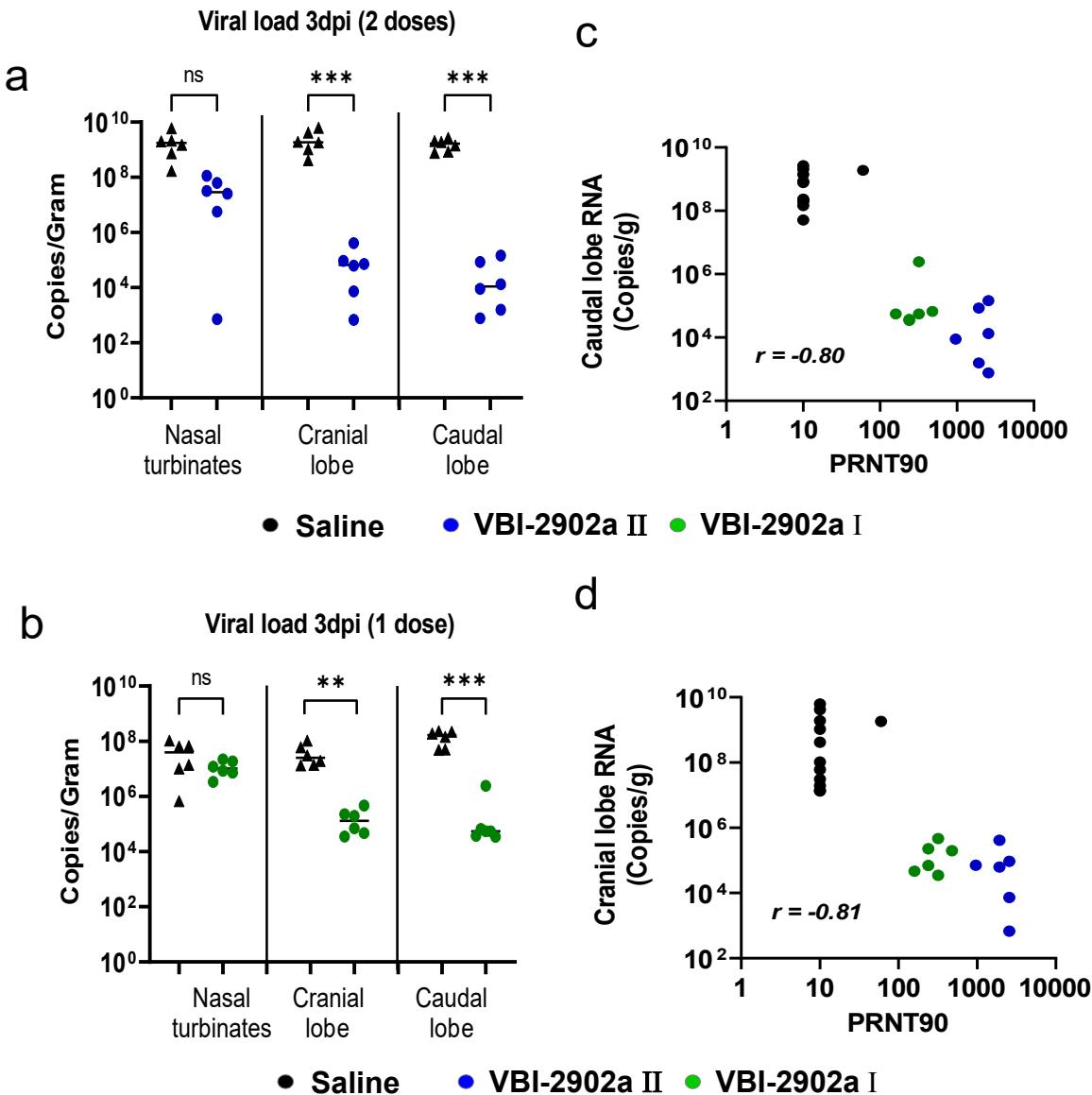
784 FIGURE 6



785 FIGURE 7



786 FIGURE 8



787 FIGURE 9

