A recombinant SARS-CoV-2 RBD antigen expressed in insect cells elicits immunogenicity and confirms safety in animal models

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27 **Abstract**

28 COVID-19 pandemic has accelerated the development of vaccines against its etiologic
29 agent, SARS-CoV-2. However, the emergence of new variants of the virus requires new
30 immunization strategies in addition to the current vaccines approved for human
31 administration. In the present report, the immunological and safety evaluation in mice
32 and hamsters of a subunit vaccine based on the RBD sub-domain with two adjuvants of
33 oil origin is described.

34 The RBD protein was expressed in insect cells and purified by chromatography until
35 >95% purity. The protein was shown to have the appropriate folding as determined by
36 ELISA and flow cytometry binding assays to its receptor, as well as by its detection by
37 hamster immune anti-S1 sera under non-reducing conditions.

38 In immunization assays in mice and hamsters, the purified RBD formulated with
39 adjuvants based on oil-water emulsifications and squalene was able to stimulate specific
40 neutralizing antibodies and confirm the secretion of IFN- γ after stimulating spleen cells
41 with the purified RBD. The vaccine candidate was shown to be safe, as demonstrated by
42 the histopathological analysis in lungs, liver and kidney. These results demonstrate the
43 potential of the purified RBD administered with adjuvants through an intramuscular
44 route, to be evaluated in a challenge against SARS-CoV-2 and determine its ability to
45 confer protection against infection.

46 **Keywords:** RBD, vaccine, SARS-CoV-2, COVID-19, squalene, adjuvant, recombinant
47 protein, SF9, baculovirus.

48

49 **Introduction**

50 In December 2019, a phylogenetically related SARS-CoV virus, later identified as
 51 SARS-CoV-2, caused an outbreak of atypical pneumonia in Wuhan. This virus is
 52 associated with a high rate of transmission, the appearance of symptoms such as fever
 53 and respiratory difficulties leads later to pulmonary and systemic failure with an
 54 exacerbated inflammatory condition that can lead to death [1]. The high transmission
 55 and mortality, coupled with the lack of effective treatment, justify the urgent
 56 development of vaccine candidates.

57 SARS-CoV-2 recognizes the Angiotensin Converting Enzyme-2 (ACE-2), which
 58 belongs to the surface of several types of human cells. The glycosylated Spike (S)
 59 protein gives the virus the ability to bind to the cell membrane and then fuse for the
 60 entry of viral RNA. The Spike protein has the S1 domain, and at its most distal end has
 61 a receptor binding sub-domain (RBD) [2]. The RBD is responsible for the binding of
 62 the virus to the ACE-2 receptor of host cells [3,4]. The amino acid sequences of RBD
 63 protein are being subjected to a positive selective pressure, which is conferring greater
 64 affinity to the ACE-2 receptor, this is due to the change in the structural conformation
 65 of ACE-2 binding [5]. An important mechanism of neutralization is the blockade of
 66 ACE-2 binding to the virus, so candidate vaccines based on the RBD domain induce a
 67 strong immune response, generating a remarkable humoral and cellular response [6–8].
 68 Several vaccine candidates use the baculovirus expression system. Researchers widely
 69 use this system due to its easy manipulation and the ability to produce complex proteins
 70 with suitable glycosylation patterns [9]. Currently, human and veterinary vaccines
 71 produced in this system are widely commercialized [10]. Additionally, to the successful
 72 production of some large-scale vaccine candidates for clinical trials [11,12]. However,
 73 these vaccines require appropriate adjuvants to stimulate a strong immune response.

74 There are several types of adjuvants on the market, which have an immunogenic effect
 75 when inoculated in animals and humans: those that are based on Alum [3], as well as
 76 emulsions based on mineral oils or non-mineral [14], which are the most widely used
 77 and approved for use in humans [15]. Alum-based adjuvants are not highly effective in
 78 stimulating the cellular immune response of either Th1 or Th2 [16]. These adjuvants
 79 require improvements in their concentration and the type of aluminum used to generate
 80 a cellular-type immune response; however, these could cause necrosis or tissue damage
 81 in the inoculation area [17]. This has led to the use of emulsions based on squalene-in-
 82 water, which come in formulations according to the interface where they are prepared:
 83 oil-in-water (O/W), which are microdroplets of oil in the aqueous phase together with
 84 the antigen; and water-in-oil (W O), microdroplets of water containing the antigen, in an
 85 oily phase [18].

86 In the present study, purified RBD administered through an intramuscular route with
 87 two different oil adjuvants was evaluated for immunogenicity and safety in mice and
 88 hamsters.

89

90 **Material and methods**

91 **Animals**

92 This study used thirty-five female albino mice (*Mus musculus*) strain BALB/c of 5-8
 93 weeks-old and 5 female Golden Syrian hamsters (*Mesocricetus auratus*) of 8-10 weeks-
 94 old obtained from the Universidad peruana Cayetano Heredia (UPCH) and the Instituto
 95 Nacional de Salud (INS-Peru), respectively.

96 **Adjuvants**

97 An oil-in-water (ESSAI 1849101) hereinafter defined as A1, and modified adjuvant
98 resulting from a mix of water-in-oil adjuvant and squalene, hereinafter defined as A3
99 were used.

100 **Ethics statement**

101 The use of animals was aligned to ethical protocols approved by the Bioethics
102 Committee of the Universidad Nacional Hermilio Valdizán and the animal's ethical
103 Committee at the Universidad Peruana Cayetano Heredia, registered as approval
104 certificates of Research Project No. 1, 2, and 10 and E011-06-20, respectively.

105 **Recombinant RBD expression in Sf9 cells**

106 **Recombinant baculovirus generation**

107 The amino acid sequence of the SARS-CoV-2 spike protein was obtained from the
108 SARS-CoV-2 reference genome Wuhan-Hu-1 (Genbank accession number:
109 NC_045512.2). For the design of RBD construct, the Pro330-Ser530 region was
110 selected. The sequence was optimized for expression in insect cells, the gp67 secretion
111 signal peptide was added at the N-terminal and a 10xHis-tag in the C-terminal region.
112 The resulting sequence was chemically synthesized by (GenScript Laboratories, USA)
113 and cloned at the EcoRI/HindIII sites of pFastBac1 (Thermo Fisher Scientific, USA)
114 under the control of the polyhedrin promoter and upstream of the SV40 polyadenylation
115 sequence. Transformation of competent DH10BAC cells and transfection of Sf9 cells
116 were performed with the Bac-to-Bac technology following the manufacturer's
117 instructions (Thermo Fisher Scientific, USA).

118

119 **Propagation of baculovirus and expression of RBD in Sf9 insect cells**

120 **culture**

121 The recombinant baculovirus was amplified in Sf9 cells (Thermo Fisher Scientific,
122 USA) to a density of 2×10^6 cells/mL in ExCell 420 medium (Sigma Aldrich, USA)
123 supplemented with 5% fetal bovine serum (Gibco, USA). Cultures were infected at a
124 multiplicity of infection (MOI) of 0.4. At 48 hours post infection (hpi), cultures were
125 centrifuged at 4500 rpm for 15 minutes. The supernatants were collected and titrated by
126 plaque assay. Viral stocks were stored at 4°C until use.

127 For protein production, 7 L of Sf9 cell culture at a density of 2×10^6 cells/mL were
128 infected with the baculovirus at a MOI of 3 using a Biostat B plus bioreactor (Sartorius,
129 Germany). The following conditions were maintained during the culture period:
130 temperature at 28°C, pH at 6.2, 50% dissolved oxygen (DO) with an oxygen flow rate
131 of 0.1 vvm via micro sparger and agitation at 150 rpm. At 48 hours post-infection, the
132 cultures were centrifuged at 4500 rpm for 15 minutes and the supernatant was filtered
133 through a 0.22 µm membrane.

134

135 **Recombinant RBD purification**

136 **Tangential filtering**

137 Tangential filtration was conducted on a Hydrosart cassette (Sartorius, Germany) with 5
138 kDa of nominal molecular weight cutoff (MWCO) was used in a SARTOFLOW
139 Advanced (Sartorius, Germany) tangential flow system. The supernatant was retained
140 and concentrated to a volume of 2 L. Subsequently, the retentate was diafiltered into a
141 saline phosphate buffer (PBS) at pH 6.3 and concentrated again to a volume of 1 L,
142 filtered as filtered through 0.22 µm membrane and stored at 4°C until use.

143

144 **Affinity chromatography**

145 As a first step, an immobilized metal affinity chromatography (IMAC) was performed
146 using a HisTrap Excel column (1.6 x 2.5 cm) on an AKTA Pure 25L system (Cytiva,
147 Sweden). Desalting and buffer exchange were performed on a Hiprep 26/10 desalting
148 column (Cytiva, Sweden) using PBS pH 7.4 throughout the elution phase. The desalted
149 protein was concentrated on an Amicon 10,000 MWCO (Merck, Germany) and filtered
150 through a 0.22 µm membrane.

151

152 **Size exclusion chromatography**

153 As a second step, a size exclusion chromatography was performed on a Superdex 200
154 increase 10/300 GL column (Cytiva, Sweden) using PBS pH 7.4 during the entire
155 process. Protein fractions were collected and analyzed by SDS-PAGE under reducing
156 conditions and Western blot using a commercial anti-His monoclonal antibody. The
157 pool of selected fractions was concentrated using an Amicon 10,000 MWCO (Merck,
158 Germany) and filtered through a 0.22 µm membrane. The concentration of purified RBD
159 was determined using the Bradford assay (Merck, Germany).

160

161 **Recombinant RBD characterization in vitro**

162 **RBD binding to human ACE-2**

163 A 96-well plate was coated overnight at 4°C with 100 µL of a recombinant human
164 ACE-2 fused to a Fc fragment (GenScript Laboratories, USA) at 1 µg/mL in carbonate
165 buffer (pH 9.6). The plate was blocked with 3% skimmed milk for 1 hour at room
166 temperature and then washed five times with PBS 0.05% Tween 20 (PBS-T). Serial

167 dilutions (1:2) of purified RBD were performed in PBS, starting from 2 µg/mL and
 168 ending to 1.9 ng/mL. Dilutions were added to the wells and incubated for 2 hours at
 169 37°C. Five washing steps with PBS-T were performed, 100 µL of rabbit IgG polyclonal
 170 anti-spike antibody (SinoBiological, China) was added to the wells (1:5000) in 1%
 171 skimmed milk and incubated for 1 hour at 37°C. The plate was washed five times with
 172 PBS-T. Then, 100 µL of anti-rabbit IgG HRP conjugated (GenScript, USA) (1:30,000)
 173 in 1% skimmed milk was added to the wells. The plates were incubated at 37°C for 1
 174 hour. Finally, the plates were washed with PBS-T five times, and 100 µL of TMB
 175 (Sigma Aldrich, USA) were added to the wells and incubated for 15 minutes at room
 176 temperature. The reaction was stopped with 50 µL of 2N sulfuric acid and the
 177 absorbance at 450 nm was read with an Epoch 2 microplate reader (Biotek, USA).

178

179 **RBD binding to Vero-E6 cells**

180 Vero-E6 cells (Cod. CRL-1586™, ATCC®, USA), which were previously cultured in
 181 DMEM/F12 (HyClone, USA) + 10% fetal bovine serum (FBS) (HyClone, USA), were
 182 harvested and washed with DPBS with 5% FBS (FACS buffer). Approximately 10⁶
 183 cells were blocked with FACS buffer and 5% of normal mouse serum (Abcam, USA)
 184 for 30 min at 37°C. Then, the cells were incubated with the purified RBD (8 µg/mL) for
 185 2 h at 37°C. To remove the excess of RBD not attached to Vero E6, the cells were
 186 washed with FACS buffer twice. After that, the mix was marked with rabbit monoclonal
 187 antibody anti-SARS-CoV-2 S1 (1:200) (Sino Biological, China) as the primary antibody
 188 for 1 h at 37°C, followed by the addition of the secondary goat anti-rabbit IgG antibody
 189 conjugated with Alexa Fluor 488 (1:200) (Abcam, USA). Finally, cells were acquired
 190 by the BD FACSCanto™ II flow cytometer (BD Biosciences, USA). The data was
 191 analyzed using the software FlowJo v.10.6 (BD Biosciences, USA), and the graphics

192 were generated with GraphPad Prism 8.0.1. For the interpretation of results, the
193 percentage of positive cells indicates of binding of RBD to Vero E6 cells.

194

195 **RBD recognition by immunized sera**

196 Purified RBD was loaded at 0.2 µg/well and electrophoretically separated by SDS-
197 PAGE under non-reducing conditions and transferred to nitrocellulose membranes using
198 an e-blot device (GenScript Laboratories, USA). The membranes were blocked with 5%
199 (w/v) non-fat milk in PBS with 0.1% of Tween 20 at pH 7.4 and incubated overnight at
200 room temperature. Then, membranes were washed three times for 5 minutes each with
201 Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) and incubated for two
202 hours at room temperature with serum of a hamster immunized with a New Castle
203 disease virus expressing the S1 sub-unit of SARS-CoV-2 [19] (1:250) in 5% non-fat
204 milk. After three washes with TBS-T, anti-Hamster IgG antibody conjugated to HRP
205 (Abcam, USA) was added to the membrane at 1:5000 dilution in 5% non-fat milk and
206 incubated for two hours at room temperature. Finally, the membranes were washed
207 three times with TBST-0.1%, incubated with luminol (Azure Biosystems, USA) as a
208 substrate and revealed with a CCD camera (Azure Biosystems, USA).

209

210 **Immunization and samples collection in mice**

211 Female BALB/c mice (18-25g) were immunized intramuscularly (i.m.) with 20 and 50
212 µg/mice of purified RBD mixed with 50 µL of A1 or A3 (1:1, 100 µL final volume).
213 Two boosters were administered at 15 and 30 days post-immunization (DPI) with the
214 same dose (Fig. 1). As a control, mice were immunized with PBS mixed with A1 or A3,
215 an unvaccinated group was maintained during the experiment. Serum of each animal

216 was collected on 0, 15, 30 and 45 DPI by low-speed centrifugation of blood at 2500 rpm
217 for 5 minutes. All animals were euthanized at 45 DPI and organs (lung, liver and
218 kidney) were collected for histopathological analysis.

219 Figure 1. Mice immunization flow chart. Mice were immunized by the intramuscular
220 route using a prime-boost regimen with a booster on days 15 and 30. Seven groups of
221 mice were included: group 1 (20 µg RBD/A1, n=5), group 2 (50 µg RBD/A1, n=5),
222 group 3 (20 µg RBD/A3, n=5), group 4 (50 µg RBD/A3, n=5), group 5 (only A1 n=5),
223 group 6 (only A3 n=5) and group 7 (no immunization).

224 **Immunization and samples collection in hamsters**

225 Five Golden Syrian hamsters were immunized intramuscularly, each one with 30 µg of
226 purified RBD mixed with oil adjuvant A3 (1:1) (which had the best adjuvant tested in
227 mice) in a final volume 100 µL (Fig 2). Five animals received only adjuvant A3 and
228 were considered as the group. At 15 DPI, all groups received a booster at the same dose.
229 Hamsters were bled at 0, 15 and 30 DPI to evaluate the specific and neutralizing
230 antibody (nAbs) titers. Serum from each sample was obtained by centrifugation of
231 blood at 2500 rpm for 5 minutes.

232 Figure 2. Hamster immunization flow chart. Hamsters were immunized by the
233 intramuscular route with 30 µg of purified RBD in adjuvant A3 using a prime-boost
234 regimen with a booster on day 15.

235 **Evaluation of humoral immunity**

236 **Detection of specific antibodies by ELISA**

237 Nunc MaxiSorp 96-well flat bottom plates (Sigma-Aldrich, USA) were coated with 100
238 µL of SARS-CoV-2 RBD (1 µg/mL) (GenScript, USA) in carbonate bicarbonate buffer

(pH 9.6) and incubated at 4°C overnight. The next day, the wells were washed six times with PBS containing 0.05% (v/v) Tween-20 (PBS-T) and blocked with 3% (w/v) Difco Skim Milk (BD Biosciences, USA) in PBS-T for 2 h in agitation at RT. The plates were then washed six times with PBS-T. Then, 100 µL of each collected serum sample diluted 1:100 with 1% (w/v) Difco Skim Milk (BD Biosciences, USA) was added to each plate for 1 h at 37°C. The wells were washed six times with PBS-T and incubated with 100 µL (1:10000) of Goat Anti Mouse IgG (Genscript, USA) or Anti Hamster IgG (Abcam, USA) conjugated to HRP diluted in 1% Difco Skim Milk in PBS-T for 1h at 37°C. The plates were washed six times and were incubated with 100 µL of TMB for 15 min at RT. Finally, the reaction was stopped by adding 50 µL per well of 2 N H₂SO₄, and the plates were read at 450 nm using an Epoch 2 microplate reader (Biotek, USA). The negative control was obtained from serum samples of the control group.

Detection of neutralizing antibodies

Hamster serum samples were processed to assess neutralizing antibodies (nAbs) titers against SARS-CoV-2 at 0, 15, and 30 days post immunization. All Neutralization assays were performed with the surrogate virus neutralization test (sVNT) (GenScript, USA), following the manufacturer's instructions. Plates were read for absorbance at 450 nm using an Epoch 2 microplate reader (Biotek, USA). The optical density results were converted into percentage of inhibition, by the formula provided by the manufacturer. The positive and negative cut-off points for the detection of SARS-CoV-2 nAbs were set as follows: positive, if percentage of inhibition $\geq 30\%$ (neutralizing antibody detected) and negative, if percentage of inhibition $<30\%$ (neutralizing antibody not detectable).

262

263 **Evaluation of cellular immunity**

264 **Extraction of mononuclear cells from mouse spleen**

265 The mice vaccinated with the purified RBD and the control group (adjuvant only) were
 266 euthanized at 45 days post immunization, and spleens were removed. aseptically. The
 267 organs were transferred to Petri dishes with 5 mL cold RPMI medium (Sigma Aldrich,
 268 USA) and two pieces of 41 µm nylon net (Merck, USA), where the organ was disrupted
 269 using a 3 mL syringe plunger. The cell suspension was filtered and placed in a
 270 centrifuge tube containing 2 mL of Histopaque® 1077 (Sigma Aldrich, USA). The
 271 samples were centrifuged at 300 x g for 30 minutes without brake. The buffy coat
 272 containing mononuclear cells was removed, placed in cold RPMI medium, and washed
 273 twice. Cells were resuspended in 1 mL of complete RPMI medium and counted by
 274 hemocytometer. Cells were resuspended in fetal bovine serum (HyClone, USA) with
 275 10% dimethyl sulfoxide (Sigma Aldrich, USA) and frozen in liquid nitrogen until use.

276 **ELISPOT for IFN-γ secretion in spleen mononuclear cells.**

277 Mononuclear cells were cultured in 96-well plates with a PVDF membrane, previously
 278 coated with anti-mouse IFN-γ (clone RMMG-1, Merck, USA) and blocked with 1%
 279 bovine serum albumin (BSA) (Sigma Aldrich, USA). Cells were stimulated with the
 280 purified RBD (4 µg/mL) for 24 hours at 37°C at 5% CO₂. Concanavalin A (Sigma
 281 Aldrich, USA) was used as a positive control. The cells were removed by successive
 282 washes with water and PBS with 0.1% Tween. The wells were incubated with
 283 biotinylated anti-mouse IFN-γ (clone R4-6A2, Biolegend, USA) for 16 hours at 4° C.
 284 After washing, the wells were incubated with streptavidin-alkaline phosphatase (SAP)
 285 (Sigma Aldrich, USA) for one hour at room temperature. After washing, the
 286 chromogen-substrate, NBT/BCIP (Abcam, USA), was added. The spots formed were

counted with an AID EliSpot plate reader (Advanced Imaging Devices, v. 7.0, Germany).

Intracellular labeling of cellular immune response cytokines.

The mononuclear cells were stimulated with or without purified RBD (8 µg/mL) for 21 hours at 37° C at 5% CO₂ hours, in the last 5 hours of culture a protein transport inhibitor Brefeldin A (1µL/mL) was added (BD Biosciences, USA). Cells were fixed using the BD Cytofix/Cytoperm® kit (BD Biosciences, USA) following the manufacturer's instructions, and then labeled with conjugated antibodies to surface antigens (PerCP-Cy®5.5 anti-mouse CD3, FITC anti-mouse CD4, APC-Cy®7 mouse anti-CD8, all from BD Biosciences, USA; LIVE/DEAD™ Fixable Yellow Dead Cell Stain, Invitrogen, USA) and intracellular cytokines (PE anti-mouse IFN-γ, PE-Cy®7 anti-mouse TNF-α, APC anti-mouse IL-2, all from BD Biosciences, USA). The labeled cells were acquired with the BD FACSCanto™ II flow cytometer and analyzed with the program FlowJo v.10.6.2 (BD Biosciences).

Immunophenotype of spleen mononuclear cells.

Mononuclear cells were directly labeled with conjugated antibodies to surface antigens (PerCP-Cy®5.5 anti-mouse CD3, clone, FITC anti-mouse CD4, APC-Cy®7 anti-mouse CD8, for T lymphocyte phenotype, all from BD Biosciences, USA; LIVE/DEAD™ Fixable Yellow Dead Cell Stain, for cell viability, cat. No. L34959, invitrogen, USA). These cells were acquired with the BD FACSCanto™ II flow cytometer, and the analysis was performed with the program FlowJo v 10.6.2 (BD Biosciences).

Histopathological analysis

310 For safety analysis, organs were obtained from euthanized mice at 45 days post
 311 immunization and fixed with 10% buffered formalin for 48 hours. Then, organs were
 312 reduced and placed in a container for 24 hours with buffered formalin. The containers
 313 with the organs were passed to an automatic tissue processor (Microm brand)
 314 conducting the following processes: dehydration, diaphanating, rinsing, and
 315 impregnation; within an average of 8 hours. Organs included in paraffin were sectioned
 316 to a thickness of 5 microns (Microtome Leica RM2245) and placed in a flotation
 317 solution in a water bath and then fixed on a slide sheet, dried in the stove at 37°C for 1
 318 to 2 hours. The staining was done with the Hematoxylin and Eosin staining method
 319 (H&E). Samples were mounted in a microscope slide with Canada Balm (glue) and
 320 dried at 37°C for 12 to 24 hours, for further labeling. The colored slides with H&E
 321 were taken and analyzed under an AxioCam MRc5 camera and AxioScope.A1
 322 microscope (Carl Zeiss, Germany) at 20x magnification by a board-certified veterinary
 323 pathologist.

324 **Statistical analysis**

325 All quantitative data were analyzed using GraphPad Prism version 6.1 (GraphPad
 326 Software, San Diego, CA, USA). Student t-test was used to evaluate cellular immunity.
 327 For EC50 estimation, a regression model of four parameter logistic curve (4PL) was
 328 used. Two-way ANOVA analysis was performed to determine significant difference in
 329 ELISA results. A 5% statistical significance was considered in all cases.

330

331

332 **Results**

333 **Recombinant SARS-CoV-2 RBD production**

334 Recombinant RBD was expressed and secreted into the extracellular medium by
335 infected Sf-9 cells. A single band of ~28KDa was detected by western blot using Anti-
336 his and Anti-spike antibodies (Fig 3B). In bioreactor conditions, the highest protein
337 expression level was observed at 68 hours post-infection. After the purification
338 processes, a productivity level of 0.8 mg/mL of RBD was obtained at a purity level >
339 90% (Fig 3C).

340 Figure 3. RBD expression and purification. (A) Design of the expression cassette
341 integrated into the recombinant baculovirus. (B) Detection of RBD from infected
342 culture supernatants using an anti-His (left) and anti-spike (right) antibody. Bv-WT:
343 Wild type baculovirus; Bv-RBD: RBD expressing baculovirus. (C) SDS-PAGE of
344 purified RBD after the affinity chromatography purification step (Lane 1) and size
345 exclusion chromatography (Lane 2).

346

347 **Recombinant SARS-CoV-2 RBD characterization**

348 To determine the correct conformational state of RBD, ACE-2 receptor binding assays
349 were performed. ACE-2 binding dependent on RBD concentration was observed, with a
350 half maximal effective concentration (EC50) of 46.8 ng/mL (Fig 4A). Similarly,
351 through flow cytometry, bounded RBD to Vero E6 cell surface at different
352 concentrations, with a 60% binding level (Fig 4C). Based on the main fluorescence
353 intensity (MFI), the difference between the cells treated with purified RBD and those
354 treated with FACS buffer as a negative control was significant. On the other hand,
355 commercially available recombinant RBD was used as a positive control. Although its

356 binding was slightly higher than RBD (78%), the difference was not statistically
357 significant. This trend was observed in all the concentrations evaluated.

358 The importance of disulfide bonds for the correct folding of the RBD sub-domain is
359 known. Therefore, an additional way to verify the correct folding of the recombinant
360 RBD was evaluating its detection under reducing and non-reducing conditions using a
361 serum from a hamster immunized with a New Castle Disease virus (NDV) expressing
362 the S1 Domain [19] (Fig 4B). In this way, by Western blot RBD could be detected by
363 the serum only under non-reducing conditions, demonstrating that it conserves the
364 folding of the RBD sub-domain occurring in the Spike protein.

365 Figure 4. RBD binding and folding characterization in vitro. (A) Dose dependent curve
366 of RBD binding to human ACE-2 by ELISA, dashed lines represent the EC50 value.
367 Dots and error bars represent the mean value of three independent experiments and the
368 standard deviation, respectively. (B) Disulfide bond dependent recognition of RBD by
369 hamsters immunized serum by western blot. Lane1: RBD under non-reducing
370 conditions; Lane 2: RBD under reducing conditions. (C) RBD binding to Vero E6 cell
371 surface. The binding values are represented as the percentage of cells bound to RBD
372 (left diagram) and the Mean Fluorescence Intensity (MFI) of each group was evaluated
373 (right diagram). Two repetitions were performed per group, except in the FACS buffer
374 group. Student *t*-test was used to compare the MFI values. ns: not significant ($P>0.05$);
375 **: significant ($P<0.01$).

376

377 **Humoral Immunity**

378 Specific antibodies were detected in both groups immunized with each adjuvant. In
379 group A1, antibody levels with both doses of FAR-RBD were similar at 15 and 30 days

after immunization. However, after the second booster, there was a slight decrease in the antibodies detected in the sub-group immunized with 20 µg of RBD, while those immunized with 50 µg increased slightly. Regarding group A3, it was observed that the antibodies generated were higher in the dose of 50 µg at all times of evaluation. Moreover, at 45-day post-immunization the antibodies detected remained at the same level prior to the second booster. In addition, group A3 generated higher levels of antibodies at 15 days post-immunization compared to group A1. Control groups immunized with each adjuvant had baseline reactivities throughout the evaluation time (Fig 5).

Figure 5. Detection of specific antibodies against RBD in mice. Immunized mice were bled at 0, 15, 30 and 45 days post immunization. All sera were isolated by low-speed centrifugation. Serum samples were processed to detect specific antibodies against SARS-CoV-2 RBD protein using indirect ELISA assay. (A) Group immunized with RBD mixed with adjuvant 1 and (B) Group immunized with RBD mixed with adjuvant 3. Two-way ANOVA and post-hoc Tukey's test were performed. **: P<0.01

Since with adjuvant 3 the maximum levels of antibodies were obtained with a single boost and in less time, this adjuvant was used to immunize the hamsters. In this way, a significant increase in specific antibody levels was observed from day 15 post-immunization until day 30 in all the individuals tested (Fig 6A). The neutralization assays using the surrogate virus neutralization test (sVNT) detected neutralizing antibodies only at day 30 post-immunization, where the sera from hamsters vaccinated showed a mean percentage of inhibition of the RBD-ACE2 union above 30%. Sera of the control group remained below 30% and did not show neutralizing antibodies (Fig 6B).

404 Figure 6. Detection of specific antibodies against RBD and neutralizing antibodies in
405 hamsters. (A) Immunized hamsters were bled at 0, 15 and 30 days post immunization.
406 Serum samples were processed to detect specific antibodies against SARS-CoV-2 RBD
407 protein using indirect ELISA assay. (B) Serum samples were processed to evaluate the
408 neutralizing antibody titers against SARS-CoV-2 using sVNT. The cut-off for
409 positive/negative neutralizing antibodies in the sample was 30% of inhibition of RBD
410 binding to ACE-2. Two-way ANOVA and post-hoc Tukey's test were performed. **: $P < 0.01$. ****: $P < 0.0001$.

412

413 **Cellular immunity**

414 The cellular immunity stimulated by the purified RBD in mice was evaluated on day 45
415 after the first immunization. The percentage of CD4⁺ and CD8⁺ T cells is observed for
416 each adjuvant evaluated. For adjuvant A1, the percentage of CD4⁺ and CD8⁺ T cells
417 increased proportional to the dose of RBD administered (Fig 7A). For adjuvant A3, the
418 percentage of cells decreased when the dose of RBD was increased. Although the
419 differences were not significant for all groups compared with the control group. When
420 reviewing the production of Th1-type cytokines (IFN- γ , TNF- α and IL-2) (Fig 7C), the
421 increase in CD8⁺ T cells secreting IFN- γ (for A1 and A3), TNF- α (for A1 and A3) and
422 IL-2 (for A1 and A3) decreased according to the administered dose of RBD. Regarding
423 the secretion of IFN- γ in splenocytes stimulated with purified RBD using the ELISPOT
424 technique (Fig 7B), the adjuvant A3 stimulated a greater number of cells directly
425 proportional to the administered dose.

426 Figure 7. Evaluation of cellular immunity in mice vaccinated with purified RBD. Mice
427 were immunized with 20 and 50 μ g of RBD using two different adjuvants (A1 and A3)

at 0, 15 and 30 days post immunization. On day 45 post-immunization mice were sacrificed and spleens were processed. (A) Percentage of CD4 and CD8 positive cells by flow cytometry, between the groups immunized (n=3, except the adjuvant control). (B) IFN- γ ELISPOT of splenocytes between the groups immunized with purified RBD using A1 or A3 adjuvant (n=3, except the adjuvant control). (C) Intracellular staining of Th1 cytokines (IFN- γ , TNF- α and IL-2) of splenocytes stimulated with RBD (n=3, except the adjuvant control). ns: not significant ($P>0.05$), *: $P<0.05$.

Safety

Histopathological analysis of the of the groups of mice immunized with purified RBD mixed or mixed with A1 or A3, including the unvaccinated group not showed signs of serious injury or damage. Lungs not showed clinical appearance of pneumonia and there was no evidence of kidney symptoms. Although in liver a slight vacuolar degeneration was identified, this was observed in all the groups tested, including the control group. (Fig. 8).

Figure 8. Histopathological analysis of mice inoculated with purified RBD and control. Organs were obtained 45 days after the first immunization and stained with hematoxylin-eosin (H&E). These images are representative slides from vaccinated mice and negative control mice. (A) Lung sections. (B) Liver sections. (C) Kidney sections. All the images are in a 200X magnification.

450 Discussion

451 SARS-CoV-2 continues to be a problem worldwide. As an immediate response to the
452 emergence of new variants and their dissemination, the constant development and
453 evaluation of vaccines are necessary. In the present study, production of a recombinant
454 sub-domain RBD expressed in insect cells, and immunization together with two oil-
455 based adjuvants, elicited an immune response in mice and hamsters while demonstrated
456 to be safe.

457 Currently, most of the approved and candidate vaccines are based on the complete spike
458 protein. However, there are several vaccine candidates based on the single RBD
459 antigen, ongoing pre-clinical and clinical phase [20]. Although, in some reports the
460 complete spike has shown greater immunogenicity [21], the single RBD remains as a
461 strong vaccine candidate because it comprises the most important epitopes to which
462 neutralizing antibodies should target. In addition, it generates antibodies with enhanced
463 neutralizing activity [22–24]. The greater accumulation of mutations in the S1 and S2
464 domains can destabilize the protein, hindering its production and the yields obtained as
465 a purified protein [25,26]. On the other hand, RBD has demonstrated an easier
466 production [27], and results in a more conserved antigen. Recent studies have
467 bioengineered RBD variants with improved stability and higher immune response in
468 mice compared to the current Wuhan-Hu-1 vaccine [28]. Likewise, a thermotolerant
469 RBD fused to a trimerization motif has generated high neutralization titers in guinea
470 pigs and mice, as well as protection in hamsters from viral challenge [29].

471 Despite that the purified RBD evaluated in this study comprised 22 amino acids less
472 than the generally recognized RBD region (Arg319-Phe541) [30], it was structurally
473 and functionally viable as demonstrated by the binding assays by ELISA and flow

474 cytometry. This functionality was maintained because the expressed region comprises
475 the residues that form the disulfide bonds that give stability to the nucleus and the key
476 external sub-domains of the RBD [3,23] maintaining the integrity of the receptor
477 binding motif, which ultimately is the main region that directly interacts with the ACE2
478 receptor. This was confirmed by the lack of RBD recognition of the hamster anti-S1
479 immune sera under reducing conditions, but the strong recognition of RBD by the
480 immune sera under non-reducing conditions. This suggests, that the disulphide bonds
481 are present and are favoring a correct folding and 3D structure of the RBD antigen, that
482 may be presenting appropriate conformational epitopes, as most of the immune
483 antibodies targets tertiary epitopes spanning the exposed sites of the RBD in the trimeric
484 pre-fusion Spike [31].

485 The production level of RBD in this study, was relatively low (0.8 mg/L), compared to
486 previous reports of expression of the same domain using the baculovirus expression
487 system [27], it is likely that this is due to the baculovirus type used, which is not
488 optimized for secreted expression, or to the second purification step required to obtain a
489 higher degree of purity. These levels could be optimized using baculoviruses lacking the
490 *v-cath* and *chiA* genes [32] or through optimization strategies of the amino acid
491 sequence that have been proven to improve expression levels and immunogenicity of
492 the RBD [28,33].

493 The chemical composition of an adjuvant is important because its components may
494 interfere with organism responses. Most oil-water (O/W) adjuvants that contain
495 squalene, also have other components (Tween 80, Span 85, polyethylene glycol or
496 derivatives), which when emulsified in an aqueous phase, generate a stable chemical
497 structure that allows the transport of antigens for their recognition by cells such as
498 macrophages or dendritic cells [18,34]. In the immunization experiments conducted in

599 this study, adjuvant 3 (W/O + squalene) was associated to higher levels of anti-RBD
 600 antibodies than adjuvant 1 (O/W) at 15 days post immunization. However, after the
 601 second booster was administered (45 DPI), the antibody levels for both adjuvants were
 602 not significantly different. This could be explained by the fact that O/W emulsions, as
 603 an adjuvant for mice, generates higher levels of antibodies while directing the cellular
 604 immune response to the Th2 type [34]. Also, it is known that O/W emulsions stimulate
 605 a strong production of TNF- α [16,35] and do not generate local inflammation reactions
 606 when injected subcutaneously or intramuscularly [36]. On the other hand, the W/O
 607 adjuvant formulations are not effective enough to induce strong humoral responses, as
 608 they can generate inflammatory responses and the formation of granulomas [36,37]. In
 609 contrast, adjuvant A3, which is a novel composition, demonstrates the stimulation of a
 610 strong humoral response. We believe further studies are necessary to clarify and
 611 confirm these observations

612 When adjuvant A1 was administered with the purified RBD, the formulation did not
 613 generate IFN- γ , IL-2 nor TNF- α in the evaluation by ICS. However, an increase in the
 614 percentage of CD4+ and CD8+ T cells was observed. This observation is in agreement
 615 with a previous study, where Arunachalam et al. [38] found that adjuvant A1 (Essai
 616 O/W 1849101, Seppic) added to RBD nanoparticles did not elicit a strong antibody
 617 response nor protection as expected in Rhesus monkeys. Nevertheless, when A1 was
 618 used with alpha-tocopherol it produced a stronger level of neutralizing antibodies and
 619 protection against infection with SARS-CoV-2. However, the use of this adjuvant
 620 generated an inflammatory response, associated with a high expression of TNF- α and
 621 IL-2. We found that the novel adjuvant A3 stimulated the secretion of greater IFN- γ
 622 levels in splenocytes compared to adjuvant A1, as well as IL-2 and TNF- α in CD8+ T

523 cells. This is consistent with the possible inflammatory effect generated by adjuvants
524 based on W/O emulsions [37].

525 The generation of neutralizing antibodies in hamsters was observed at 30 days post
526 immunization. Although the surrogate test does not directly determine the neutralization
527 of virus invasiveness in cells, it has been shown that it has a high correlation index with
528 classic viral neutralization tests [8]. In addition, various studies have demonstrated a
529 relationship between the development of neutralizing antibodies with the protection of
530 re-infection in humans, as well as in challenge tests in hamsters [39,40].

531 Due to limitations in space and the availability of animals, this trial was conducted with
532 5 individuals per group, and the heterogeneity was evident as previously reported in a
533 similar protocol [41]. Unfortunately, it was not possible to establish clear conclusions
534 about the tendency of the population when stimulated with the two different adjuvants,
535 as there was no significant difference between the controls and the immunized groups. It
536 is important to perform additional studies with a greater sample size to perform a better
537 evaluation of cellular and humoral immunity [13,16,23,28] to 8 per group [35,42].

538 In conclusion, the RBD vaccine candidate presented in this study, administered through
539 an intramuscular route, was shown to be safe and able to induce humoral and cellular
540 immunity as well as neutralizing antibodies in mice and hamsters. Further studies are
541 required to evaluate protection in a challenge trial.

542

543 **Ethics statement**

544 This study was approved by the Bioethics Committee of the Universidad Nacional
545 Hermilio Valdizán registered as approval certificates of Research Project No. 1, 2 and

546 10. Animal immunizations and procedures were performed by qualified personnel
547 following the ARRIVE guidelines [43]

548

549 **Authors' contributions**

550 MFD, MZ, RCG, APA contributed to conception and designed the research. RCG,
551 APA, RM, KGM, AM, DRM, SQG, MCM, AAA, IRO, MCO, EHG, YRL, NPM, GIR,
552 YSA, DVP, KVS performed experiments. RCG, RMM, APA and DRM acquired the
553 data of the study. RCG, APA and DRM analyzed and interpreted the data. RCG, RMM,
554 APA, DRM and SQG wrote the paper, and all authors revised it, read and approved the
555 final manuscript.

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557 **References**

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