TITLE:

Immunogenicity and safety in pigs of PHH-1V, a SARS-CoV-2 RBD fusion heterodimer vaccine candidate

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ABSTRACT

The continuing high global incidence of COVID-19 and the undervaccinated status of billions of persons strongly motivate the development of a new generation of efficacious vaccines. We have developed an adjuvanted vaccine candidate, PHH-1V, based on a protein comprising the receptor binding domain (RBD) of the Beta variant of SARS-CoV-2 fused in tandem with the equivalent domain of the Alpha variant, with its immunogenicity, safety and efficacy previously demonstrated in mouse models. In the present study, we immunized pigs with different doses of PHH-1V in a prime-and-boost scheme showing PHH-1V to exhibit an excellent safety profile in pigs and to produce a solid RBD-specific humoral response with neutralising antibodies to 7 distinct SARS-CoV-2 variants of concern, with the induction of a significant IFNγ^+ T-cell response. We conclude that PHH-1V is safe and elicits a robust immune response to SARS-CoV-2 in pigs, a large animal preclinical model.

KEYWORDS:

SARS-CoV-2; COVID-19; RBD; recombinant vaccine; humoral immune response; IFNγ.

47 # INTRODUCTION

48 Coronavirus disease-19 (COVID-19) was declared a pandemic by the World Health Organization
49 (WHO) in March 2020 due to its widespread transmission, prompting a global effort to develop
50 safe and effective vaccines against SARS-CoV-2, with more than 240 candidate vaccines currently
51 under clinical development [1]. These include messenger RNA, protein-based and viral vector-
52 based vaccines targeting the viral spike protein or its Receptor Binding Domain (RBD), and
53 conventional inactivated vaccines targeting the entire virus [2]. Several vaccines have shown
54 great efficacy in protecting from severe disease, and have been deployed and administered in
55 an unprecedented global vaccination effort. However, the continued emergence of new viral
56 variants with increasing immune escape and transmissibility characteristics compromises the
57 intended control of the pandemic [3]. In addition, an unequal distribution of currently available
58 vaccines is present, with over 80% of the population in low- and middle-income countries not
59 having received any SARS-CoV-2 vaccine dose (as of July 2022) [1].

60 With these motivations, we have developed the adjuvanted PHH-1V vaccine candidate based on
61 a fusion heterodimer protein containing, in tandem, the RBD of the B.1.351 (Beta) and B.1.1.7
62 (Alpha) SARS-CoV-2 variants at its N- and C-terminus, respectively. As a result, a key feature of
63 PHH-1V distinguishing it from other adjuvanted protein-based subunit vaccines is the inclusion
64 of sequences corresponding to two heterologous variants of concern (VOCs) as part of the same
65 protein molecule. The safety, immunogenicity and efficacy of the PHH-1V vaccine candidate
66 were previously tested in mouse models [4]. Recently, EMA's human medicines committee
67 (CHMP) has recommended authorising the PHH-1V vaccine (Bimervax) as a booster in people
68 aged 16 years and above who have been vaccinated with an mRNA COVID-19 vaccine.In the
69 present study we have further investigated its safety and immunogenicity in pigs - a large animal
70 model involving an immune system with physiologically relevant similarities to humans [5].

MATERIALS AND METHODS

PHH-1V vaccine

PHH-1V is a bivalent recombinant protein vaccine based on a heterodimer containing the receptor binding domain (RBD) of two SARS-CoV-2 variants, Beta and Alpha. The SARS-CoV-2 Wuhan-Hu-1 Spike RBD sequence corresponds to amino acids 319-541, according to the code P0DTC2 [6]. Based on this sequence, a dimer of RBDs was designed containing the mutations of the SARS-CoV-2 Beta variant (B.1.351) at its N-terminus and the mutation of the Alpha variant (B.1.1.7) at its C-terminus. The RBD heterodimer is expressed in CHO cells with stable expression (clone 57) and adjuvanted with SQBA, an oil-in-water emulsion based on squalene, in which the oil particles are dispersed to the aqueous phase. SQBA adjuvant contains per 0.5 mL dose: squalene (9.75 mg), polysorbate 80 (1.18 mg), sorbitan trioleate (1.18 mg), sodium citrate (0.66 mg), citric acid (0.04 mg), and water for injection.

Animal study design

Large White x Landrace hybrid pigs were used as a model to assess immune responses to PHH-1V. In the first study (Study 1), three groups of six pigs (3 females and 3 males) were administered with three different doses of PHH-1V vaccine (Group A: 10 µg; Group B: 20 µg; Group C: 40 µg), and one group of four pigs (two females and two males) received PBS (Group D) **(Figure 1)**. In the second study (Study 2) for safety and cellular immune response analysis, ten male pigs were distributed into one group of six pigs administered 40 µg PHH-1V per dose (Group 1) and a second group of four pigs administered PBS (Group 2). In both studies, pigs received an intramuscular injection in a total volume of 0.5 mL of test product on days 0 (D0) and 21 (D21) **(Figure 1)**. Safety was assessed by monitoring general clinical signs (recorded daily) and rectal temperatures (recorded one day before each dosage, at vaccination, 4 and 6 hours after that, and daily for 5 days after the first injection and 2 days after the second injection) and local reactions (inflammation and nodules) at the injection site (observed daily) throughout the study. Blood samples for the different analyses were extracted from the external jugular vein of the animals.

***Ex vivo* assays**

ELISA determinations for SARS-CoV-2 RBD-binding antibodies

MaxiSorp plates (Nunc) were coated with 100 µL/well of 1 µg/mL of SARS-CoV-2 RBD protein (Sino Biologicals) O/N at 4 ºC, then washed with 300 µL/well of CIVTEST solution (Laboratorios HIPRA) and blocked with 250 µL/well of StabilBlock Immunoassay Stabilizer (Surmodics) for 1 hour at room temperature. Wells were incubated with 100 µL of serial dilutions of the serum samples, then washed again, and after that, the bound total IgG specific antibodies were

107 detected by 100 µL of peroxidase-conjugated AffiniPure Goat Anti-Swine IgG (H+L) (Jackson
108 ImmunoResearch) incubated for 1 hour at 37 ºC. After washing, an incubation with 100 µL of
109 TMB Substrate (Neogen) during 15 minutes at room temperature protected from light was done.
110 Then 100 µL of TMB stop solution was added and measurement of absorbance at 450 nm in a
111 Versamax instrument (Molecular Devices) was performed. The end-point titre of RBD-bound
112 total IgG antibodies was established as the reciprocal of the last serum dilution that yielded
113 three times the mean optical density of the negative control.

114

115 ***Pseudovirus-based neutralisation assays (PBNA)***

116 Pseudoviruses were generated by cotransfection of Expi293F cells (Thermo Fisher Scientific)
117 with the pNL4-3.Luc.R-.E- vector (NIH AIDS repository) and a pcDNA3.1(+) plasmid coding for
118 SARS-CoV-2 S protein (GeneArt) with a deletion of the C-terminal 19 amino acids and human-
119 codon optimised. The same procedure was followed to generate expression plasmids for the
120 Alpha, Beta, Gamma, Delta, Delta plus and Omicron (BA.1) variants of the SARS-CoV-2 spike
121 protein. Transfections were carried out using ExpiFectamine Reagent (Thermo Fisher Scientific)
122 according to manufacturer instructions at an 8:1 ratio (HIV:Spike). After 48 hours of culture at
123 37 ºC and 8% CO_2, supernatants were harvested, filtered through 0.45 µm filters, frozen, and
124 subsequently titrated on HEK293T cells overexpressing human ACE-2 (Integral Molecular).

125 The neutralization assay has been previously validated in a large subset of samples with a
126 replicative viral inhibition assay [7]. Neutralization assays were performed in duplicate in Nunc
127 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ of pseudovirus were
128 preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma
129 samples for 1 hour at 37 ºC in a total volume of 150 µL. Then, $2x10^4$ HEK293T/hACE2 cells treated
130 with DEAE-Dextran (Sigma-Aldrich) were added to each well. After 48 hour-incubation at 37 ºC
131 and 5% CO_2, luminiscence was determined using the EnSight Multimode Plate Reader and
132 BriteLite Plus Luciferase reagent (PerkinElmer). Percentage neutralisation was calculated as
133 (RLUmax–RLUexp)/(RLUmax–RLUmin)*100, with RLUexp being the experimental infectivity
134 calculated from infected cells treated with each serum, RLUmax the maximal infectivity
135 calculated from untreated infected cells, and RLUmin the minimal infectivity calculated from
136 uninfected cells. IC_{50} values were calculated by fitting the neutralisation values and the log of
137 plasma dilution to a 4-parameter equation using Prism 9.0.2 (GraphPad Software, USA).

138

139 ***RBD-induced IFNγ ELISpot assay***

140 A total of 5×10^5 peripheral blood mononuclear cells (PBMCs) per well in 100 µL/well were plated
141 and *ex vivo* stimulated with other 100 µL/well of a 1:1 mixture of RBD-overlapping peptides from
142 the SARS-CoV-2 B.1.1.7 and B.1.351 lineages (1 µg/mL per peptide, final concentration), with
143 complete RPMI (negative control), or with 2.5 ng/mL PMA plus 250 ng/mL ionomycin (positive

144 control; both from Sigma). After 18-20 hours incubation at 37 ºC, plates were developed using
145 reagents and following procedures and incubation periods described in the pig IFNγ ELISpot Plus
146 kit (3130-4HPW-10, Mabtech). Spots were counted under a dissection microscope (Leica GZ6).
147 Data were expressed as the number of RBD-specific IFNγ-secreting cells per 1×10^6 PBMCs.

148 **Statistical analysis**

149 Statistical analyses and plots were generated using R (version 4.0.5) or GraphPad Prism (version
150 9). All plots depict individual data points for each animal, along with the sample mean and
151 standard deviation. The exact number (n) used in each experiment is indicated in the caption
152 below each figure. Data transformations were conducted to ensure a better distribution for
153 appropriate model fitting: for the analysis of log-normal variables (IgG and neutralising
154 antibodies) data was $\log_{10}$-transformed, while for the analysis of percentage variables (ELISpot
155 and intracellular staining) data was arcsine-square root-transformed. Linear mixed effects
156 models of repeated measures were fitted with the longitudinal data. In these models, the
157 treatment group, the timepoint and the group-by-timepoint interaction were introduced as
158 fixed effects and the animal ID as a random effect. Non-significant terms (at the 5% level) were
159 sequentially removed from the models (backward elimination). Linear models were employed
160 for the analysis of the temperature and humoral and cellular immunity data. For the
161 temperature and IgG data, mixed effects models were used to account for the repeated
162 measures design of the experiments, considering time and treatment group (and the two-way
163 interaction, if supported by the model) as fixed factors and the animal identifier as random
164 effect. For the analysis of the neutralising antibodies, a mixed effects model was used to account
165 for the repeated measures design of the experiment, considering the treatment group and the
166 SARS-CoV-2 variant as fixed factors and the animal identifier as a random effect. Also for
167 neutralising antibodies, vaccinated groups were analysed with an ANOVA and the pooled data
168 (groups A, B, C) was compared against the PBS group by means of a one-sample t-test (null
169 hypothesis=1.31). On the other hand, generalised least squares models were fitted for the
170 cellular immunity data to account for data heteroskedasticity. For all models, parameter
171 estimation was performed using restricted maximum likelihood and assumptions were tested
172 graphically.

173 Statistically significant differences between groups are indicated with a line on top of each
174 group: ** $p<0.01$; * $p<0.05$; $^{+}$ $0.05<p<0.1$.

175 # RESULTS

176 **PHH-1V displays a good safety profile in pigs**

177 Rectal temperatures showed no differences between groups (**Supplementary Figure 1**). Local
178 reactions at the injection site or general clinical signs were not observed in any animal
179 throughout the study. Furthermore, in the Study 1, the maximum individual increases after first
180 and second administration were 0.59 ºC and 0.68 ºC, respectively, in both cases in group B. On
181 the other hand, in the Study 2, the maximum individual temperature increases after first and
182 second administration were 1.16 ºC and 0.43 ºC, respectively, in both cases in group 1.

183

184 **PHH-1V elicits a robust humoral response**

185 After acclimation, pigs were immunized (day 0) and boosted (day 21) with three different doses
186 of PHH-1V. Seven days after the second vaccination (day 28), the sera from all the animals were
187 analysed by ELISA to determine the RBD-binding antibodies. Immunization induced significant
188 levels of RBD-specific IgG antibodies in all vaccinated groups compared to the control group
189 (**Figure 2A and Supplementary Table 1**). In all dosage groups, the antibody titres remained at
190 stably elevated levels until the end of the study on day 42, with significant differences compared
191 to control animals (**Figure 2A**).

192

193 **PHH-1V elicits neutralising antibodies to all SARS-CoV-2 variants of concern**

194 A prime-boost of PHH-1V (40 µg/dose) elicited significant titres of neutralising antibodies (nAbs)
195 against all VOCs analysed by PBNA compared to non-vaccinated animals in sera collected on day
196 42 post-prime dose (**Figure 2B**). When compared to the Alpha and Beta variants, the neutralising
197 titre of the sera was slightly reduced against Gamma, Mu, Delta and Omicron BA.1 variants
198 (**Figure 2B and Supplementary Table 2**). No significant differences were observed between PHH-
199 1V doses in the neutralising titres against Alpha variant on day 42 (**Figure 2C**).

200

201 **PHH-1V elicits a robust cellular immune response**

202 To determine the cellular immune response (Study 2), peripheral blood mononuclear cells
203 (PBMCs) were isolated on day 40 and tested by IFNγ ELISpot *ex vivo* for their reactivity towards
204 a pool of RBD peptides representing Alpha and Beta SARS-CoV-2 variant epitopes. The PBMCs
205 from vaccinated pigs (40 µg RBD fusion heterodimer/dose) showed a significant higher
206 percentage of IFNγ^{+} T-cells than in control animals after *in vitro* re-stimulation (**Figure 3A**).

207 In addition, in PHH-1V immunised pigs a tendency was observed in the activation antigen-
208 specific Th1 response of CD4^{+} and CD8^{+} T cells expressing IFNγ and/or IL-2 and/or TNF-α after *in*

209 *vitro* re-stimulation **(Figures 3B and 3C)**. Supplementary Figure 3 shows individual frequencies
210 of cytokine expressing T cells.

211 **DISCUSSION**

212 An unprecedented effort has enabled the development of a substantial number of vaccines and
213 several therapies against SARS-CoV-2, a novel virus that has caused an enormous impact on lives
214 [1]. Many vaccine candidates have been designed, using broadly diverse platforms and
215 technologies, which has afforded the production of the large numbers of doses required for
216 mass vaccination programs. Despite the phenomenal success and effectiveness of several of
217 these vaccines, the continuing pandemic, the insufficient distribution of vaccines to many
218 developing countries, and the emergence of new immune escape variants endowed with
219 increasing transmissibility [8], must be urgently addressed with an end to the pandemic as a goal
220 - including the development of a new generation of affordable vaccines featuring higher efficacy
221 against emerging variants and improved safety [3].

222 In the present study we have examined the safety and immunogenic potential of the RBD
223 subunit-based vaccine candidate, PHH-1V, in pigs which is a large animal presenting an immune
224 system with significant similarities to humans [5], and a useful pre-clinical model to for assessing
225 the immunogenicity of upcoming SARS-CoV-2 vaccine candidates [9]. We have found that two
226 vaccination doses are safe and induce high and specific humoral and cellular immune responses.
227 As expected, PHH-1V elicited strong RBD-binding IgG antibodies after two doses. Notably, it also
228 generated significant neutralisation activity, determined by pseudovirus-based assays, against
229 all tested VOCs (Alpha, Beta, Gamma, Delta and Omicron BA.1). The results of ELISpot assaying
230 showed that 40 µg of PHH-1V induces significantly higher percentage of T-cells expressing IFNγ
231 than mock-vaccinated pigs, being this dose a candidate to induce both humoral and cellular
232 responses.

233 Neutralising antibody levels correlate well with vaccine protective efficacy [10]. As the RBD bears
234 immunodominant neutralisation epitopes [11], a vaccine consisting of RBD, rather than a full-
235 length S protein, is expected to induce more effectively RBD-focused antibodies [12, 13]. A
236 second crucial feature of PHH-1V is the simultaneous presence in the same protein of RBDs for
237 two heterologous VOCs, Beta and Alpha, in a two-domain tandem fusion configuration [14]. We
238 have found that PHH-1V induces neutralising antibodies that are cross-reactive against the
239 majority VOCs with the strongest presence to date, including the highly immunoevasive Beta,
240 Gamma and Omicron BA.1 variants, after two doses. The presence of known immune escape
241 mutations on PHH-1V, such as K417N, E484K or N501Y [15, 16], may be partially responsible for
242 the neutralisation cross-reactivity observed against variants sharing these mutations, such as
243 Alpha (N501Y), Beta (K417N, E484K, N501Y), Gamma (E484K, N501Y), Mu (E484K) or Omicron
244 (K417N, N501Y), although epitopes not containing such immune escape mutations must play a
245 role in eliciting neutralising antibodies against the Delta SARS-CoV-2 variant [17-19].

246 A limitation of the present study is that a challenging analysis after the PHH-1V vaccine is not
247 feasible, as the pig model is not susceptible to SARS-CoV-2 [20]. Nevertheless, our previous
248 challenge studies in mice models and non-human primates have demonstrated efficacy, safety

249 and protection from the viral challenge of PHH-1V [4]. Future studies should address additional
250 relevant aspects, such as the immune response to booster doses or the induction of B and T cell
251 memory.

252

253 CONCLUSIONS

254 In conclusion, PHH-1V exhibited an excellent safety profile and a strong and specific immune
255 response in pigs, showing a broadly cross-reactive neutralisation activity against heterologous
256 VOCs, and IFNγ^{+} T-cells activation. These results and those observed in other pre-clinical models,
257 support the implementation of the PHH-1V vaccine as a primary or booster vaccination schedule
258 in humans to prevent the consequences of SARS-CoV-2 infection. Further Phase IIb and III clinical
259 trials are ongoing to support the potential of this candidate vaccine as a heterologous booster
260 (NCT05142553, NCT05246137) [21, 22].

261 ## FIGURE LEGENDS

262 **Figure 1. Experimental procedure schematics.** Two-dose prime-and-boost intra-muscular
263 immunization on days 0 and 21 (0.5 mL/dose), with a 42-day follow-up. Two independent studies
264 were performed. Study 1: For safety and humoral response monitoring, three experimental
265 groups (A-C) were inoculated (n = 6/group; 3 female and 3 male) with 10 µg, 20 µg or 40 µg of
266 PHH-1V. A control group (D; n = 4; 2 female and 2 male) was inoculated with PBS. Study 2: For
267 safety and cellular response monitoring, pigs of group 1 (n = 6; 6 male) were inoculated with 40
268 µg of PHH 1V. Control group 2 (n = 4; 4 male) was inoculated with PBS.

269

270 **Figure 2. Vaccination of pigs with PHH-1V elicits SARS-CoV-2 RBD-specific humoral and**
271 **neutralising antibody responses against SARS-CoV-2 variants of concern (VOCs). A.**
272 Longitudinal analysis of RBD-specific IgG determined in samples collected on days 0, 21, 28, 35
273 and 42. Group A: 10 µg PHH-1V RBD fusion heterodimer; Group B: 20 µg PHH-1V RBD fusion
274 heterodimer; Group C: 40 µg PHH-1V RBD fusion heterodimer; D: Control PBS. Each data point
275 represents an individual pig with bars denoting the mean ± standard deviation per group. IgG
276 longitudinal data have been analysed by means of a linear mixed effects model of repeated
277 measures with the $\log_{10}$-transformed titres. * $p<0.05$; ** $p<0.01$, indicating statistically
278 significance vs. Control group (D). **B.** Neutralisation activity by sera collected on day 42 after the
279 first vaccine dose from Group C (40 µg PHH-1V) vs. Group D (PBS) against 7 different VOCs: α
280 (B.1.1.7), β (B.1.351), γ (P.1), µ (B.1.621), δ (B.1.617.2), δ+ (B.1.617.2.1) and o (BA.1).
281 Pseudovirus neutralisation assays were conducted, yielding titres expressed as $\log_{10}$ IC_{50}. Each
282 data point represents an individual pig with bars denoting the mean ± standard deviation per
283 group. PBNA IC_{50} longitudinal data have been analysed by means of a linear mixed effects model
284 of repeated measures with the $\log_{10}$-transformed titres. ** $p<0.01$. Titre values are provided in
285 **Table S2**. **C.** Neutralization activity by sera collected on day 42 after the first vaccine dose from
286 Group A: 10 µg PHH-1V RBD fusion heterodimer; Group B: 20 µg PHH-1V RBD fusion
287 heterodimer; Group C: 40 µg PHH-1V RBD fusion heterodimer; and D: Control PBS. Pseudovirus
288 neutralisation assays were conducted, yielding titres expressed as $\log_{10}$ IC_{50}. Each data point
289 represents an individual pig with bars denoting the mean ± standard deviation per group. PBNA
290 data in vaccinated groups were compared with an ANOVA and the pooled data (groups A, B, C)
291 was compared against the PBS group by means of a one-sample t-test (null hypothesis =1.31)..
292 Differences between vaccinated groups were not significant but the three groups were
293 significantly different from the control group. ** $p<0.01$ indicating statistically significance vs.
294 Control group (D).

295

296 **Figure 3. Vaccination of pigs with PHH-1V elicits cellular response. A.** Induction of IFNγ$^+$ T-cells
297 determined by ELISpot assays in PBMCs from vaccinated pigs, sampled on day 40 and stimulated
298 *ex vivo* with an RBD peptide pool representing Alpha and Beta SARS-CoV-2 variant epitopes.

299 Group 1: 40 µg PHH-1V RBD fusion heterodimer; Group 2: Control PBS. Each data point
300 represents an individual pig with bars denoting the mean ± standard deviation per group.
301 ELISpot data have been analysed by means of a generalised least squares model with the arcsine-
302 square root-transformed proportion of IFNγ -positive cells * $p<0.05$. **B-C.** $CD4^+$ (**B)** and $CD8^+$ **(C)**
303 T cells expressing IFNγ and/or IL-2 and/or TNF-α was measured by intracellular cytokine staining
304 (ICS) after the stimulation of PMBCs with a pool of peptides from SARS-CoV-2 RBD of Alpha and
305 Beta variants. Cytokine expression of PBMCs stimulated with medium was considered as
306 background and subtracted from the responses measured from the RBD peptide pool for each
307 individual pig. Group 1: PHH-1V vaccine 40 µg RBD fusion heterodimer/dose; Group 2: control
308 group (PBS). Each data point represents an individual pig with bars denoting the mean ±
309 standard deviation per group. ICS data have been analysed by means of a generalised least
310 squares model with the arcsine-square root-transformed proportion of positive cells $^+$
311 $0.05<p<0.1$.

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DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A.Mo., A.P., A.B., E.P., A.F., M.C., L.G., C.G., T.P.C., R.M., and L.F. are employees of HIPRA. E.P. reports travel reimbursement from Gilead. J.B. reports consultancy fees from Albajuna Therapeutics; grants (outside the submitted work) from HIPRA, Grifols, Nesapor, and MSD; and speaking fees and travel reimbursement from Gilead. P.C.R., M.S.-O., and A.Me. reports grants (outside the submitted work) from HIPRA. S.M. declares no conflicts of interest.

333 **REFERENCES**

334 [1] WHO. WHO Coronavirus (COVID-19) Dashboard. 2022. https://covid19.who.int/. (Accesed
335 July 4th, 2022)
336 [2] Mellet J, Pepper MS. A COVID-19 Vaccine: Big Strides Come with Big Challenges. Vaccines
337 (Basel). 2021;9. https://doi.org/10.3390/vaccines9010039.
338 [3] Altmann DM, Boyton RJ. COVID-19 vaccination: The road ahead. Science. 2022;375:1127-32.
339 https://doi.org/10.1126/science.abn1755.
340 [4] Barreiro A, Prenafeta A, Bech-Sabat G, Roca M, Perozo Mur E, March R, et al. Preclinical
341 evaluation of a COVID-19 vaccine candidate based on a recombinant RBD fusion heterodimer of
342 SARS-CoV-2. iScience. 2023;26:106126. https://doi.org/10.1016/j.isci.2023.106126.
343 [5] Pabst R. The pig as a model for immunology research. Cell Tissue Res. 2020;380:287-304.
344 https://doi.org/10.1007/s00441-020-03206-9.
345 [6] Portal ESBR. Spike glycoprotein OS=Severe acute respiratory syndrome coronavirus 2. 2023.
346 https://viralzone.expasy.org/resources/Coronav/Wuhan-Hu-1_Spike.fasta. (Accesed
347 03/28/2023, 2023)
348 [7] Trinité B, Tarrés-Freixas F, Rodon J, Pradenas E, Urrea V, Marfil S, et al. SARS-CoV-2 infection
349 elicits a rapid neutralizing antibody response that correlates with disease severity. Scientific
350 Reports. 2021;11:2608. https://doi.org/10.1038/s41598-021-81862-9.
351 [8] DeGrace MM, Ghedin E, Frieman MB, Krammer F, Grifoni A, Alisoltani A, et al. Defining the
352 risk of SARS-CoV-2 variants on immune protection. Nature. 2022;605:640-52.
353 https://doi.org/10.1038/s41586-022-04690-5.
354 [9] Lara-Puente JH, Carreño JM, Sun W, Suárez-Martínez A, Ramírez-Martínez L, Quezada-
355 Monroy F, et al. Safety and Immunogenicity of a Newcastle Disease Virus Vector-Based SARS-
356 CoV-2 Vaccine Candidate, AVX/COVID-12-HEXAPRO (Patria), in Pigs. mBio. 2021;12:e0190821.
357 https://doi.org/10.1128/mBio.01908-21.
358 [10] Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing
359 antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2
360 infection. Nat Med. 2021;27:1205-11. https://doi.org/10.1038/s41591-021-01377-8.
361 [11] Goel RR, Painter MM, Apostolidis SA, Mathew D, Meng W, Rosenfeld AM, et al. mRNA
362 vaccines induce durable immune memory to SARS-CoV-2 and variants of concern. Science.
363 2021;374:abm0829. https://doi.org/10.1126/science.abm0829.
364 [12] Burton DR, Walker LM. Rational Vaccine Design in the Time of COVID-19. Cell Host Microbe.
365 2020;27:695-8. https://doi.org/10.1016/j.chom.2020.04.022.
366 [13] Liu Z, Xu W, Xia S, Gu C, Wang X, Wang Q, et al. RBD-Fc-based COVID-19 vaccine candidate
367 induces highly potent SARS-CoV-2 neutralizing antibody response. Signal Transduct Target Ther.
368 2020;5:282. https://doi.org/10.1038/s41392-020-00402-5.
369 [14] Wang CY, Hwang KP, Kuo HK, Peng WJ, Shen YH, Kuo BS, et al. A multitope SARS-CoV-2
370 vaccine provides long-lasting B cell and T cell immunity against Delta and Omicron variants. J
371 Clin Invest. 2022;132. https://doi.org/10.1172/jci157707.
372 [15] Jangra S, Ye C, Rathnasinghe R, Stadlbauer D, Krammer F, Simon V, et al. SARS-CoV-2 spike
373 E484K mutation reduces antibody neutralisation. Lancet Microbe. 2021;2:e283-e4.
374 https://doi.org/10.1016/s2666-5247(21)00068-9.
375 [16] Liu Y, Liu J, Plante KS, Plante JA, Xie X, Zhang X, et al. The N501Y spike substitution enhances
376 SARS-CoV-2 infection and transmission. Nature. 2022;602:294-9.
377 https://doi.org/10.1038/s41586-021-04245-0.
378 [17] Debes AK, Xiao S, Egbert ER, Caturegli P, Sitaras I, Pekosz A, et al. Comparison of total and
379 neutralizing SARS-CoV-2 spike antibodies against omicron and other variants in paired samples
380 after two or three doses of mRNA vaccine. medRxiv. 2022.
381 https://doi.org/10.1101/2022.01.26.22269819.
382 [18] Wratil PR, Stern M, Priller A, Willmann A, Almanzar G, Vogel E, et al. Three exposures to the
383 spike protein of SARS-CoV-2 by either infection or vaccination elicit superior neutralizing

immunity to all variants of concern. Nat Med. 2022;28:496-503. https://doi.org/10.1038/s41591-022-01715-4.
[19] Goel RR, Painter MM, Lundgreen KA, Apostolidis SA, Baxter AE, Giles JR, et al. Efficient recall of Omicron-reactive B cell memory after a third dose of SARS-CoV-2 mRNA vaccine. Cell. 2022;185:1875-87.e8. https://doi.org/10.1016/j.cell.2022.04.009.
[20] Vergara-Alert J, Rodon J, Carrillo J, Te N, Izquierdo-Useros N, Rodríguez de la Concepción ML, et al. Pigs are not susceptible to SARS-CoV-2 infection but are a model for viral immunogenicity studies. Transbound Emerg Dis. 2021;68:1721-5. https://doi.org/10.1111/tbed.13861.
[21] Leal L, Pich J, Ferrer L, Nava J, Martí-Lluch R, Esteban I, et al. Safety and Immunogenicity of a Recombinant Protein RBD Fusion Heterodimer Vaccine against SARS-CoV-2: preliminary results of a phase 1-2a dose-escalating, randomized, double-blind clinical trial. medRxiv. 2022:2022.08.09.22278560. https://doi.org/10.1101/2022.08.09.22278560.
[22] Corominas JG, C. Prenafeta, A. Moros, A. Cañete, M. Barreiro, A. . Safety and immunogenicity of the protein-based PHH-1V compared to BNT162b2 as a heterologous SARS-CoV-2 booster vaccine in adults vaccinated against COVID-19: a multicentre, randomised, double-blind, non-inferiority phase IIb trial. Lancet Reg Health Eur. 2023. https://doi.org/10.1016/j.lanepe.2023.100613

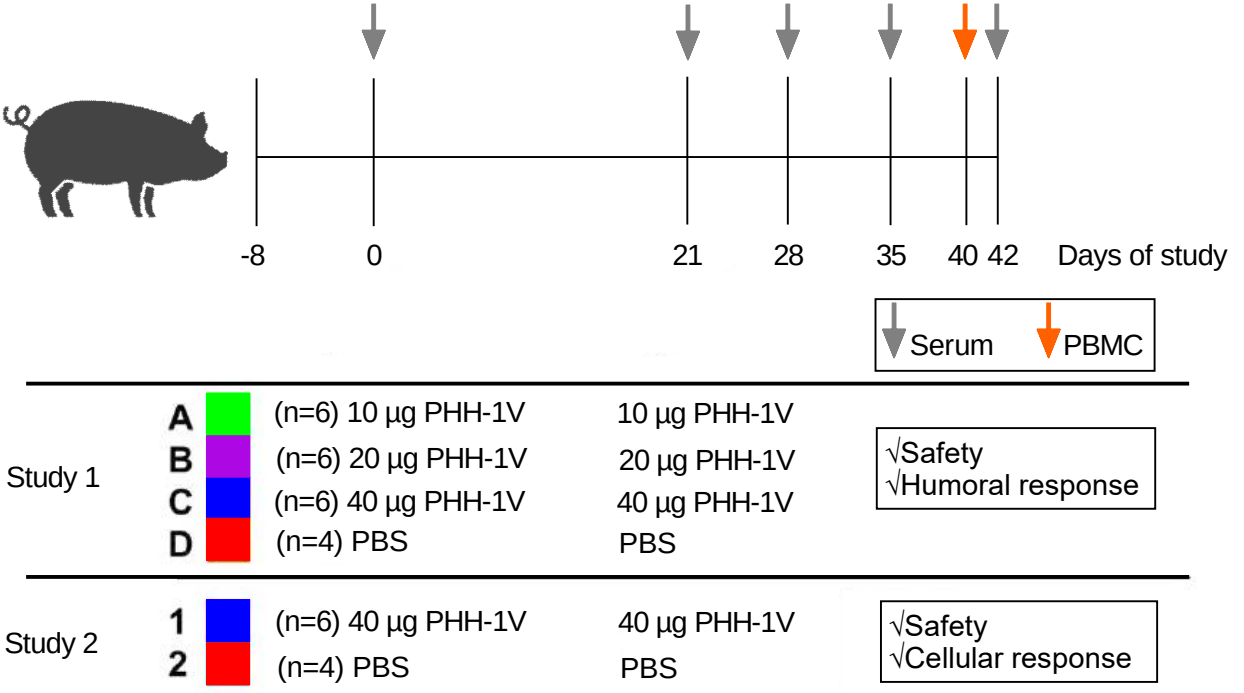
-8
0
21
28
35
40
42
Days of study
Serum
PBMC
Study 1
A
B
C
D
(n=6) 10 µg PHH-1V
(n=6) 20 µg PHH-1V
(n=6) 40 µg PHH-1V
(n=4) PBS
10 µg PHH-1V
20 µg PHH-1V
40 µg PHH-1V
PBS
√Safety
√Humoral response
Study 2
1
2
(n=6) 40 µg PHH-1V
(n=4) PBS
40 µg PHH-1V
PBS
√Safety
√Cellular response

Figure 1

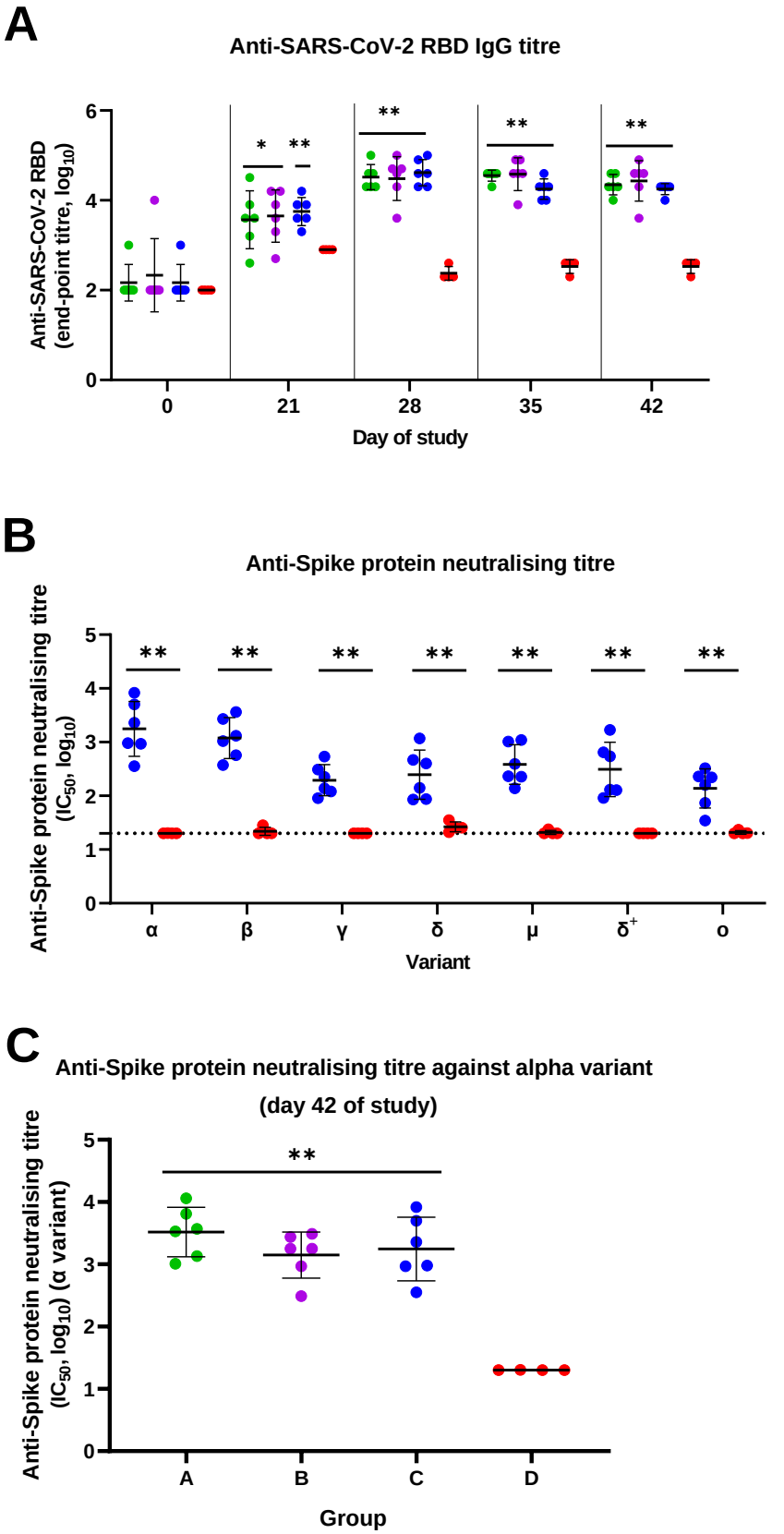
A
Anti-SARS-CoV-2 RBD IgG titre
Anti-SARS-CoV-2 RBD (end-point titre, log10)
Day of study
0
21
28
35
42
B
Anti-Spike protein neutralising titre
Anti-Spike protein neutralising titre (IC50, log10)
Variant
α
β
γ
δ
μ
δ+
ο
C
Anti-Spike protein neutralising titre against alpha variant (day 42 of study)
Anti-Spike protein neutralising titre (IC50, log10) (α variant)
Group
A
B
C
D
A: PHH-1V (10 µg RBD fusion heterodimer/dose)
B: PHH-1V (20 µg RBD fusion heterodimer/dose)
C: PHH-1V (40 µg RBD fusion heterodimer/dose)
D: Control (PBS)

Figure 2

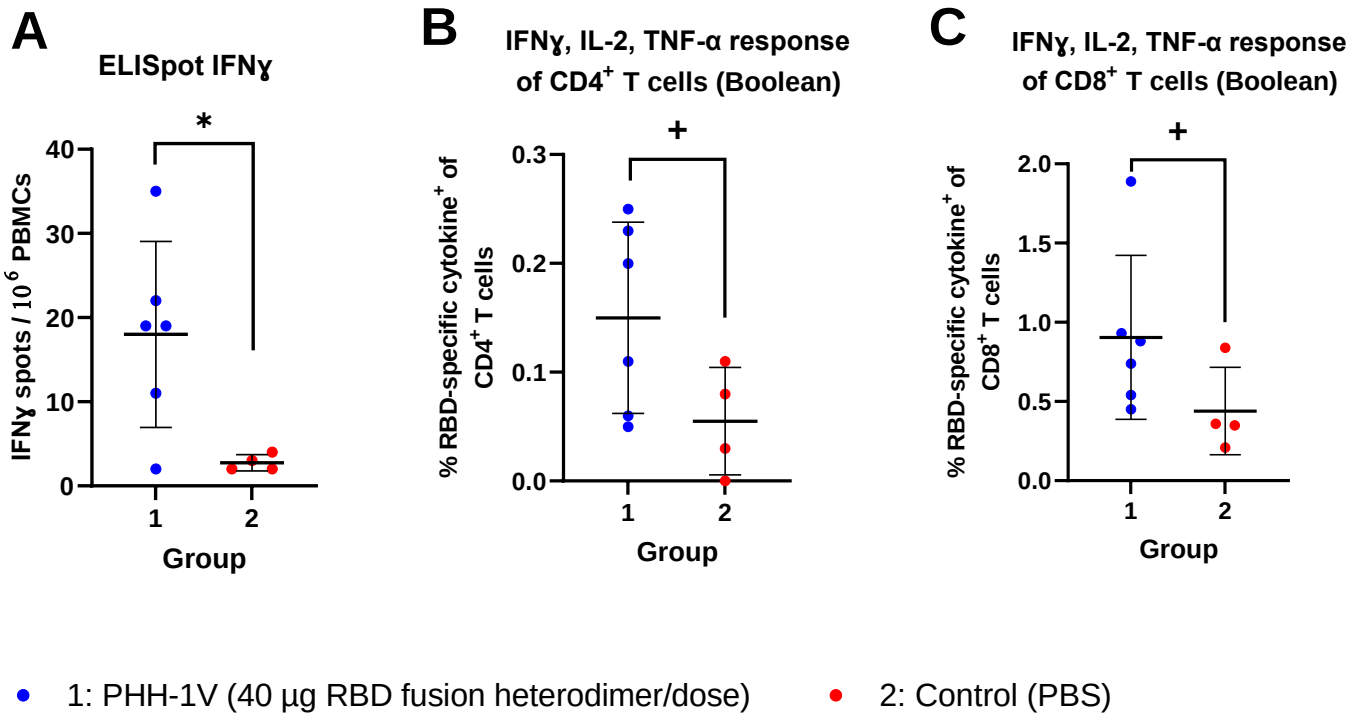
A
ELISpot IFNɣ
*
IFNɣ spots / 10^6 PBMCs
40
30
20
10
0
1
2
Group
B
IFNɣ, IL-2, TNF-α response of CD4+ T cells (Boolean)
+
% RBD-specific cytokine+ of CD4+ T cells
0.3
0.2
0.1
0.0
1
2
Group
C
IFNɣ, IL-2, TNF-α response of CD8+ T cells (Boolean)
+
% RBD-specific cytokine+ of CD8+ T cells
2.0
1.5
1.0
0.5
0.0
1
2
Group
1: PHH-1V (40 µg RBD fusion heterodimer/dose)
2: Control (PBS)

Figure 3