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.

48 # INTRODUCTION

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

61 With these motivations, we have developed the adjuvanted PHH-1V vaccine candidate based on
62 a fusion heterodimer protein containing, in tandem, the RBD of the B.1.351 (Beta) and B.1.1.7
63 (Alpha) SARS-CoV-2 variants at its N- and C-terminus, respectively. As a result, a key feature of
64 PHH-1V distinguishing it from other adjuvanted protein-based subunit vaccines is the inclusion
65 of sequences corresponding to two heterologous variants of concern (VOCs) as part of the same
66 protein molecule. The safety, immunogenicity and efficacy of the PHH-1V vaccine candidate
67 were previously tested in mouse models [4]. PHH-1V is currently under rolling review by the
68 European Medicines Agency (EMA).

69 In the present study we have further investigated its safety and immunogenicity in pigs - a large
70 animal model involving an immune system with physiologically relevant similarities to humans
71 [5].

72 # MATERIALS AND METHODS

73 **PHH-1V vaccine**

74 The PHH-1V vaccine is composed of a recombinant protein formed by the fusion of the RBD
75 domain of the Beta variant of SARS-CoV-2 (B.1.351) at its N-terminus and the RBD domain of the
76 Alpha variant (B.1.1.7) at its C-terminus, expressed in CHO cells with stable expression (clone
77 57), adjuvanted with SQBA, an oil-in-water emulsion based on squalene, as previously described
78 elsewhere [4]. Phosphate-buffered saline (PBS) was inoculated as a negative control.

79

80 **Animal study design**

81 Large White x Landrace hybrid pigs were used as a model to assess immune responses to PHH-
82 1V. Twenty-four pigs (12 females and 12 males) were distributed into three dosage groups
83 (Group A: 10 µg; Group B: 20 µg; Group C: 40 µg), and one group of four pigs (2 females and 2
84 males) received PBS (Group D) **(Figure 1)**. In a second arm of the study for safety and cellular
85 immune response analysis, 10 male pigs were distributed into one group of 6 pigs administered
86 40 µg PHH-1V per dose (Group 1) and a second group of 4 pigs administered PBS (Group 2). Pigs
87 received an intramuscular injection in a total volume of 0.5 mL of test product on days 0 (D0)
88 and 21 (D21) in both arms of the study **(Figure 1)**. Safety was assessed by monitoring general
89 clinical signs (recorded daily) and rectal temperatures (recorded one day before each dosage, at
90 vaccination, 4 and 6 hours after that, and daily for 5 days after the first injection and 2 days after
91 the second injection) and local reactions (inflammation and nodules) at the injection site
92 (observed daily) throughout the study.

93

94 ***Ex vivo* assays**

95 Materials and methods for ELISA determinations of SARS-CoV-2 RBD-binding antibodies,
96 pseudovirus-based neutralisation assays (PBNA), and RBD-induced IFNγ ELISpot assay are
97 explained in detail in **Supplementary Data**.

98

99 **Statistical analysis**

100 Statistical analyses and plots were generated using R (version 4.0.5) or GraphPad Prism (version
101 9). Unless otherwise specified, all plots depict individual data points for each animal, along with
102 the sample mean and standard deviation. When required, data was either $\log_{10}$- or arcsine-
103 transformed (i.e. log-normal and percentage variables, respectively). For testing the effect of
104 one or two factors, linear models were generally employed: generalized least squares or mixed
105 effects models depending on the dependence of the observations. Parameter estimation was
106 performed using restricted maximum likelihood and assumptions were tested graphically.

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

109 # RESULTS

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

111 Rectal temperatures showed no differences between groups. Local reactions at the injection site
112 or general clinical signs were not observed in any animal throughout the study (data not shown).

113

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

115 After acclimation, pigs were immunized (day 0) and boosted (day 21) with 3 different doses of
116 PHH-1V. Seven days after the second dose (day 28), the sera from all vaccination groups had
117 significant levels of RBD-specific IgG antibodies (**Figure 2A and Supplementary Table 1**). In all
118 dosage groups, the antibody titres remained at stably elevated levels until the end of the study
119 on day 42, with highly significant differences compared to control animals (**Figure 2A**).

120

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

122 A prime-boost of PHH-1V (40 µg/dose) elicited significant titres of neutralising antibodies (nAbs)
123 against all VOCs analysed compared to non-vaccinated animals in sera collected on day 42 post-
124 prime dose (**Figure 2B**). When compared to the Alpha and Beta variants, the neutralising titre of
125 the sera was slightly reduced against Gamma, Mu, Delta and Omicron BA.1 variants (**Figure 2B**
126 **and Supplementary Table 2**).

127

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

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

135 In addition, in PHH-1V immunised pigs a tendency was observed in the *in vitro* stimulation of
136 PBMCs by predominantly activating antigen-specific CD4$^+$ T helper cells and CD8$^+$ T cytotoxic
137 cells expressing Th1 cytokines (IFNγ and/or IL-2 and/or TNF-α) at 19 days after the second dose
138 (**Figures 3B and 3C)**.

DISCUSSION

An unprecedented effort has enabled the development of a substantial number of vaccines and several therapies against SARS-CoV-2, a novel virus that has caused an enormous impact on lives [1]. Many vaccine candidates have been designed, using broadly diverse platforms and technologies, which has afforded the production of the large numbers of doses required for mass vaccination programs. Despite the phenomenal success and effectiveness of several of these vaccines, the continuing pandemic, the insufficient distribution of vaccines to many developing countries, and the emergence of new immune escape variants endowed with increasing transmissibility [6], must be urgently addressed with an end to the pandemic as a goal - including the development of a new generation of affordable vaccines featuring higher efficacy against emerging variants and improved safety [3].

In the present study we have examined the safety and immunogenic potential of the RBD subunit-based vaccine candidate, PHH-1V, in pigs - a large animal presenting an immune system with significant similarities to humans [5], and a useful pre-clinical model to for assessing the immunogenicity of upcoming SARS-CoV-2 vaccine candidates [7]. We have found that two vaccination doses are safe and induce high and specific humoral and cellular immune responses. As expected, PHH-1V elicited strong RBD-binding IgG antibodies after two doses. Notably, it also generated significant neutralisation activity, determined by pseudovirus-based assays, against all tested VOCs (Alpha, Beta, Gamma, Delta and Omicron BA.1). The results of ELISpot assaying showed that 40 µg of PHH-1V induces significantly higher percentage of T-cells expressing IFNγ than mock-vaccinated pigs - the 40-µg dose being a proper dose candidate to induce significant cellular responses.

Neutralising antibody levels correlate well with vaccine protective efficacy [8]. As the RBD bears immunodominant neutralisation epitopes [9], a vaccine consisting of RBD, rather than a full-length S protein, is expected to induce more effectively RBD-focused antibodies [10, 11]. A second critical feature of PHH-1V is the simultaneous presence in the same protein of RBDs for two heterologous VOCs, Beta and Alpha, in a two-domain tandem fusion configuration. [12]We have found that PHH-1V induces neutralising antibodies that are cross-reactive against all VOCs, including the highly immunoevasive Beta, Gamma and Omicron BA.1 variants, after two doses. The presence of known immune escape mutations on PHH-1V, such as K417N, E484K or N501Y [13, 14], may be partially responsible for the neutralisation cross-reactivity observed against variants sharing these mutations, such as Alpha (N501Y), Beta (K417N, E484K, N501Y), Gamma (E484K, N501Y), Mu (E484K) or Omicron (K417N, N501Y), although epitopes not containing such immune escape mutations must play a role in eliciting neutralising antibodies against the delta SARS-CoV-2 variant.

It has been shown that a third ("booster") vaccine dose, administered in homologous or heterologous immunization schedules, can at least partially restore neutralisation activity to Omicron [15]. The ability of a booster dose to restore the neutralising activity against Omicron

177 is not limited to mRNA-based or vector-based vaccines and has also been observed for
178 inactivated and subunit vaccines or a combination of infection with prior VOCs and vaccination
179 [16]. Such cross-reactive responses may be explained by the expansion of persisting clones of
180 memory B cells, including a response to more conserved regions of the RBD, after three
181 exposures to the SARS-CoV-2 Spike [17]. Given the relative maintenance of neutralisation
182 potency against Omicron BA.1 and Delta provided by PHH-1V we anticipate that a third or
183 booster dose of this vaccine, included as part of a heterologous vaccine scheme, is likely to elicit
184 potent neutralisation and protective immunity against these variants.

185 A limitation of the present study is that a challenging analysis after the PHH-1V vaccine is not
186 feasible, as the pig model is not susceptible to SARS-CoV-2 [18]. Nevertheless, our previous
187 challenge studies in mice models and non-human primates have demonstrated efficacy, safety
188 and protection from the viral challenge of PHH-1V [4]. Future studies should address additional
189 relevant aspects, such as the immune response to booster doses or the induction of B and T cell
190 memory.

191

192 CONCLUSIONS

193 In conclusion, PHH-1V exhibited an excellent safety profile and a strong and specific immune
194 response in pigs, showing a broadly cross-reactive neutralisation activity against heterologous
195 VOCs, and IFNγ^+ T-cells activation. These results and those observed in other pre-clinical models,
196 support the implementation of the PHH-1V vaccine as a primary or booster vaccination schedule
197 in humans to prevent the consequences of SARS-CoV-2 infection. Further Phase IIb and III clinical
198 trials are ongoing to support the potential of this candidate vaccine as a heterologous booster
199 (NCT05142553, NCT05246137).

200 ## FIGURE LEGENDS

201 **Figure 1. Experimental procedure schematics.** Two-dose prime-and-boost intra-muscular
202 immunization on days 0 and 21 (0.5 mL/dose), with a 42-day follow-up. For safety and humoral
203 response monitoring, 3 experimental groups (A-C) were inoculated (n = 6/group; 3 female and
204 3 male) with 10 µg, 20 µg or 40 µg of PHH-1V. A control group (D; n = 4; 2 female and 2 male)
205 was inoculated with PBS. For safety and cellular response monitoring, pigs of group 1 (n = 6; 6
206 male) were inoculated with 40 µg of PHH 1V. Control group 2 (n = 4; 4 male) was inoculated with
207 PBS.

208

209 **Figure 2. Vaccination of pigs with PHH-1V elicits SARS-CoV-2 RBD-specific humoral and**
210 **neutralising antibody responses against SARS-CoV-2 variants of concern (VOCs). A.**
211 Longitudinal analysis of RBD-specific IgG determined in samples collected on days 0, 21, 28, 35
212 and 42. Group A: 10 µg PHH-1V RBD fusion heterodimer; Group B: 20 µg PHH-1V RBD fusion
213 heterodimer; Group C: 40 µg PHH-1V RBD fusion heterodimer; D: Control PBS. Each data point
214 represents an individual pig with bars denoting the mean ± standard deviation per group. IgG
215 longitudinal data have been analysed by means of a linear mixed effects model of repeated
216 measures with the $\log_{10}$-transformed titres. * $p<0.05$; ** $p<0.01$.**B.** Neutralisation activity by sera
217 collected on day 42 after the first vaccine dose from Group C (40 µg PHH-1V) vs. Group D (PBS)
218 against 7 different VOCs: α (B.1.1.7), β (B.1.351), γ (P.1), µ (B.1.621), δ (B.1.617.2), δ+
219 (B.1.617.2.1) and o (BA.1). Pseudovirus neutralisation assays were conducted, yielding titres
220 expressed as $\log_{10}$ IC_{50}. Each data point represents an individual pig with bars denoting the mean
221 ± standard deviation per group. PBNA IC_{50} longitudinal data have been analysed by means of a
222 linear mixed effects model of repeated measures with the $\log_{10}$-transformed titres. ** $p<0.01$.
223 Titre values are provided in **Table S2**.

224

225 **Figure 3. Vaccination of pigs with PHH-1V elicits cellular response. A.** Induction of IFNγ^+ T-cells
226 determined by ELISpot assays in PBMCs from vaccinated pigs, sampled on day 40 and stimulated
227 *ex vivo* with an RBD peptide pool representing Alpha and Beta SARS-CoV-2 variant epitopes.
228 Group 1: 40 µg PHH-1V RBD fusion heterodimer; Group 2: Control PBS. Each data point
229 represents an individual pig with bars denoting the mean ± standard deviation per group.
230 ELISpot data have been analysed by means of a generalised least squares model with the arcsine-
231 square root-transformed proportion of IFN γ -positive cells * $p<0.05$. **B-C.** $CD4^+$ (**B)** and $CD8^+$ **(C)**
232 T-cell response was measured by intracellular cytokine staining (ICS) after the stimulation of
233 PMBCs with a pool of peptides from SARS-CoV-2 RBD of Alpha and Beta variants. Cytokine
234 expression of PBMCs stimulated with medium was considered as background and subtracted
235 from the responses measured from the RBD peptide pool for each individual pig. Group 1: PHH-
236 1V vaccine 40 µg RBD fusion heterodimer/dose; Group 2: control group (PBS). Each data point
237 represents an individual pig with bars denoting the mean ± standard deviation per group. ICS

238 data have been analysed by means of a generalised least squares model with the arcsine-square
239 root-transformed proportion of positive cells $^{+}$ $0.05<p<0.1$.

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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.

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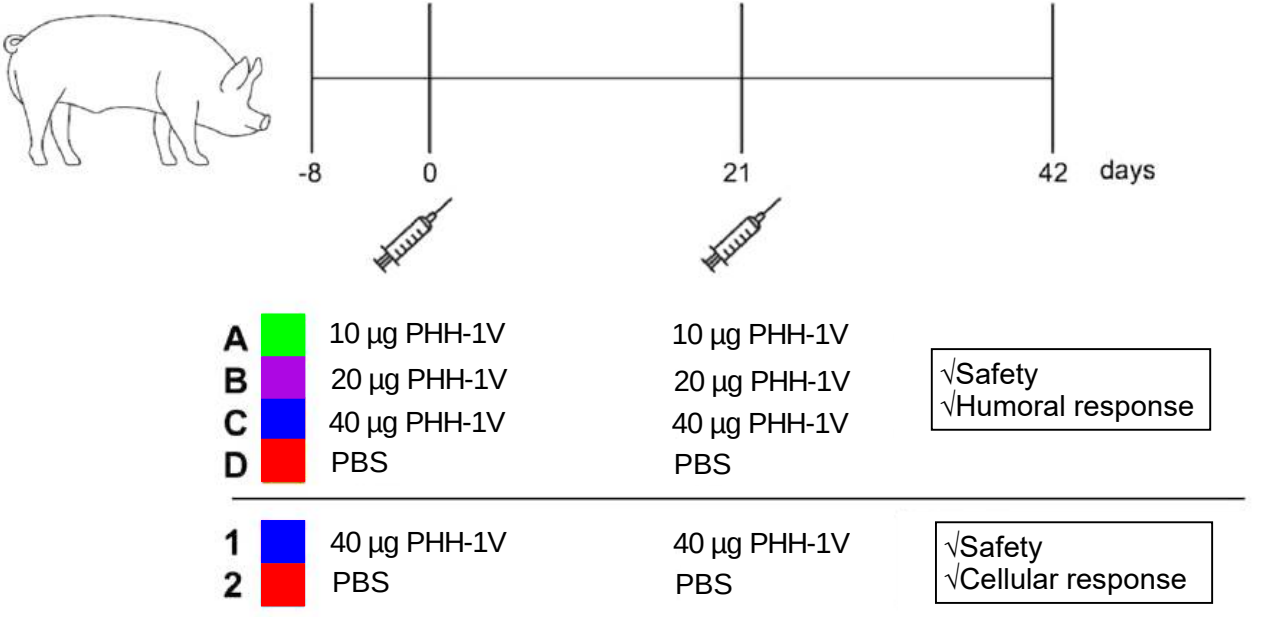
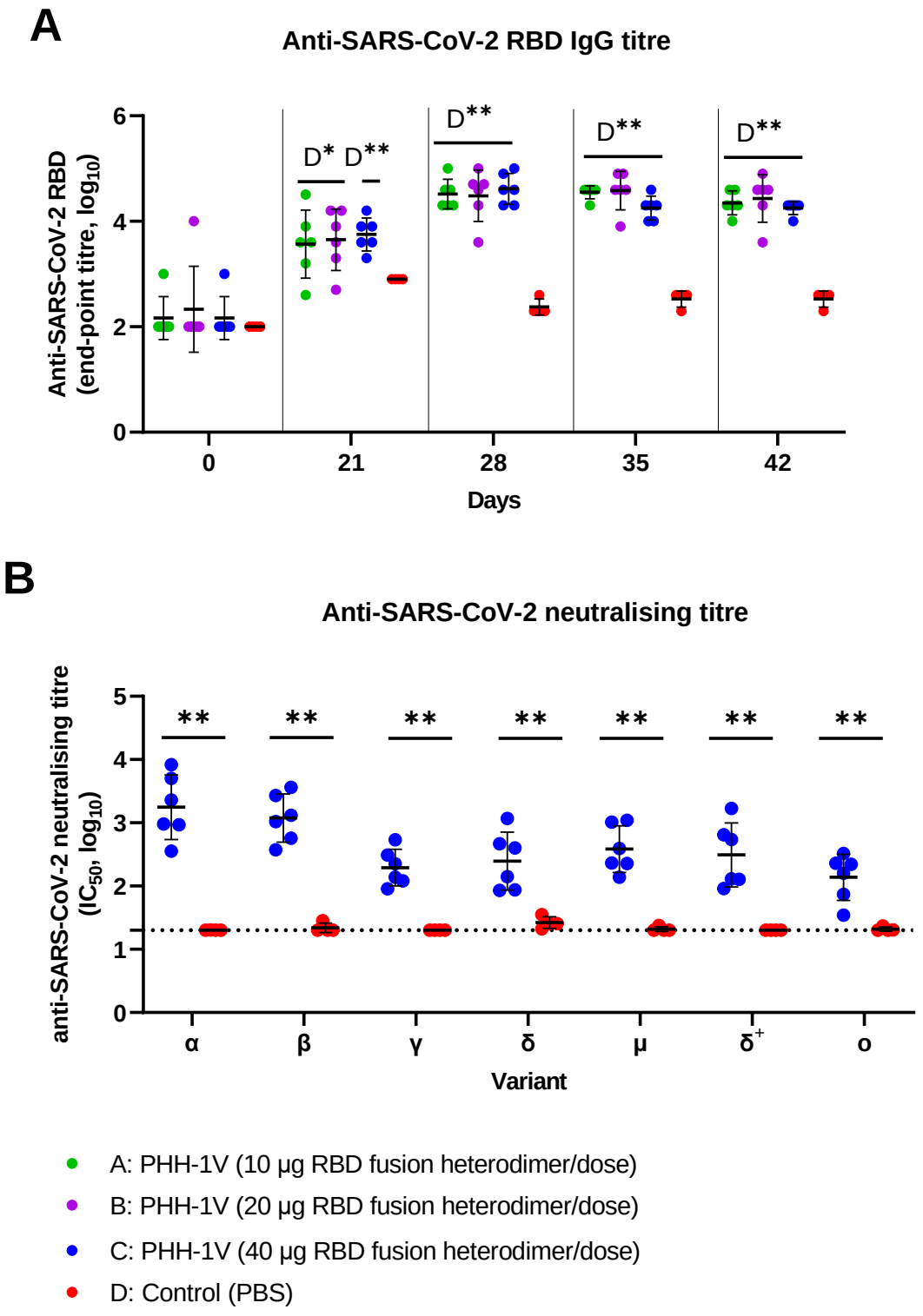

-8
0
21
42
days
A
10 µg PHH-1V
10 µg PHH-1V
B
20 µg PHH-1V
20 µg PHH-1V
C
40 µg PHH-1V
40 µg PHH-1V
D
PBS
PBS
√Safety
√Humoral response
1
40 µg PHH-1V
40 µg PHH-1V
2
PBS
PBS
√Safety
√Cellular response

Figure 1


A
Anti-SARS-CoV-2 RBD IgG titre
Anti-SARS-CoV-2 RBD (end-point titre, log10)
6
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D* D**
D**
D**
D**
0
21
28
35
42
Days
B
Anti-SARS-CoV-2 neutralising titre
anti-SARS-CoV-2 neutralising titre (IC50, log10)
5
4
3
2
1
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α
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Variant
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

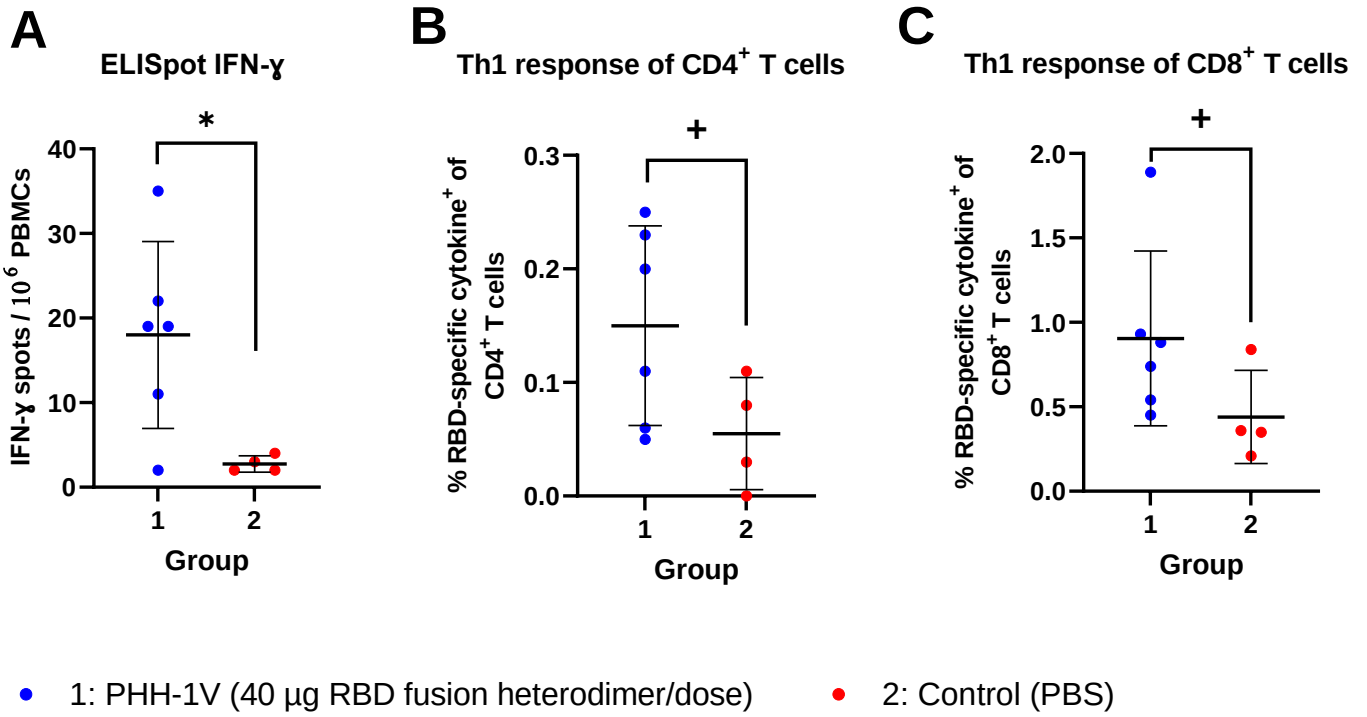
A
ELISpot IFN-γ
*
IFN-γ spots / 10^6 PBMCs
40
30
20
10
0
1
2
Group
B
Th1 response of CD4+ T cells
+
% RBD-specific cytokine+ of CD4+ T cells
0.3
0.2
0.1
0.0
1
2
Group
C
Th1 response of CD8+ T cells
+
% RBD-specific cytokine+ of CD8+ T cells
2.0
1.5
1.0
0.5
0.0
1
2
Group
1: PHH-1V (40 µg RBD fusion heterodimer/dose)
2: Control (PBS)

Figure 3