Neutralising antibodies against the SARS-CoV-2 Delta variant induced by Alhydroxyquim-II-adjuvanted trimeric spike antigens

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25 **ABSTRACT**

26 Global control of COVID-19 will require the deployment of vaccines capable of inducing long-

27 term protective immunity against SARS-CoV-2 variants. In this report, we describe an

28 adjuvanted subunit candidate vaccine that affords elevated, sustained and cross-variant SARS-

29 CoV-2 neutralising antibodies (NAbs) in multiple animal models. Alhydroxiquim-II is a

30 TLR7/8 small-molecule agonist chemisorbed on aluminium hydroxide. Vaccination with

31 Alhydroxiquim-II combined with a stabilized, trimeric form of the SARS-CoV-2 spike protein

32 (termed CoVac-II) resulted in high-titre NAbs in mice, with no decay in responses over an 8-

33 month period. NAbs from sera of CoVac-II-immunized mice, horses and rabbits were broadly

34 neutralising against SARS-CoV-2 variants. Boosting long-term CoVac-II-immunized mice

35 with adjuvanted spike protein from the Beta variant markedly increased levels of NAb titres

36 against multiple SARS-CoV-2 variants; notably high titres against the Delta variant were

37 observed. These data strongly support the clinical assessment of Alhydroxiquim-II-adjuvanted

38 spike proteins to protect against SARS-CoV-2 variants of concern.

39

40 **MAIN TEXT**

41 COVID-19 vaccines have had a remarkable impact on controlling the pandemic in high and

42 middle-income countries. However, global access to affordable COVID-19 vaccines remains

43 a critical issue[1]. Neutralising antibodies (NAbs) are considered the key determinant of SARS-

44 CoV-2 protective immunity[2,3], yet in both natural infection and vaccination the levels of NAbs

45 decay over time[4,5]. This issue is compounded by the emergence of SARS-CoV-2 variants

46 circulating that show partial resistance to current vaccines[6,7], highlighting the need for next-

47 generation vaccines that display strong and persistent immunity.

48

49 Subunit vaccines represent a safe, affordable platform for COVID-19 vaccines, with positive

50 outcomes for some candidates from clinical trials[8,9]. To develop a subunit vaccine that is

51 suitable for global distribution, large-scale antigen manufacture and selection of adjuvants are

52 critical issues. To address these issues, we used stabilized, trimeric SARS-CoV-2 spike protein

53 produced with a fully scalable, chemically defined and GMP-compliant production process[10].

54 To create the CoVac-II vaccine candidate, trimeric spike protein was combined with

55 Alhydroxiquim-II (AHQ-II); a small molecule toll-like receptor 7/8 agonist that is chemisorbed

56 to alum. AHQ-II is the adjuvant used in the Covaxin COVID-19 vaccine, which has received

57 emergency use approval in multiple countries[11]. Importantly, recombinant spike trimer

58 expressed and purified from Chinese hamster ovary (CHO) cells was stable when stored at

59 multiple temperatures and resistant to repeated freeze-thaw cycles (Fig. S1). Following

60 vaccination of mice with CoVac-II (Fig. 1A), high-titre NAbs were apparent in plasma as early

61 as 2 weeks after the first immunization (Fig. 1B). NAb titres did not drop over the time course

62 and reached their peak at 252 days post-vaccination. This corresponds with the excellent

63 stability of both the spike antigen (Fig. S1) and adjuvant used; AHQ-II is stable for at least one

64 year at room temperature (not shown). When compared to spike alone, AHQ-II increased NAb

65   titres by approximately 1000-fold; this increase compares favourably to the effect seen with

66   adjuvants Matrix-M (approximately 10-fold increase) and AS03 (approximately 500-fold) used

67   in other SARS-CoV-2 subunit vaccines[12,13]. Spike plus Alhydrogel (SpK^{Alum}) resulted in NAb

68   titres well above that elicited by spike alone, however titres were approximately 100-fold lower

69   at day 252 compared to those from CoVac-II-vaccinated animals (Fig.1B).

70

71   To determine the pattern of T cell immunity induced by the CoVac-II vaccine, we assessed the

72   level of spike-specific multifunctional CD4$^+$ T cells in peripheral blood mononuclear

73   cells (PBMC) from vaccinated mice. High frequency of CD4$^+$IFN$^+$IL-2$^+$TNF$^+$ were seen after

74   CoVac-II delivery, that were absent from mice vaccinated with spike alone or SpK^{Alum} (Fig.

75   1C). This corresponds with the Th1-polarising effect of AHQ-II when delivered with

76   inactivated SARS-CoV-2 in animal models and humans[11,14]. We also noted a significant

77   correlation between NAb titres and the level of multifunctional CD4$^+$ T cells produced in

78   individual mice (Fig. S2). To further dissect vaccine-induced immunity, we examined the

79   cellular make-up of the draining lymph nodes 7 days after vaccination. Both Alum^{SpK} and

80   CoVac-II induced expansion of spike-specific B cells (CD19$^+$MHCII$^+$; Fig. 1D) and T

81   follicular helper cell (Tfh) cells (CD4$^+$CXCR5$^+$PD1$^+$; Fig. 1E). The total numbers of antigen

82   specific B cells (Fig. 1F), and Tfh cells (Fig. 1G) were significantly increased in CoVac-II-

83   vaccinated mice compared to immunization with Alum^{SpK}. Thus, delivery of trimeric spike

84   antigen with AHQ-II results in strong B and T cell anti-SARS-CoV-2 immune response.

85

86   The ability of CoVac-II-induced antibodies to neutralise SARS-CoV-2 variants of concern

87   (VOCs) was examined using isolates of ancestral (Wuhan), as well as Alpha and Beta variants[4].

88   All plasma samples from CoVac-II-immunized mice neutralised both Alpha and Beta VOCs

89   however titres were reduced for Beta compared to the ancestral virus (8-fold reduction), which

90   is a similar reduction as seen for other COVID-19 vaccines (Fig. 2A)[7]. NAb titres using plasma

91   from SpK^{Alum}-vaccinated mice were reduced to the limit of detection against Beta (Fig 2B).

92   Cross-species neutralisation of VOCs was apparent after immunization of rabbits (Fig. 2C) or

93   horses (Fig.2D) with CoVac-II. NAb titres were maintained against the Alpha variant in both

94   species compared to ancestral virus, and high titres against Beta in rabbits (4.7-fold reduction

95   compared to ancestral) and horses (2.7-fold). Thus AHQ-II can adjuvant vaccine

96   immunogenicity across multiple animal models, adding to its already proven immunogenicity

97   in humans as part of the Covaxin vaccine[11].

98

99   Although CoVac-II immunisation affords some level of cross-neutralisation against the Beta

100   variant, vaccines currently in use display reduced efficacy against this variant when assessed

101   in placebo-controlled or test-negative control trials[8,15,16]. We therefore used our established

102   systems for rapid and high-level production of trimeric viral antigens[10,17] to produce the Beta

103   spike antigen. Mice vaccinated with CoVac-II eight months earlier (or control groups) were

104   boosted with a single dose of Beta spike formulated in AHQ (CoVac351). Two weeks later,

105   the ability of their plasma to neutralize Beta spike-pseudotyped virus was determined[18]. The

106   increase of NAbs in response to this booster injection was greatest in mice previously

107   vaccinated with SpK^{Alum} or CoVac-II (approximate increase of 16-fold compared to pre-boost

108   levels), however responses were maximal in the CoVac-II prime, CoVac351 boosted group

109   (Fig. 2E). High numbers of spike-specific, multifunctional Th1 CD4$^+$ cells were observed in

110   PBMCs from the CoVac-II/CoVac351 group (Fig. S3). To determine breadth of cross

111   neutralisation afforded by boosting with the Beta variant, plasma from CoVac-II-primed,

112   CoVac351-boosted mice was examined for neutralisation of 'wild-type' virus and three VOCs;

113   Alpha, Beta and Delta. In pre-boost samples, all VOCs were neutralised, however titres were

114   reduced approximately 10-fold against Beta compared to 'wild-type' virus (Fig. 2F). However

115   boosting with CoVac351 resulted in enhanced NAb titres against all VOCs, with greatest

116   increase seen against the Beta variant (Fig 2F). Notably, neutralisation titres against the Delta

117   variant were high ($>10^4$) with only a small reduction in titre (approximately 2.2-fold) compared

118   to 'wild-type' virus. Of note, these NAb titres are at least 1 order of magnitude higher than the

119   average human convalescent response, which we have previously assessed with similar

120   methodology, and are comparable with that of 'elite' neutralisers[4].

121

122   In conclusion, the CoVac-II subunit vaccine we described in this report demonstrates

123   remarkable longevity of immune responses (no decay in NAbs up to 8 months post-vaccination

124   in mice) and is highly immunogenic in multiple animal models, including rabbits and horses.

125   The waning of immunity observed in convalescent patients[4] and with current vaccines, coupled

126   with low NAb titres correlating with breakthrough infections[19], suggests that maintenance of

127   humoral immunity will be critical to ensure prolonged vaccine-induced protection against

128   disease. NAbs developed in all immunised species are able to effectively neutralise SARS-

129   CoV-2 variants of concern, which can be augmented by boosting with variant-specific spike

130   vaccines. CoVac-II-immunity compares favourably with other vaccines tested in the same

131   models, that have subsequently shown high-levels of protection in humans[13,20,21]. The excellent

132   safety profile and immunogenicity demonstrated by a AHQ-II-adjuvanted inactivated SARS-

133   CoV-2 vaccine[11], coupled with our ability to manufacture multiple, high quality antigens at

134   scale [10], suggests that AHQ-II/spike protein combinations could constitute safe, affordable and

135   mass-manufacturable COVID-19 vaccines for global distribution.

136

METHODS

Immunization

Female C57BL/6 mice (6-8 weeks of age) were purchased from Australian BioResources (Moss Vale, Australia), and housed at the Centenary Institute in specific pathogen-free conditions. All mouse experiments were performed according to ethical guidelines as set out by the Sydney Local Health District (SLHD) Animal Ethics and Welfare Committee.

SARS-CoV-2 full-length spike stabilized, trimeric proteins were expressed in CHO cells and purified as previously described[10]. Mice (n=3-5) were vaccinated subcutaneously (s.c) once with 5 μg of spike combined with 100 μg of Alhydrogel (Alum; Invivogen, California, USA) or 100 μg Alhydroxiquim-II and boosted three weeks after the first vaccination. In some experiments, mice were further boosted with 5 μg B.1.351/Beta spike protein in 100 μg Alhydroxiquim-II. Mice were bled fortnightly after the first immunization and plasma was collected after centrifugation at 300 x *g* for 10 min. Remaining blood was resuspended in PBS Heparin 20 U/mL, stratified on top of Histopaque 10831 (Sigma-Aldrich, Missouri, USA) and the PBMC layer collected after gradient centrifugation.

Rabbit and horse immunization experiments were performed at Envigo/Cocalico Biologicals (Reamstown, PA, USA), and at East Tennessee Clinical Research, Inc. (Rockwood, TN, USA), respectively, in accordance with institutional guidelines. Adult New Zealand White rabbits (n=4 per group) were bled for pre-immune sera via the marginal vein of the pinna, and immunized intramuscularly (i.m) in the flank region on Days 1, and 15 (Prime + One-boost regimen) with 5 μg of spike protein, adjuvanted with 200 μg (Al content) of either Alhydroxiquim-II, or Alhydrogel, each formulated in 0.2 mL saline. Horses (n=3) were

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161   immunized i.m. in the neck region on Days 1, and 15 with 20 µg spike/500 µg Alhydroxiquim-

162   II, or Alhydrogel.

163

164   **Flow cytometry assays**

165   To examine T and B cell populations, cells were collected from popliteal lymph nodes 7 post

166   immunization and surface stained (2×10^6 cells) with Fixable Blue Dead Cell Stain (Life

167   Technologies), spike-AF647 (1 µg) and/or the antibodies listed in Supplementary Table 1.

168   Where required, Cells were then fixed and permeabilized using fixation/permeabilization kit

169   (ThermoFischer) according to the manufacturer's protocol. To assess spike-specific cytokine

170   induction by T cells, murine PBMCs were stimulated for 4 hrs with spike (5 µg/mL) and then

171   supplemented with Protein Transport Inhibitor cocktail (Life Technologies, California, USA)

172   for a further 10-12 hrs. Cells were surface stained with Fixable Blue Dead Cell Stain (Life

173   Technologies) and marker-specific fluorochrome-labeled antibodies (Supplementary Table 1).

174   Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm™ kit according to

175   the manufacturer's protocol and intracellular staining was performed to detect cytokines IFN-

176   γ, IL-2, TNF, IL-17 (see Supplementary Table 1 for details). All samples were acquired on a

177   BD LSR-Fortessa (BD) or a BD-LSRII and assessed using FlowJo™ analysis software v10.6

178   (Treestar, USA).

179

180   **Live SARS-CoV-2 and pseudovirus neutralisation assay**

181   High-content fluorescence microscopy was used to assess the ability of plasma from mice to

182   inhibit live SARS-CoV-2 infection and the resulting cytopathic effect in live permissive cells

183   (VeroE6, final MOI=0.05), using previously described methodology[4]. Alpha (B.1.1.7), Beta

184   (B1.351) and Delta (B.1617.2) variants were compared against 'wild-type' D614G virus from

185   the same clade (B.1.319), or ancestral virus (Wuhan). The cut-off for determining the

186   neutralisation endpoint titre of diluted serum samples was set to ≥50% neutralisation.

187   Neutralising antibody titers in rabbit and horse immune sera were quantified at Virovax using

188   an automated, liquid-handler-assisted, high-throughput, microfocus neutralisation/high-content

189   imaging methods developed at ViroVax. Briefly, serially-diluted sera (paired pre-immune and

190   immune) and SARS-CoV-2 virus (final MOI of 10) were added to 10^6/mL Vero cells (ATCC®

191   CCL-81) containing 50 μg/mL of propidium iodide (PI) and plates were then loaded in an

192   IncuCyte S3 high-content imaging system (Essen Bioscience/Sartorius, Ann Arbor, MI).

193   Longitudinal image acquisition and processing for virus-induced cytopathic effect (CPE) and

194   cell death (PI uptake) were performed every six hours, until cell death profiles had crested and

195   stabilized (3.5 days). Neutralising antibody titers (expressed as IC_{50}) were obtained from four-

196   parameter logistic curve-fits of cell death profiles using OriginPro 9 (Origin Lab Corp.,

197   Northampton, MA).

198

199   Replication-deficient SARS-CoV-2 Spike pseudotyped lentivirus particles were generated by

200   co-transfecting GFP-luciferase vector and ancestral[4] or Beta[18] spike expression constructs with

201   lentivirus packaging and helper plasmids into 293T cells using Fugene HD (Promega) as

202   previously described [22]. To determine neutralising antibody titres, pseudovirus particles were

203   incubated with serially diluted plasma samples at 37°C for 1 hr prior to spinoculation (800xg)

204   of ACE2 over-expressing 293T cells[22]. 72 hr post-transduction, cells were fixed and stained

205   with Hoechst 33342 (NucBlue™ Live ReadyProbes™ Reagent, Invitrogen) as per the

206   manufacturers instruction's, imaged used an Opera Phenix high content screening system

207   (Perkin Elmer) and the percentage of GFP positive cells was enumerated (Harmony® high-

208   content analysis software, Perkin Elmer).

209

210   **Statistical analysis**

211 The significance of differences between experimental groups was evaluated by one-way

212 analysis of variance (ANOVA), with pairwise comparison of multi-grouped data sets achieved

213 using Dunnett's *post-hoc* test. Differences were considered statistically significant when $p \leq$

214 0.05.

215

216 **Data availability**

217 The results supporting the findings in this study are available upon request from the

218 corresponding authors.

219

220 **Acknowledgements**

235

Contributions

236

237 CC, MJW, SGT, MS, FMW, SAD and JAT designed the study. CC, PP, AOS, CA, HL, NDB,

238 AA, MS, SD performed the experiments. All authors contributed to data

239 analysis/interpretation. TT, BMD, NRL provided support in methodology and resources for

240 this study; JAT wrote the first manuscript draft and all authors provided revision to the

241 scientific content of the final manuscript.

242

Competing interests

243

244 ViroVax has intellectual property on Alhydroxiquim-II. ExcellGene uses a registered CHO cell

245 line as substrate for transfections (CHOExpress®) and proprietary methods in cell line

246 generation and production of proteins.

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303   **Figure Legends**

304   **Figure 1. Sustained neutralising antibody titres and generation of multifunctional CD4$^+$**

305   **T cells responses after vaccination with Alhydroxiquim-II-adjuvanted spike antigen. A**.

306   C57BL/6 mice were vaccinated s.c at day 1 and day 21 with PBS, SpK (5 μg ancestral spike),

307   SpK^{Alum} (5 μg spike/100 μg Alhydrogel) or CoVac-II (5 μg spike/100 μg Alhydroxiquim-II).

308   **B.** Neutralising antibody (NAb) titres (IC$_{50}$) in plasma were determined using ancestral spike-

309   pseudotyped virus. Dotted line shows the limit of detection. **C.** PBMCs taken 1 week post-

310   boost were restimulated with 5 μg/mL of SARS-CoV-2 spike and the number of cytokine-

311   expressing CD4$^+$ T cells determined by flow cytometry. **D-G**. C57BL/6 mice were vaccinated

312   as in **A** and 7 days the frequency of spike-specific B cells (**D**, CD19$^+$MHCII$^+$) or T follicular

313   helper T cells (Tfh) (**E**, CXCR5$^+$PD-1$^+$) determined by flow cytometry. The total number of

314   spike$^+$ B cells (**F**) and Tfh cells (**G**) is also shown. Data presented as geometric mean ±

315   geometric SD (**B**) or mean ± SEM (**C, F, G**). Significant differences between groups were

316   determined by one-way ANOVA.

317

318   **Figure 2. Alhydroxiquim-II-adjuvanted vaccines afford cross-species neutralisation of**

319   **variants of concern, that is augmented by a variant-specific booster vaccine.** Mice (n=4 to

320   5) were vaccinated as in Figure 1 and 3 weeks post-vaccination, plasma from CoVac-II (**A**) or

321   SpK^{Alum} (**B**) tested for neutralising activity against live SARS-CoV-2 infection of Vero E6 cells

322   (ancstrl=ancestral virus). **C.** Rabbits (n=3) were immunized i.m. twice with CoVac-II

323   (ancestral 5 μg spike/200 μg Alhydrogel) and NAb titres against live SARS-CoV-2 viruses

324   determined.   **D.** Horses (n=3) were immunized i.m. twice with CoVac-II (ancestral 20 μg

325   spike/500 μg Alhydrogel) and NAb titres against live SARS-CoV-2 viruses determined. **E.**

326   Mice vaccinated 250 days previously were boosted with a single dose of CoVac351 (5 μg Beta

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327   spike/100 μg Alhydroxiquim-II) and NAb titres against Beta spike-pseudovirus determined at

328   one week post-boost. Data presented as geometric mean ± geometric SD. **F.** Plasma from

329   CoVac351-boosted mice was tested for neutralising activity against live SARS-CoV-2

330   infection of Vero E6 cells. The dotted line shows the limit of detection. Significant differences

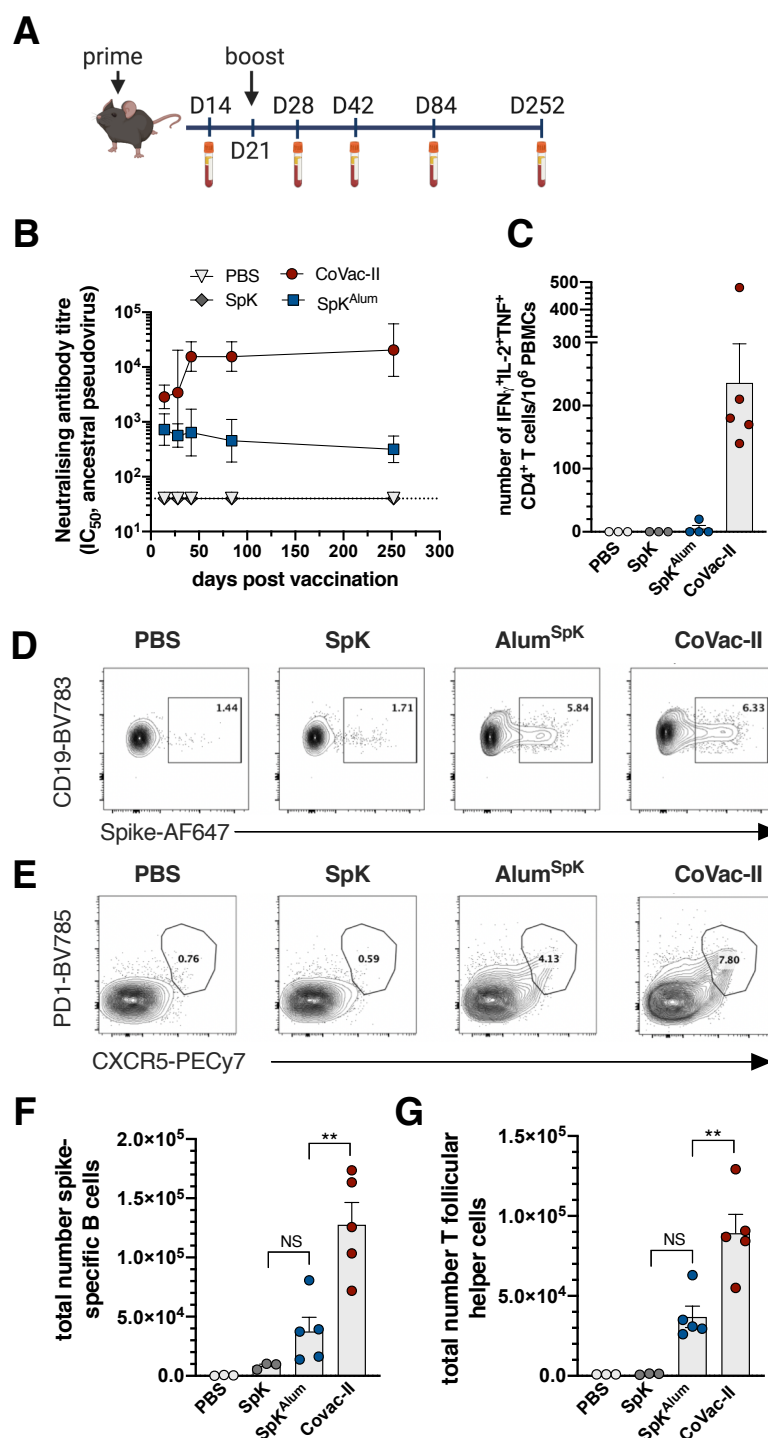331   between groups were determined by one-way ANOVA.

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Figure 1

Figure 1. Sustained neutralising antibody titres and generation of multifunctional CD4$^+$ T cells responses after vaccination with Alhydroxiquim-II-adjuvanted spike antigen. A. C57BL/6 mice were vaccinated s.c at day 1 and day 21 with PBS, SpK (5 mg ancestral spike), SpK^{Alum} (5 μg spike/100 mg Alhydrogel) or CoVac-II (5 μg spike/100 mg Alhydroxiquim-II). **B.** Neutralising antibody (NAb) titres (IC$_{50}$) in plasma were determined using ancestral spike-pseudotyped virus. Dotted line shows the limit of detection. **C.** PBMCs taken 1 week post-boost were restimulated with 5 μg/mL of SARS-CoV-2 spike and the number of cytokine-expressing CD4$^+$ T cells determined by flow cytometry. **D-G.** C57BL/6 mice were vaccinated as in **A** and 7 days the frequency of spike-specific B cells (**D**, CD19$^+$MHCII$^+$) or T follicular helper T cells (Tfh) (**E**, CXCR5$^+$PD-1$^+$) determined by flow cytometry. The total number of spike$^+$ B cells (**F**) and Tfh cells (**G**) is also shown. Data presented as geometric mean ± geometric SD (B) or mean ± SEM (C, F, G). Significant differences between groups were determined by one-way ANOVA.
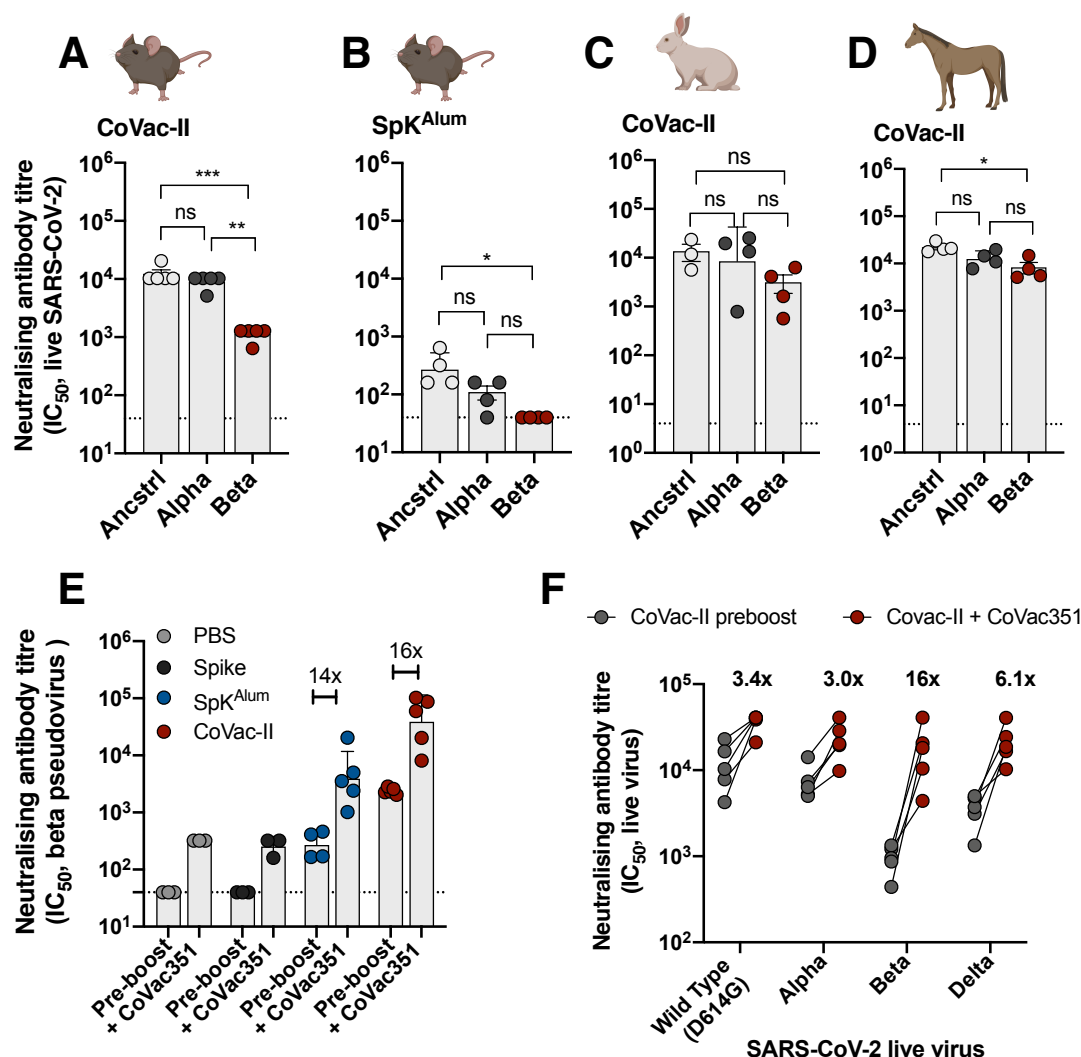
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Figure 2

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352 **Figure 2. Alhydroxiquim-II-adjuvanted vaccines afford cross-species neutralisation of variants of concern,**
353 **that is augmented by a variant-specific booster vaccine.** Mice (n=4 to 5) were vaccinated as in Figure 1 and 3
354 weeks post-vaccination, plasma from CoVac-II (**A**) or SpK^{Alum} (**B**) tested for neutralising activity against live
355 SARS-CoV-2 infection of Vero E6 cells (ancstrl=ancestral virus). **C.** Rabbits (n=3) were immunized i.m. twice
356 with CoVac-II (ancestral 5 μg spike/200 μg Alhydrogel) and NAb titres against live SARS-CoV-2 viruses
357 determined. **D.** Horses (n=3) were immunized i.m. twice with CoVac-II (ancestral 20 μg spike/500 μg
358 Alhydrogel) and NAb titres against live SARS-CoV-2 viruses determined. **E.** Mice vaccinated 250 days
359 previously were boosted with a single dose of CoVac351 (5 μg Beta spike/100 μg Alhydroxiquim-II) and NAb
360 titres against Beta spike-pseudovirus determined at one week post-boost. Data presented as geometric mean ±
361 geometric SD. **F.** Plasma from CoVac351-boosted mice was tested for neutralising activity against live SARS-
362 CoV-2 infection of Vero E6 cells. The dotted line shows the limit of detection. Significant differences between
363 groups were determined by one-way ANOVA.
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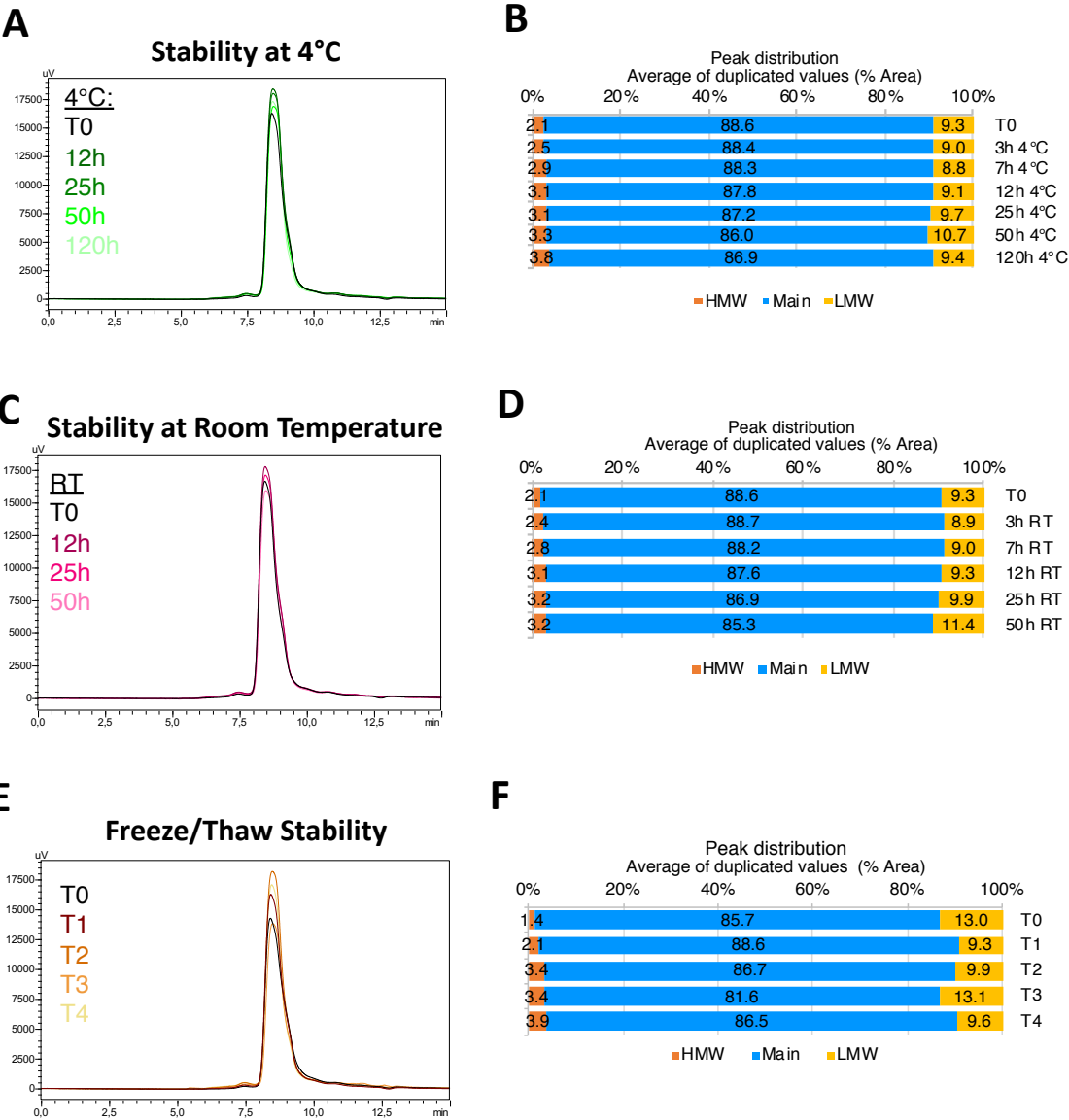
Supplementary Figure 1. Stability of trimeric spike protein produced in CHO cells. Chromatographs (**A, C, E**) and peak distribution (**B, D, F**) of spike protein after storage at 4°C (**A, B**) and room temperature (RT, **C, D**) or after 4 cycles of freeze-thawing (**E, F**).

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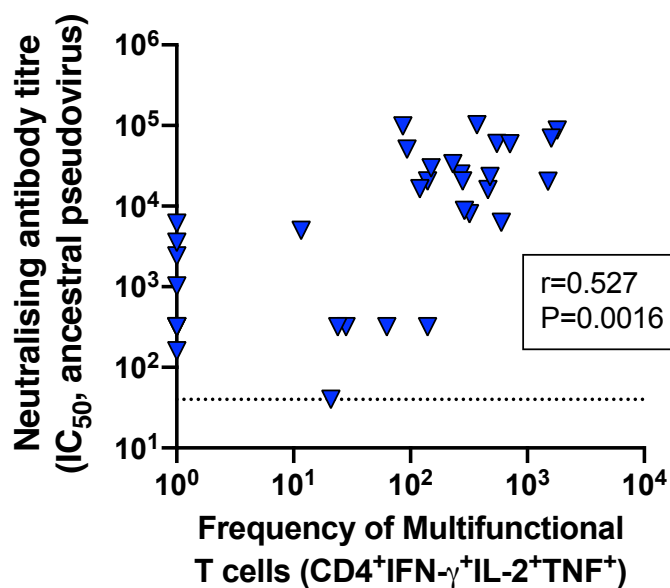
Supplementary Figure 2. Spearman correlation of neutralizing antibody titre and multifunctional CD4^{+} T cells after vaccination with AHQ-adjuvanted spike vaccines. The dotted line shows the limit of detection.

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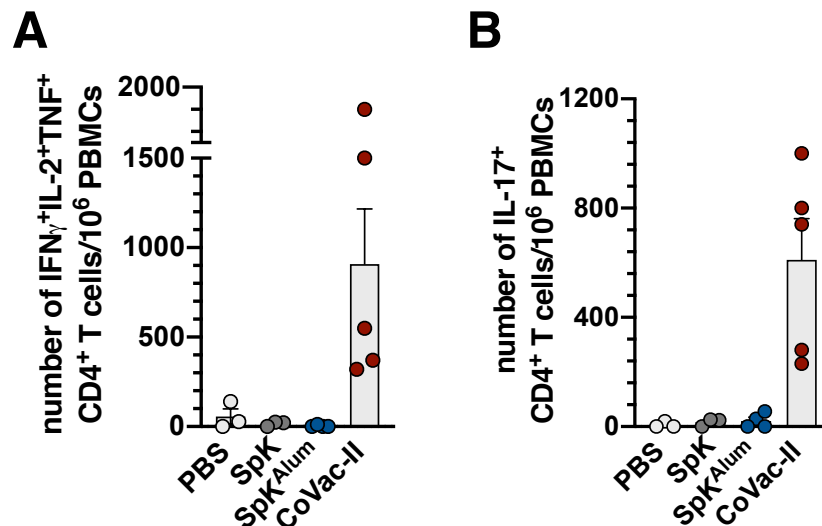
Supplementary Figure 3. Mice vaccinated 250 days previously were boosted with a single dose of CoVac351 (5 μg Beta spike/100 μg Alhydroxiquim-II). PBMCs isolated one week post-boost were restimulated *ex vivo* with 5 μg/mL of Beta spike and the number of circulating CD4$^+$ expressing IFN-γ, IL-2 and TNF (**A**) or IL-17 (**B**) determined by flow cytometry.

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