

1 **Title:**

2 **Immunological and pathological outcomes of SARS-CoV-2 challenge after**
3 **formalin-inactivated vaccine immunisation of ferrets and rhesus macaques**

4 **Authors:**

5 Kevin R. Bewley¹, Karen Gooch¹, Kelly M. Thomas¹, Stephanie Longet¹, Nathan
6 Wiblin¹, Laura Hunter¹, Kin Chan¹, Phillip Brown¹, Rebecca A. Russell², Catherine
7 Ho¹, Gillian Slack¹, Holly E. Humphries¹, Leonie Alden¹, Lauren Allen¹, Marilyn
8 Aram¹, Natalie Baker¹, Emily Brunt¹, Rebecca Cobb¹, Susan Fotheringham¹, Debbie
9 Harris¹, Chelsea Kennard¹, Stephanie Leung¹, Kathryn Ryan¹, Howard Tolley¹,
10 Nadina Wand¹, Andrew White¹, Laura Sibley¹, Charlotte Sarfas¹, Geoff Pearson¹,
11 Emma Rayner¹, Xiaochao Xue², Teresa Lambe³, Sue Charlton¹, Sarah Gilbert³,
12 Quentin J. Sattentau², Fergus Gleeson⁴, Yper Hall¹, Simon Funnell¹, Sally Sharpe¹,
13 Francisco J. Salguero¹, Andrew Gorringe^{1*} and Miles Carroll^{1,5*}

14

15 ¹Public Health England, Porton Down, Salisbury SP4 0JG

16 ²The Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE

17 ³The Jenner Institute, Nuffield Department of Medicine, University of Oxford, Oxford
18 OX3 7DQ

19 ⁴Oxford Departments of Radiology and Nuclear Medicine, Oxford University
20 Hospitals NHS Foundation Trust, Oxford OX3 7LE

21 ⁵Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine,
22 University of Oxford, Oxford, OX3 7LG

23

24 ***Joint corresponding authors**

25 Professor Andrew Gorringe

26 Public Health England, Porton Down, Salisbury SP4 0JG

27 Email Andrew.gorringe@phe.gov.uk

28 **Running title: SARS-CoV-2 enhanced disease**

29

30 **Key words: SARS-CoV-2, COVID-19, vaccine-enhanced disease, ferret, rhesus**
31 **macaque, coronavirus**

32

33 **Abstract**

34 There is an urgent requirement for safe and effective vaccines to prevent novel
35 coronavirus disease (COVID-19) caused by SARS-CoV-2. A concern for the
36 development of new viral vaccines is the potential to induce vaccine-enhanced
37 disease (VED). This was reported in several preclinical studies with both SARS-CoV-
38 1 and MERS vaccines but has not been reported with SARS-CoV-2 vaccines. We
39 have used ferret and rhesus macaques challenged with SARS-CoV-2 to assess the
40 potential for VED in animals vaccinated with formaldehyde-inactivated SARS-CoV-2
41 (FIV) formulated with Alhydrogel, compared to a negative control vaccine in ferrets or
42 unvaccinated macaques. We showed no evidence of enhanced disease in ferrets or
43 rhesus macaques given FIV except for mild transient enhanced disease seen at
44 seven days post infection in ferrets. This increased lung pathology was observed

45 early in the infection (day 7) but was resolved by day 15. We also demonstrate that
46 formaldehyde treatment of SARS-CoV-2 reduces exposure of the spike receptor
47 binding domain providing a mechanistic explanation for suboptimal immunity.

48

49 **Introduction.**

50 Novel coronavirus disease (COVID-19) caused by SARS-CoV-2 is a global
51 pandemic with a cumulative total of over 63 million cases and 1.4 million deaths
52 reported as of 2nd December 2020¹. Consequently, there is an urgent requirement to
53 develop safe and effective vaccines to prevent COVID-19². Currently 52 vaccine
54 candidates are in clinical evaluation (11 at Phase 3) with 162 listed as in pre-clinical
55 evaluation (WHO draft landscape of COVID-19 vaccines – 8 December 2020). The
56 leading vaccine candidates in Phase 3 studies include a non-replicating viral vector,
57 three inactivated virus vaccines and two vaccines based on mRNA technology³.
58 Promising clinical results from phase 2/3 studies are available for mRNA-based
59 vaccines^{4 5}, the adenovirus vaccine (ChAdOx1nCoV-19/AZD1222)⁶, which
60 expresses a codon-optimised full-length spike protein (S) and whole virus vaccines
61 grown in Vero cells and inactivated with β -propiolactone^{7 8 9}. These vaccines have
62 also been evaluated for protection in non-human primates following challenge with
63 SARS-CoV-2. The virus induces only mild to moderate disease in macaques but
64 these vaccines reduce viral loads and pathology in the upper and lower respiratory
65 tracts to varying degrees^{10 8 7 11}.

66 A concern for the development of new viral vaccines is the potential to induce
67 vaccine enhanced disease¹² (VED) which has been associated with prior pre-clinical
68 studies of both SARS and MERS vaccines. The most studied mechanism of VED is

69 antibody-dependent enhancement (ADE) of disease, reviewed recently by Arvin *et*
70 *al.*¹³. It has been suggested that ADE could be a consequence of low affinity
71 antibodies that bind to viral proteins but have limited neutralising activity¹⁴. The
72 vaccine enhancement of disease by ADE mechanisms was described in children
73 given formaldehyde-inactivated respiratory syncytial virus (RSV) vaccines in the
74 1960s¹⁵, measles vaccines¹⁶ and in dengue haemorrhagic fever due to secondary
75 infection with a heterologous dengue serotype¹⁷.

76 There is limited evidence of ADE with SARS-CoV-1 vaccines in animal models and
77 whilst it has not been reported in the majority of vaccine studies, a study that used
78 formalin or ultraviolet-inactivated SARS-CoV-1 observed that older mice developed
79 pulmonary pathology with an eosinophil infiltrate¹⁸. A further study demonstrated
80 protection in mice following immunisation with formalin or ultraviolet light-inactivated
81 SARS-CoV-1, but animals developed eosinophilic pulmonary infiltrates¹⁹. A modified
82 vaccinia virus Ankara expressing S protein (MVA-S) was not protective in ferrets
83 challenged with SARS-CoV-1, but liver inflammation was noted²⁰. Formalin-
84 inactivated SARS-CoV-1 vaccines were protective in rhesus macaques²¹, but also
85 promoted lymphocytic infiltrates and alveolar oedema with fibrin deposition after
86 challenge²². Likewise, MVA expressing S protein showed protection in one study²³,
87 but greater occurrence of diffuse alveolar damage than seen in control animals
88 following challenge. Fortuitously, VED has not been reported in the numerous
89 SARS-CoV-2 efficacy animal challenge vaccine studies published to date. However,
90 these studies were primarily designed to assess efficacy and not VED.

91 We have developed a ferret intranasal SARS-CoV-2 infection model where viral
92 shedding and mild lung pathology is observed and re-challenged animals are fully
93 protected²⁴. We have also evaluated both rhesus and cynomolgus macaques for

94 their susceptibility to SARS-CoV-2 challenge and showed the development of
95 pulmonary lesions in both species which are equivalent to those seen in mild clinical
96 cases in humans²⁵. In order to interrogate the potential for VED in ferrets and rhesus
97 macaques to support future safety studies on novel COVID-19 vaccines, we
98 prepared a formaldehyde-inactivated SARS-CoV-2 vaccine (FIV), formulated in
99 Alhydrogel. In this study design we aim to induce a suboptimal immune response
100 which may promote VED, immunised animals are challenged with SARS-CoV-2 14
101 days after vaccination. Clinical signs, viral shedding and pathology are monitored
102 following challenge, and immune responses characterised before and after infection.
103 No enhanced pathology is observed in either species except for transient enhanced
104 pathology at 7 days post infection in ferrets and we present a possible mechanism
105 for suboptimal immunity induced by formaldehyde-inactivated SARS-CoV-2 spike.

106

107 **Materials & Methods**

108 **Viruses and cells.** SARS-CoV-2 Victoria/01/2020²⁶ was provided by The Doherty
109 Institute, Melbourne, Australia at P1 and passaged twice in Vero/hSLAM cells [ECACC
110 04091501]. Briefly, confluent monolayers of hSLAM cells were infected at a multiplicity
111 of infection (MOI) of approximately 0.0005 for 60 min in medium (see below) containing
112 no FBS at 37°C. The flasks were then filled with media supplemented with 4% heat-
113 inactivated FBS. Virus was harvested at 72 h post-infection by removal of any remaining
114 attached cells with sterile 5 mm borosilicate glass beads, clarification of the cell/media
115 supernatant by centrifugation at 1000 x g for 10 min, followed by dispensing and storage
116 at $\geq -65^{\circ}\text{C}$. Whole genome sequencing was performed, on the challenge isolate, using
117 both Nanopore and Illumina as described previously²⁴. Virus titre was determined by

118 plaque assay on Vero/E6 cells [ECACC 85020206]. Cell cultures were maintained at
119 37°C in MEM (Life Technologies, California, USA) supplemented with 10% foetal
120 bovine serum (FBS, Sigma, Dorset, UK) and 25mM HEPES (Life Technologies). All
121 Vero/hSLAM cell cultures were also supplemented with 0.4 µg/ml Geneticin (Gibco).

122

123 **Preparation of formalin-inactivated virus vaccine (FIV).** Centrifugal concentrators
124 (VivaSpin20; 300kDa cut off) were sterilised with 20 mL of 70% ethanol for 10 min
125 followed by a wash with 20mL of Dulbecco's PBS (Gibco). To reduce the concentration
126 of calf serum components in the material, concentrators were loaded with 120mL of
127 SARS-CoV-2 at a titre of 8.45×10^6 pfu/mL and centrifuged at $3000 \times g$ for 60 – 80
128 min (until the retained volume was ≤ 2 mL); the concentrators were re-filled with 20mL
129 of DPBS and centrifugation repeated for a total of three washes. After the final wash,
130 the material was pooled and made up to 30mL in sterile DPBS. Methanol-free
131 formaldehyde solution at 36% (w/v) was added to a final formaldehyde concentration
132 of 0.02% at room temperature for 72 h. The inactivated virus was subjected to a further
133 three 20mL DPBS washes to remove the residual formaldehyde, made up to 20mL in
134 DPBS and aliquoted and stored below -15°C. To confirm inactivation, virus was
135 seeded onto Vero/hSLAM cells in three flasks (100µl/flask) which were serially
136 passaged for a total of 27 days. Microscopic examination for signs of cytopathic effect
137 (CPE) and RTqPCR were used to confirm no viable virus remained.

138

139 **Assessment of inactivated SARS-CoV-2, SDS PAGE:** Samples were added to
140 Laemmli buffer (Sigma, S3401) and heated at 90°C for 5 min and loaded onto a 10-
141 well NuPAGE 4-12% Bis-Tris gel, 1.0mm (ThermoFisher). 5µL SeeBlue Plus2

142 (ThermoFisher) ladder was loaded as a marker and gels were stained with SimplyBlue
143 SafeStain (ThermoFisher). Western Blot: Samples were processed as described for
144 SDS PAGE and transferred to PVDF membrane with iBlot2 (ThermoFisher). After
145 transfer, membranes were washed with tris-buffered saline 0.1% Tween20 (TBST) for
146 5 min at room temperature, followed by 1 h in blocking buffer (TBST, 5% skimmed
147 milk powder). Membranes were washed three times for 5 min with TBST. MERS
148 convalescent neutralising serum (NIBSC S3) was diluted 1:1000 in blocking buffer and
149 incubated for 1 h at room temperature and then at 4°C overnight. Membranes were
150 washed three times for 5 min with TBST and then incubated with either anti-human
151 IgG-AP or anti-rabbit IgG-AP (1:5000 in blocking buffer) for 1 h with agitation.
152 Membranes were washed three times as above and then developed with BCIP/NBT
153 liquid substrate system (Sigma-Aldrich). The protein concentration of the FIV was
154 determined using a BCA assay (Pierce #23227) (859µg/mL). Densitometry analysis
155 (ImageQuant TL; GE Healthcare) of the Western blot revealed a band of
156 approximately 180 kDa that was only present in wild-type virus and FIV preparations.
157 The relative density of this band (20.7%) permitted estimation of the proportion of the
158 FIV total protein that was Coronavirus-specific (178µg/mL).

159

160 **Transmission electron microscopy.** Live virus was inactivated and fixed with final
161 concentrations of 4%(w/v) formaldehyde and 2.5%(w/v) glutaraldehyde at ambient
162 temperature for >16 h prior to processing. Inactivated virus was processed without any
163 additional fixation steps. Samples (approximately 10µL) were placed directly on to
164 electron microscopy grids (400 mesh copper grid, covered with a carbon reinforced
165 plastic film). After 5 min adsorption the sample was removed, and the grids were
166 negatively stained using 2% methylamine tungstate. The grids were examined using

167 a CM100 transmission electron microscope (Philips/FEI/ThermoFisher Scientific)
168 operated at 80kV.

169

170 **Animals. Ferrets:** Ten healthy, female ferrets (*Mustela putorius furo*) aged 5-7
171 months were obtained from a UK Home Office accredited supplier (Highgate Farm,
172 UK). The mean weight at the time of challenge was 1002g/ferret (range 871-1150g).
173 Animals were housed as described previously²⁴.

174 **Rhesus macaques:** Twelve rhesus macaques of Indian origin (*Macaca mulatta*) were
175 used in the study. Study groups comprised three males and three females and all were
176 adults aged 2-4 years and weighing between 3.73 and 5.52 kg at the time of challenge.
177 Animals were housed as described previously²⁵. All experimental work was conducted
178 under the authority of a UK Home Office approved project licence that had been
179 subject to local ethical review at PHE Porton Down by the Animal Welfare and Ethical
180 Review Body (AWERB).

181

182 **Vaccinations.** Animals were randomly assigned to control (Ad-GFP for ferrets, no
183 vaccine for rhesus macaques) and FIV-vaccinated groups. The weight distribution of
184 the ferrets was tested to ensure there was no difference between groups (t-test, $p >$
185 0.05). An identifier chip (Bio-Thermo Identichip, Animalcare Ltd, UK) was inserted
186 subcutaneously into the dorsal cervical region of each animal. Macaques were
187 stratified for sex and into socially compatible cohorts and then randomly assigned into
188 treatment groups. FIV was diluted in PBS to 133 μ g/mL Coronavirus-specific protein
189 and mixed 1:1 in 2% Alhydrogel (Invivogen vac-alu-250) to give a final concentration
190 of 66.7 μ g/mL in 1% Alhydrogel. Ferrets were immunised with a single intramuscular

191 dose of 10 μ g of Coronavirus-specific protein in 150 μ L divided over two sites and
192 macaques were immunised with 25 μ g in 300 μ L administered into the quadriceps
193 femoris muscle of the right leg. Vaccination was 14 days before challenge. Control
194 ferrets were immunised with a single intramuscular dose of 2.5×10^{10} virus particles
195 of Ad-GFP²⁷, a replication-deficient simian adenovirus vector containing an insert
196 unrelated to Coronavirus (Green Fluorescent Protein, GFP), 28 days prior to
197 challenge. Control macaques received no vaccine.

198

199 **SARS-CoV-2 challenge.** Prior to challenge ferrets were sedated by intramuscular
200 injection of ketamine/xylazine (17.9 mg/kg and 3.6 mg/kg bodyweight) and macaques
201 with ketamine hydrochloride (Ketaset, 100mg/ml, Fort Dodge Animal Health Ltd., UK;
202 10mg/kg). SARS-CoV-2 Victoria/01/2020²⁶ was prepared as described previously²⁴. It
203 was delivered to ferrets by intranasal instillation (1.0mL total, 0.5mL per nostril) diluted
204 in PBS. A single dose of virus (5×10^6 pfu/ferret) was delivered to Ad-GFP- (n=4) and
205 FIV- (n=6) vaccinated ferrets. Macaques were challenged with 5×10^6 delivered by the
206 intratracheal route (2ml) and intranasal instillation (1ml total, 0.5ml per nostril). The
207 schedule of euthanasia and sampling is shown in **Table 1**.

208 Nasal washes were obtained by flushing the nasal cavity with 2mL PBS. Throat swabs
209 were collected using a standard swab (Sigma Virocult[®]) gently stroked across the back
210 of the pharynx in the tonsillar area. Throat swabs were processed, and aliquots stored
211 in viral transport media (VTM) and AVL at $\leq -60^{\circ}\text{C}$ until assay. Clinical signs of disease
212 were monitored as described previously^{24 25}. The necropsy procedures were also as
213 described previously^{24 25}.

214

215 **SARS-CoV-2 virology.** RNA was isolated from nasal wash and throat swabs.
216 Samples were inactivated in AVL (Qiagen) and ethanol. Downstream extraction was
217 then performed using the BioSprint™96 One-For-All vet kit (Indical) and Kingfisher
218 Flex platform as per manufacturer's instructions.

219 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting a
220 region of the SARS-CoV-2 nucleocapsid (N) gene was used to determine viral loads
221 and was performed using TaqPath™ 1-Step RT-qPCR Master Mix, CG (Applied
222 Biosystems™), 2019-nCoV CDC RUO Kit (Integrated DNA Technologies) and
223 QuantStudio™ 7 Flex Real-Time PCR System. Sequences of the N1 primers and
224 probe were: 2019-nCoV_N1-forward, 5' GACCCCAAATCAGCGAAAT 3'; 2019-
225 nCoV_N1-reverse, 5' TCTGGTTACTGCCAGTTGAATCTG 3'; 2019-nCoV_N1-probe,
226 5' FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 3'. The cycling conditions were:
227 25°C for 2 min, 50°C for 15 min, 95°C for 2 min, followed by 45 cycles of 95°C for 3 s,
228 55°C for 30 s. The quantification standard was in vitro transcribed RNA of the SARS-
229 CoV-2 N ORF (accession number NC_045512.2) with quantification between 1×10^1
230 and 1×10^6 copies/ μ L. Positive samples detected below the limit of quantification were
231 assigned the value of 5 copies/ μ L, whilst undetected samples were assigned the value
232 of 2.3 copies/ μ L, equivalent to the assay's lower limit of detection.

233

234 **ELISA to quantify anti-S, RBD and N IgG.** A full length trimeric and stabilised version
235 of the SARS-CoV-2 Spike protein (amino acids 1-1280, GenBank: MN MN908947)
236 was developed by Florian Krammer's lab as described²⁸. Recombinant SARS-CoV-2
237 Receptor-Binding-Domain (RBD) (319-541) Myc-His was provided by MassBiologics.
238 Recombinant SARS-CoV-2 Nucleocapsid phosphoprotein (GenBank: MN908947,

239 isolate Wuhan-Hu-1) was expressed and purified from *Escherichia coli* as full-length
240 nucleoprotein (amino acids 1-419) with a C-terminal 6xHis-Tag (Native Antigen
241 Company). High-binding 96-well plates (Nunc Maxisorp, 442404) were coated with 50
242 μ l per well of 2 μ g/mL Spike trimer, RBD or N in 1x PBS (Gibco) and incubated
243 overnight at 4°C. The ELISA plates were washed five times with wash buffer (PBS
244 0.05% Tween 20) and blocked with 100 μ L/well 5% FBS (Sigma, F9665) in PBS 0.1%
245 Tween 20 for 1 h at room temperature. After washing, serum samples were serially
246 diluted in 10% FBS in PBS 0.1% Tween 20 and 50 μ l/well of each dilution was added
247 to the antigen coated plate and incubated for 2 h at room temperature. Following
248 washing, anti-ferret IgG-HRP (Novus Biologics, NB7224) diluted (1/1000) or anti-
249 monkey IgG-HRP (Invitrogen PA-84631 diluted 1:10,000) in 10% FBS in 1X PBS/0.1%
250 Tween 20 and 100 μ l/well was added to each plate, then incubated for 1 h at room
251 temperature. After washing, 1mg/mL O-phenylenediamine dihydrochloride solution
252 (Sigma P9187) was prepared and 100 μ L per well were added. The development was
253 stopped with 50 μ L per well 1M Hydrochloric acid (Fisher Chemical, J/4320/15) and
254 the absorbance at 490 nm was measured. OD_{1.0} titres were calculated using Softmax
255 Pro 7.0.

256

257 **SARS-CoV-2 neutralisation assays.** The plaque reduction neutralisation test
258 (PRNT) was performed as described previously with ferret serum²⁴. The
259 microneutralisation assay was performed with macaque serum as described for
260 human sera²⁹.

261 **Isolation of Immune Cells.** Similar to as described previously²⁴ heparinised blood
262 and spleens were removed for the isolation of immune cells; peripheral blood

263 mononuclear cells (PBMCs) and splenocytes. The spleens were dissected into small
264 pieces. Dissected spleen was dissociated using a gentleMACS. The tissue solution
265 was passed through two cell sieves (100µm then 70µm) and then layered with Ficoll®-
266 Paque Premium (GE Healthcare, Hatfield, United Kingdom). Density gradient
267 centrifugation was carried out at 400g for 30 min on dissociated tissue and on whole
268 blood. Buffy coats containing lymphocytes were collected and washed with medium
269 by pelleting cells via centrifugation at 400 g for 10 min. The cells were counted using
270 a vial-1 cassette and a Nucleocounter-200 before cryopreservation in 95% FCS/5%
271 v/v DMSO. Cryopreserved cells were then frozen at -80°C in controlled rate freezer
272 containers overnight, before transfer to liquid nitrogen (vapour phase).

273

274 **Interferon-gamma (IFN-γ) ELISpot Assay.** An IFN-γ ELISpot assay was performed
275 as described previously for ferrets²⁴ and macaques²⁵.

276 **Immunophenotyping.** Whole blood immunophenotyping assays were performed
277 using 50 µl of heparinised blood incubated for 30 min at room temperature with optimal
278 dilutions of the following antibodies: anti-CD3-AF700, anti-CD4-APC-H7, anti-CD8-
279 PerCP-Cy5.5, anti-CD95-Pe-Cy7, anti-CD14-PE, anti-HLA-DR-BUV395, anti-CD25-
280 FITC (all from BD Biosciences, Oxford, UK); anti-CD127-APC (eBioscience); anti-γδ-
281 TCR-BV421, anti-CD16-BV786, anti-PD-1-BV711, anti-CD20-PE-Dazzle (all from
282 BioLegend); and amine reactive fixable viability stain red (Life Technologies); all
283 prepared in brilliant stain buffer (BD Biosciences). Red blood cell contamination was
284 removed using a Cal-lyse reagent kit as per the manufacturer's instructions
285 (Thermofisher scientific). BD Compbeads (BD Biosciences) were labelled with the
286 above fluorochromes for use as compensation controls. Following antibody labelling,

287 cells and beads were fixed in a final concentration of 4% paraformaldehyde solution
288 (Sigma Aldrich, Gillingham, UK) prior to flow cytometric acquisition.

289

290 Cells were analysed using a five laser LSRII Fortessa instrument (BD Biosciences)
291 and data were analysed using FlowJo (version 10, Treestar, Ashland, US).
292 Immediately prior to flow cytometric acquisition, 50 µl of Truecount bead solution
293 (Beckman Coulter) was added to each sample. Leukocyte populations were identified
294 using a forward scatter-height (FSC-H) versus side scatter-area (SSC-A) dot plot to
295 identify the lymphocyte, monocyte and granulocyte populations, to which appropriate
296 gating strategies were applied to exclude doublet events and non-viable cells.
297 Lymphocyte sub populations including T-cells, NK-cells, NKT-cells and B-cells were
298 delineated by the expression pattern of CD3, CD20, CD95, CD4, CD8, CD127, CD25,
299 CD16 and the activation and inhibitory markers HLA-DR and PD-1. GraphPad Prism
300 (version 8.0.1) was used to generate graphical representations of flow cytometry data.

301

302 **Histopathology.** The following samples from each ferret were fixed in 10% neutral-
303 buffered formalin, processed to paraffin wax and 4 µm thick sections cut and stained
304 with haematoxylin and eosin (HE); respiratory tract (left cranial and caudal lung
305 lobes; 3 sections from each lung lobe: proximal, medial and distal to the primary
306 lobar bronchus), trachea (upper and lower), larynx, tonsil, liver, kidney, spleen,
307 mediastinal lymph node, and small (duodenum) and large intestine (colon). Nasal
308 cavity samples were also taken and decalcified in an EDTA solution for 3 weeks
309 before embedding. These tissues above were examined by light microscopy and
310 evaluated subjectively. Three qualified veterinary pathologists examined the tissues
311 independently and were blinded to treatment and group details and the slides

312 randomised prior to examination in order to prevent bias. A semiquantitative scoring
313 system was developed to compare the severity of the lung lesions for each individual
314 animal and among groups. This scoring system was applied independently to the
315 cranial and caudal lung lobe tissue sections using the following parameters: a)
316 bronchial inflammation with presence of exudates and/or inflammatory cell
317 infiltration; b) bronchiolar inflammation with presence of exudates and/or
318 inflammatory cell infiltration; c) perivascular inflammatory infiltrates (cuffing); and d)
319 infiltration of alveolar walls and spaces by inflammatory cells, mainly mononuclear.
320 The severity of the histopathological lesions was scored as: 0=none (within normal
321 limits), 1=minimal, 2=mild, 3=moderate, and 4=severe.

322 Tissue sections of both lung lobes, nasal cavity and gastrointestinal tract from animals
323 culled at the early timepoint (day 6/7 post-challenge) were stained using the
324 RNAscope *in situ* hybridisation (ISH) technique to identify SARS-CoV-2 RNA. Briefly,
325 tissues were pre-treated with hydrogen peroxide for 10 min (room temperature), target
326 retrieval for 15 min (98-101°C) and protease plus for 30 mins (40°C) (Advanced Cell
327 Diagnostics). A V-nCoV2019-S probe (Cat No. 848561, Advanced Cell Diagnostics)
328 was incubated on the tissues for 2 h at 40°C. Amplification of the signal was carried
329 out following the RNAscope protocol using the RNAscope 2.5 HD Detection kit – Red
330 (Advanced Cell Diagnostics).

331 In addition, immunohistochemistry was used to identify T cells (CD3⁺) in lung tissue
332 sections. Samples were cut at 4µm onto adhesive slides and stained using the Leica
333 Bond RxM (Leica Biosystems, Germany). Briefly, slides were dewaxed and treated
334 with peroxide block for 5 min. Epitope retrieval was performed using Epitope Retrieval
335 solution 2 (Leica Biosystems, Germany) for 20 min. A polyclonal rabbit anti-human

336 CD3 antibody (1:200; Agilent Technologies Inc, CA) was applied for 15 min and used
337 with Leica Polymer Refine Detection kit to complete the staining.

338 The following samples from each rhesus macaque were fixed, processed, cut and
339 stained as described above: left cranial and caudal lung lobes, trachea, larynx,
340 mediastinal lymph node, tonsil, spleen, liver, kidney, duodenum and colon.

341

342 For the lung, three sections from each left lung lobe were sampled from different
343 locations: proximal, medial and distal to the primary lobar bronchus. A scoring system
344 was used to evaluate objectively the histopathological lesions observed in the lung
345 tissue sections²⁵. The scores for each histopathological parameter were calculated as
346 the average of the scores observed in the six lung tissue sections evaluated per
347 animal.

348 Sections from the lung lobes, duodenum and colon were stained with RNAScope ISH
349 as described above. For the lung sections, digital image analysis was carried out with
350 Nikon NIS-Ar software in order to calculate the total area of the lung section positive
351 for viral RNA.

352 **In-life imaging of macaques by computed tomography (CT).** CT scans were
353 collected four weeks before vaccination and five days after challenge. CT imaging was
354 performed on sedated animals using a 16 slice Lightspeed CT scanner (General
355 Electric Healthcare, Milwaukee, WI, USA) in both the prone and supine position to
356 assist the differentiation of pulmonary changes at the lung bases caused by gravity
357 dependant atelectasis, from ground glass opacity caused by SARS-CoV-2. All axial
358 scans were performed at 120 KVp, with Auto mA (ranging between 10 and 120) and
359 were acquired using a small scan field of view. Rotation speed was 0.8 s. Images were

360 displayed as an 11 cm field of view. To facilitate full examination of the cardiac and
361 pulmonary vasculature, lymph nodes and extrapulmonary tissues, Niopam 300
362 (Bracco, Milan, Italy), a non-ionic, iodinated contrast medium, was administered
363 intravenously (IV) at 2 ml/kg body weight and scans were collected immediately after
364 injection and ninety seconds from the mid-point of injection.

365 Scans were evaluated by a medical radiologist expert in respiratory diseases,
366 including in non-human primates³⁰, blinded to the animal's clinical status, for the
367 presence of: disease features characteristic of COVID-19 in humans (ground glass
368 opacity (GGO), consolidation, crazy paving, nodules, peri-lobular consolidation;
369 distribution: upper, middle, lower, central 2/3, bronchocentric); pulmonary embolus
370 and the extent of any abnormalities estimated (<25%, 25-50%, 51-75%, 76-100%).

371

372 **CT Score system.** To provide the power to discriminate differences between
373 individual NHP's with low disease volume (i.e. <25% lung involvement), a score
374 system was applied in which scores were attributed for possession of abnormal
375 features characteristic of COVID in human patients (COVID pattern score) and for the
376 distribution of features through the lung (Zone score). The COVID pattern score was
377 calculated as sum of scores assigned for the number of nodules identified, and the
378 possession and extent of GGO and consolidation according to the following system:
379 Nodule(s): Score 1 for 1, 2 for 2 or 3, 3 for 4 or more; GGO: each affected area was
380 attributed with a score according to the following: Score 1 if area measured < 1 cm, 2
381 if 1 to 2 cm, 3 if 2 -3 cm, 4 if > 3 cm and scores for each area of GGO were summed
382 to provide a total GGO score; Consolidation: each affected area was attributed with a
383 score according to the following: 1 if area measured < 1 cm, 2 if 1 to 2 cm, 3 if 2 -3

384 cm, 4 if > 3 cm. Scores for each area of consolidation are summed to provide a total
385 consolidation score. To account for estimated additional disease impact on the host of
386 consolidation compared to GGO, the score system was weighted by doubling the
387 score assigned for consolidation. To determine the zone score, the lung was divided
388 into 12 zones and each side of the lung divided (from top to bottom) into three zones:
389 the upper zone (above the carina), the middle zone (from the carina to the inferior
390 pulmonary vein), and the lower zone (below the inferior pulmonary vein). Each zone
391 was further divided into two areas: the anterior area (the area before the vertical line
392 of the midpoint of the diaphragm in the sagittal position) and the posterior area (the
393 area after the vertical line of the mid-point of the diaphragm in the sagittal position).
394 This results in 12 zones in total where a score of one is attributed to each zone
395 containing structural changes. The COVID pattern score and the zone are summed to
396 provide the Total CT score.

397

398

399 **ELISA to characterise ligand binding to formaldehyde-treated and untreated S**
400 **trimer and RBD.** Antigens and ligands. The S trimer expression plasmid was obtained
401 from R Shattock and P. McCay (Imperial College London, UK), and expressed the
402 Wuhan-Hu-1 sequence (NCBI Reference NC_045512.2) in the context of a functional
403 S1S2 cleavage site, and with the addition of a C-terminal trimerization domain and 6x
404 his and Myc tags. The RBD-Fc expression plasmid was also based on the Wuhan-Hu-
405 1 sequence and was obtained from the Krammer lab (Mount Sinai, NY, USA). Proteins
406 were expressed in 293F cells and purified by nickel column (Thermofisher) for S trimer
407 and protein A column (Thermofisher) for RBD-Fc. Soluble ACE2-Fc was based on a

408 published sequence³¹ and was obtained from H. Waldmann and manufactured by
409 Absolute Antibody Inc, and RBD-binding mAbs CR3022³² and EY6A3³³ were obtained
410 from T. Tan and K-Y. Huang respectively, and were biotinylated using NHS-LC-biotin
411 following manufacturer's instructions (ThermoFisher).

412 For S trimer capture ELISA, high protein binding ELISA plates (PerkinElmer) were
413 coated overnight at 4°C with anti-myc antibody 9E10 at 4 µg/mL. After washing and
414 blocking in PBS/2% BSA/0.05% tween 20, S trimer at 1 µg/mL was added in 50µL/well
415 in PBS/1% BSA/0.05% tween 20 (ELISA buffer, EB) for 2 h at room temperature. For
416 RBD capture ELISA, high protein binding ELISA plates (PerkinElmer) were coated
417 overnight at 4°C with rabbit anti-human IgG (Jackson Laboratories) at 5 µg/mL in PBS.
418 After washing and blocking, RBD-Fc at 1 µg/mL was added in 50µL/well in EB for 2h
419 at room temperature. After washing, wells requiring formaldehyde (FA) treatment were
420 incubated with 50µL 0.02% methanol-free formaldehyde (ThermoFisher Scientific) in
421 PBS for 72 h, and untreated wells with PBS for the same amount of time. After
422 washing, S-captured plates were incubated with soluble ACE2-Fc and mAbs
423 CR3022³² and EY6A³³ in EB, and binding detected using donkey anti-human HRP
424 (Jackson) at 1:5000 in 50µL EB. RBD-captured plates were incubated with biotinylated
425 ligands soluble ACE2-Fc, CR3022 and EY6A, and binding detected using streptavidin-
426 HRP (GE Healthcare) diluted 1:4000 in 50µL EB. Plates were incubated for 1h at room
427 temperature, washed, developed in 50µL TMB ELISA substrate (ThermoFisher) and
428 the reaction stopped with 50µL 0.5M H₂SO₄. Absorbance at 450 and 570 nm was read
429 on a SpectraMax M5 plate reader (Molecular Devices) and data analysed in GraphPad
430 Prism v7.

431

432 **Molecular modelling.** The structure shown is PDB 6lzg, and the views shown were
433 generated in Pymol 2.3.5 (Schrodinger LLC).

434

435 **Results**

436 **Characterisation of formaldehyde-inactivated vaccine (FIV).** Transmission
437 electron microscopy (**Fig. 1**) showed that the washing and formaldehyde inactivation
438 procedure resulted in virus particles that appeared similar to typical coronavirus
439 morphology with a complete ring of peplomers/spikes on each particle (**Fig. 1A and**
440 **B**). SDS-PAGE analysis (Supplementary Figure 1A) shows that the majority of protein
441 bands seen in the medium only (lane 2) are also seen in the live wild-type virus (lane3)
442 and the FIV (lane 4), indicating that these proteins are likely to be components of the
443 culture medium, including FBS and host cell proteins. The medium-only protein
444 species are visibly reduced in intensity following washing using the centrifugal
445 concentrator in the FIV even though this was six-fold concentrated. Notably, protein
446 species in the 62 to 100kDa range were almost absent in FIV. The Western blot
447 analysis (**Supplementary Figure 1B and 1C**) confirms that both wild-type live virus
448 and FIV react with antibodies to both SARS-CoV-2 spike-RBD and nucleocapsid.
449 Western blot with NIBSC, SARS-CoV-2 neutralising, MERS convalescent serum (S3)
450 standard has limited reactivity with proteins found in the medium (Supplementary
451 Figure 1B). However, a virus-specific band corresponding to the spike-RBD band on
452 the specific antisera blot is detected at approximately 160kDa in unwashed virus
453 preparation and at 180kDa in FIV with the increase molecular weight likely to be a
454 consequence of formaldehyde-fixation cross linking this protein.

455

456 **SARS-CoV-2 infection in control and FIV-vaccinated ferrets.** Ferrets vaccinated
457 with either a recombinant adenovirus expressing GFP (Ad-GFP) or FIV were
458 challenged intranasally with 1mL of Victoria/1/2020 SARS-CoV-2 at 5×10^6 PFU²⁶.
459 The sampling schedule is shown in **Table 1**. The high titre stock of challenge virus
460 was prepared (passage 3), and quality control sequencing showed it was identical to
461 the original stock received from the Doherty Institute and did not contain a commonly
462 reported 8 amino acid deletion in the furin cleavage site³⁴. Both groups displayed
463 similar viral genome copies in nasal wash samples which continued to be detected
464 until the end of the experiment at day 15 pc (**Fig. 2A**). Similar to previous studies²⁴
465 the peak in viral RNA shedding was seen between day 2 and 4 pc for all ferrets in
466 both groups. The majority of virus detected in nasal wash occurred between
467 challenge and day 8 pc. Interestingly, viral RNA detected in nasal wash and throat
468 swab samples was also shown to be approximately 3-fold higher in the FIV group at
469 day 2 pc. A similar trend was seen in both groups during the first week after
470 challenge although the RNA genome copies measured were substantially lower in
471 the throat swab than in the nasal wash samples (**Fig. 2B**). Overall, there were no
472 significant differences between groups in virus shedding from either the nose or
473 throat. Neither group of animals showed weight loss due to the infection
474 (**Supplementary Fig. 2**). The apparent difference in weight gain in the FIV-
475 vaccinated group was due to the necropsy-sampling of the two lightest animals from
476 this group on day 7. No fever was seen at any time in either group of ferrets post
477 infection (**Supplementary Fig. 2**).

478

479 **Pathology following SARS-CoV-2 infection in ferrets.** We performed sequential
480 culls on days 6-7 and 13-14 in order to study the potential for VED during and after

481 resolution of SARS-CoV-2 infection. The lung histopathology scores for individual
482 Ad-GFP- and FIV-vaccinated ferrets are shown in the heat map in **Fig 3A**. Samples
483 were obtained from 2 animals from each group early in the infection (Ad-GFP at day
484 6 pc and FIV at day 7 pc). The remaining animals were euthanised at days 13-14 pc
485 (Ad-GFP) and day 15 pc (FIV). All assessments, including bronchiolar, bronchial and
486 interstitial infiltrates together with perivascular cuffing, were scored as minimal or
487 mild in the Ad-GFP-vaccinated animals with a greater number of mild or moderate
488 scores in the FIV-vaccinated ferrets at the early time point (6/7 days pc). One animal
489 from the Ad-GFP group showed mild lesions compatible with acute bronchiolitis and
490 perivascular/peribronchiolar cuffing (**Fig. 3F,G**). The other animal from this group
491 showed only occasional minimal bronchiolar infiltrates. Both animals from the FIV
492 group at 7 days pc showed more remarkable changes, with mild to moderate
493 bronchiolitis (infiltrates within the bronchioles and occasionally bronchi) and
494 inflammatory foci within the parenchyma (**Fig. 3B**). Moreover, perivascular cuffing
495 was observed frequently (**Fig. 3C**), with the infiltrates being mostly mononuclear
496 cells, including CD3⁺ T lymphocytes identified by immunohistochemistry (IHC)
497 staining (**Fig. 3D**). Occasionally, neutrophils and eosinophils were also present (**Fig.**
498 **3C, insert**). The cuffing also affected numerous airways (**Fig. 3C**). Due to the small
499 numbers of animals, the differences in scores observed between FIV and Ad-GFP-
500 vaccinated groups did not reach significance.

501 In contrast, at 13-15 days pc, the lesions observed were minimal to mild with no
502 obvious differences between groups (**Fig. 3A**).

503 RNAScope ISH technique was used to detect viral RNA in lung and nasal cavity tissue
504 sections. Only very few occasional scattered cells were found positive to viral RNA in
505 the lung at day 6/7, which were within the alveolar walls and not related to the presence

506 of lesions. No differences were observed between groups. Viral RNA was also found
507 only as small foci of positive cells (epithelial and or sustentacular) within the olfactory
508 and respiratory mucosa in only one animal from the Ad-GFP group at day 6 pc (**Fig.**
509 **3H**).

510 No obvious lesions were observed in any other organ except for the liver, which
511 showed a variable degree of multifocal hepatitis, mild to moderate in all animals (**Fig**
512 **3E, I**).

513

514 **Immune responses to FIV in ferrets.** Ad-GFP-vaccinated animals showed no
515 immune responses before SARS-CoV-2 challenge (**Fig 4**). FIV-vaccinated animals
516 produced significant increases in IgG after vaccination against SARS-CoV-2 spike and
517 spike receptor binding domain (RBD). The response to nucleoprotein (N) was not
518 significant. Modest rises (GMT=89, $p=0.002$) in neutralising antibody titres were seen
519 in sera from the FIV-vaccinated animals with a rapid rise in neutralising antibody titres
520 after challenge indicating immune priming by the FIV. At termination, the GM titres for
521 FIV-vaccinated animals were 5356 and 453 for the two remaining Ad-GFP-vaccinated
522 animals. ELISpot assays applied to splenocytes isolated on day 6 pc (Ad-GFP-
523 vaccinated) or day 7 pc (FIV-vaccinated) show that animals vaccinated with FIV made
524 more pronounced responses to whole live virus, membrane, and nucleocapsid peptide
525 pools with both groups showing similar low responses to virus spike peptides
526 (**Supplementary Fig. 3A**). This early interferon gamma response post-infection is
527 consistent with an anamnestic response in the FIV-vaccinated animals following
528 priming with viral antigens. The lack of cellular immune response to spike is interesting

529 and might indicate that FIV promotes a skewed immune response. Responses
530 measured in PBMCs were much lower than those seen in splenocytes.

531

532 **SARS-CoV-2 infection in control and FIV-vaccinated rhesus macaques.**

533 Following infection with 5×10^6 PFU Victoria/1/2020 SARS-CoV-2 given in 2ml by the
534 intratracheal route and 1ml intranasally, viral RNA was quantified in nasal wash and
535 throat swab samples. Viral RNA was detected in both samples during the experiment
536 with peak values detected by day three and a decline thereafter (**Fig5A and B**). There
537 was no difference between viral copies detected in macaques that received FIV or no
538 vaccine. Bronchioalveolar lavage (BAL) was obtained on necropsy at day 7 and lower
539 geometric mean viral RNA copies per ml ($p=0.168$) were measured in macaques that
540 received FIV than no vaccine controls (**Fig 4C**). No significant changes in body
541 temperature (**Supplementary Fig. 2C**) were observed. Slight weight loss was
542 observed in both groups (**Supplementary Fig. 2D**), but no adverse clinical signs were
543 recorded for any macaque despite frequent monitoring during the study period.

544 Images from CT scans collected five days after challenge were examined by an
545 expert thoracic radiologist with experience of non-human primate prior CT
546 interpretation and human COVID-19 CT features, blinded to the clinical status.

547 Pulmonary abnormalities that involved less than 25% of the lung and reflected those
548 characteristics of SARS-CoV-2 infection in humans were identified in three of the FIV
549 group and five of the unvaccinated group. Where reported, disease was
550 predominantly bilateral (two of three FIV, five of six unvaccinated) with a similar
551 peripheral distribution through the lung lobes reported in the FIV vaccinated and
552 unvaccinated macaques. Ground glass opacity was observed in all the macaques

553 showing abnormal lung structure, with the exception of one FIV-vaccinated animal in
554 which consolidation was identified. Other features characteristic of human COVID
555 (reverse halo, perilobular, nodules, pulmonary embolus) were not observed in any of
556 the macaques in either group. Evaluation of pulmonary disease burden using a
557 scoring system designed to discriminate differences between individual macaques
558 with low disease volume revealed a non-significant trend ($p = 0.1364$) for a reduction
559 in the total CT score in the FIV group compared to the scores attributed to macaques
560 in the unvaccinated group (**Fig. 6D**). Similarly, the FIV vaccine reduced both the
561 amount of abnormalities induced (pattern score) and distribution of disease (zone
562 score) (**Supplementary Fig. 4 H and I**).

563 **Pathology following SARS-CoV-2 infection in rhesus macaques.** Pathological
564 changes were found in the lungs of all SARS-CoV-2-infected macaques and consisted
565 of multiple areas of mild to moderate bronchiolo-alveolar necrosis, inflammatory cell
566 infiltration and type II pneumocyte proliferation. Mild perivascular and peribronchiolar
567 cuffing was also observed. The lung pathology scores for individual macaques are
568 shown in **Fig. 6** and with milder pathological changes observed in the FIV-vaccinated
569 macaques. The total pathology score for the no vaccine group was greater than the
570 FIV group (**Fig. 6B**, $p=0.013$). RNAScope analysis of the percentage of area positively
571 stained for SARS-CoV-2 RNA showed a greater lung area infected in the no vaccine
572 group than FIV-vaccinated macaques (**Fig. 6C**, $p = 0.0238$).

573 **Immune responses to FIV in rhesus macaques.** Serum from control macaques
574 obtained on the day of challenge did not show any N, RBD or S-specific IgG but rises
575 in RBD and S-specific IgG were detected in serum from the FIV-vaccinated macaques
576 (**Fig. 7**). FIV-vaccinated animals also showed a rise in neutralising antibody titre on the
577 day of challenge ($p=0.0287$). Both groups showed a rise in neutralising antibody titre

578 7 days following challenge (**Fig. 7**). Similarly, on the day of challenge, a higher
579 frequency of spike-specific IFN γ -secreting cells was measured by ELISPOT assay in
580 the FIV group compared to that determined in the unvaccinated group suggesting the
581 induction of a modest but significant ($p = 0.0433$) SARS-CoV-2-specific cellular
582 response (**Supplementary. Figure 3B**). The trend reversed six to eight days after
583 challenge when frequencies were assessed at the end of the study, with higher
584 frequencies of spike-specific IFN γ -secreting cells measured in the unvaccinated group
585 compared to the FIV group in both PBMC and spleen cells.

586 Immunophenotyping flow cytometry assays were applied to whole blood samples
587 collected immediately prior to and 14 days after FIV vaccination, as well as at days
588 three and seven after SARS-CoV-2 challenge to explore potential vaccine-induced
589 changes in the cellular immune compartment that might influence the course of the
590 immune response following infection (**Sup figure 3**). CD4 and CD8 T-cells expressing
591 the immune checkpoint signalling receptor PD-1 increased significantly following FIV
592 vaccination ($p = 0.03$), with further significant increases observed in PD-1 expressing
593 CD4 T-cell populations following SARS-CoV2 infection in both FIV vaccinated and
594 unvaccinated groups (both $p = 0.03$) (SF3 D). Similarly, FIV vaccination led to a
595 significant increase in CD4 regulatory T-cells expressing CD25 and CD127 (SP3 E),
596 indicating that alongside the proinflammatory cellular response evident in the antigen-
597 specific IFN γ ELISPOT profiles, FIV vaccination also induced T-cell populations with
598 a more tolerogenic phenotype.

599 **Effects of formaldehyde on SARS-CoV-2 spike.** Formaldehyde treatment of SARS-
600 CoV-2 virus will cross link viral proteins, of which the S glycoprotein trimer is the target
601 of most neutralising antibodies. Cross-linking of S may modify its antigenicity,
602 potentially altering elicitation of neutralising antibodies. To analyse the effects of

603 formaldehyde on the S trimer and the isolated receptor binding domain (RBD), soluble
604 antigens were captured onto ELISA plates using either anti-Myc tag (S-Myc) or anti-
605 Fc (RBD-Fc) respectively to maintain their native conformation, and treated or not with
606 formaldehyde using the same protocol as for inactivation of whole virus: 0.02% for 72
607 h at room temperature. Samples were then tested for binding of RBD ligands, either
608 soluble (s)ACE2-Fc or the RBD binding monoclonal antibodies (mAbs) CR3022 and
609 EY6A which interact with RBD surfaces non-overlapping the ACE2 binding site.
610 Binding curves revealed that ligands binding to formaldehyde-treated S protein gave
611 substantially lower maximum binding than that to the untreated S counterpart (**Fig.**
612 **8A**). Area under the curve (AUC) analysis revealed that binding was significantly
613 reduced for sACE2-Fc and CR3022, and had a trend to reduction for EY6A (**Fig. 8B**).
614 Interestingly, the reduction for sACE2 and CR3022 was almost precisely 2-fold,
615 suggesting either that the formaldehyde treatment had reduced the binding activity of
616 a subset of RBD domains, or that half of the formaldehyde-treated S trimers were in a
617 non-RBD available conformation. To differentiate between these two possibilities, we
618 tested binding directly to the isolated untreated or formaldehyde-treated RBD (**Fig.**
619 **8C**). Strikingly, formaldehyde treatment had no effect on RBD-ligand binding, with
620 AUC analysis showing near identical values for formaldehyde-treated and untreated
621 RBD (**Fig. 8D**). It therefore seems most likely that formaldehyde treatment is stabilising
622 a population of S trimers in the 'RBD down' conformation which would be unable to
623 engage ACE2. Indeed, recent cryo-EM structures imply that the S trimer is 50% one
624 RBD up and 50% all RBD down at equilibrium³⁵. Therefore, cross-linking of a
625 population of trimers would most likely fix this 1:1 equilibrium, allowing only half of the
626 trimers to expose the one-up RBD. By contrast, untreated S trimer would be free to
627 sample both conformations, allowing progressive ACE2 occupancy to maximum of the

628 one RBD-up trimer conformation over time. This would explain the 2-fold decrease in
629 occupancy of formaldehyde-treated S trimers shown above. To further interrogate this,
630 we modelled the location of lysine and arginine residues, the side chains of which are
631 targets for formaldehyde attack, in the S trimer and RBD structures. **Fig. 6E** focuses
632 on the location of lysines and arginines in the RBD-down S trimer, revealing a large
633 number proximal to the RBD-trimer interface that might be cross-linked to prevent RBD
634 movement. **Fig. 6F** shows that whilst there are lysine and arginine residues proximal
635 to the RBD-ACE2 binding interface (green), there are none within the interface,
636 implying that formaldehyde treatment would not directly affect RBD-ACE2 binding.
637 This modelling is therefore consistent with the idea that formaldehyde cross-linking will
638 lock 50% of trimers into the all RBD-down state, reducing access of the RBD to ligand
639 binding and B cell recognition. Such modified antigenicity would probably translate into
640 reduced RBD immunogenicity, reducing antibody production against this neutralising
641 antibody-eliciting surface.

642

643 **Discussion**

644 Rapid development of vaccines to prevent COVID-19 disease is in progress². Safety
645 is of primary importance for vaccines that are administered to healthy people. Thus
646 vaccines must be thoroughly assessed for reactogenicity and longer term safety³⁶,
647 but also to ensure they do not cause any enhancement of disease³⁷. Vaccine-
648 enhanced disease (VED) which can be mediated by antibody-dependent
649 enhancement (ADE) have been described for respiratory virus vaccines. The
650 potential risks from COVID-19 vaccines have been described by Graham³⁸.
651 Enhanced disease can potentially be mediated by antibodies that bind virus without

652 neutralising activity, which cause disease through increased viral replication, or via
653 formation of immune complexes that deposit in tissues and activate complement
654 pathways associated with inflammation¹². T helper 2 cell (Th2)-biased immune
655 responses have also been associated with ineffective vaccines that can lead to
656 enhanced disease following infection^{39 40}. In order to understand the risks of VED
657 caused by SARS-CoV-2 vaccines it will be of great benefit to produce a positive
658 control vaccine that enhances disease in an animal model following challenge so this
659 endpoint can be defined and the mechanisms can be understood and avoided in
660 vaccines prepared for human use.

661 As examples of VED have been observed following the use of a formaldehyde
662 inactivated vaccine, e.g. RSV⁴¹ and measles¹⁶, we have prepared a killed SARS-
663 CoV-2 vaccine by formaldehyde-fixation of the virus. Formaldehyde-fixed SARS-
664 CoV-1 vaccines have been shown to induce enhanced disease^{18 19 21 42} although the
665 mechanism for this is not understood. It has been suggested that non-viral
666 components of formaldehyde-inactivated preparations, such as cellular components
667 and debris or medium constituents may also play a role in enhanced disease. In a
668 cotton rat model of RSV VED, cell culture contaminants were a major driver of lung
669 pathology which was exacerbated by the formaldehyde-inactivated vaccine and
670 RSV challenge⁴³. The SARS-CoV-2 FIV prepared in this study also contained cell
671 and medium components although the content was significantly reduced by washing
672 using a centrifugal concentrator. Another factor in the FIV vaccine design was to use
673 Alhydrogel as an adjuvant which is known to induce Th2-biased immune
674 responses⁴⁴. In addition, as a sub-optimal immune response has been suggested to
675 be associated with VED¹⁴, we chose to challenge the ferrets and rhesus macaques
676 14 days after intramuscular delivery of a single dose of FIV.

677 The SARS-CoV-2 infection in the Ad-GFP- or FIV-vaccinated ferrets followed a
678 similar course to that observed in our previous study²⁴ with peak viral RNA shedding
679 between 2 and 4 days pc. It was interesting to note that higher viral loads were
680 detected in the upper respiratory tract of FIV-vaccinated animals at day 2 pc but after
681 this sampling time, very similar genome copy values were obtained. There were no
682 differences in temperature, weight (Supplementary figure 2) or any other clinical
683 signs between the two groups.

684 Consistent with the higher viral load at day 2, the lung histopathology from the two
685 FIV-vaccinated animals necropsied at day 7 was more severe than the two Ad-GFP-
686 vaccinated animals necropsied at day 6. The semiquantitative scoring system was
687 used to discriminate the severity of lesions between animals and groups. Even
688 though the number of animals was small, and although lung pathology was not
689 severe in any case, we observed some unique differences in the FIV-vaccinated
690 ferrets. At 6-7 days pc, a higher severity was observed in animals from the FIV group
691 (combined total score 24), compared to the Ad-GFP control (combined total score
692 10). This included eosinophilic infiltrate and perivascular cuffing that was not
693 observed in the control-vaccinated ferrets. The lung pathology in the ferret model²⁴
694 was quite transient and thus at 13-15 days pc, whilst there was some individual
695 variability present, both groups showed mild pathology.

696 Multifocal mild to moderate hepatitis has also been described as a potential adverse
697 effect of SARS-CoV-1 vaccines⁴⁵. However, these lesions are found as a
698 background finding for this species in many experimental studies, although viral
699 infections, systemic or in the gastrointestinal tract, have also been related to the
700 presence of these periportal inflammatory infiltrates⁴⁶. Due to the variability in

701 severity and the fact that naïve ferrets frequently show some degree of hepatitis, the
702 interpretation of this lesion must be taken cautiously.

703 Following this observation of mild transient enhanced disease in the two FIV-
704 vaccinated ferrets culled at day 7, we have tested the same FIV in 6 macaques
705 along with 6 unvaccinated controls. We have previously compared the course of
706 SARS-CoV-2 infection in both rhesus and cynomolgus macaques and showed virus
707 replication in the upper and lower respiratory tract with pulmonary lesions resembling
708 mild COVID-19 in humans²⁵. Macaques allow for a more detailed examination of
709 lung pathology using precise scoring system devised in our recent study. We also
710 have considerable experience of in life CT scanning of macaques^{47 30 25} which allows
711 further aspects of lung pathology to be characterised at time points before necropsy.
712 This detailed analysis has revealed no evidence of enhanced disease in macaques
713 at any time point, but rather that the FIV provided some protection against the mild-
714 to-moderate lung pathology observed in the unvaccinated control macaques.

715 The presence of inflammatory infiltrates, and particularly perivascular cuffing, has
716 been described as a feature potentially related to VED in SARS-CoV-1 preclinical
717 vaccine trials^{19 21 22}. In our study these infiltrates were always of mild to moderate
718 severity.

719

720 Characterisation of the immune response to the FIV vaccine prior to challenge in both
721 species confirmed the expectation of modest immunity to SARS-CoV-2 spike. A
722 significant rise in anti-S IgG, but not anti-N IgG was detected by ELISA. The geometric
723 mean neutralising titre of 89 seen in ferrets and 61 in macaques was low compared to
724 that observed in primate studies with candidate vaccines^{10 8 7 11} and in clinical trials²⁹

725 7⁴⁸ 4⁵. However, the larger rise in neutralising antibody titre in FIV-vaccinated ferrets
726 and macaques following challenge, compared to control animals indicated that priming
727 had been mediated by the FIV. The SARS-CoV-2-specific interferon γ response
728 measured in splenocytes using an ELISpot assay 6 or 7 days after challenge showed
729 a greater response in the FIV-vaccinated animals to live virus, membrane and
730 nucleocapsid peptide pools but very little response to the S protein pools, indicating a
731 poor cellular response to the S antigen with FIV. Conversely, spike peptide-specific
732 IFN γ SFU measured in macaques increased significantly following FIV vaccination
733 and evaluation of the spike-specific interferon- γ response in splenocytes and PBMC
734 collected from macaques at a similar time after SARS-CoV2 challenge revealed the
735 reverse pattern to that observed in ferrets, with greater responses measured in the
736 unvaccinated group relative to the FIV-vaccinated animals. This may reflect that
737 improved priming of the T-cell mediated response contributed to the protection
738 afforded by the FIV vaccine in this species if response magnitude is driven by antigenic
739 load following infection.

740 The expression of checkpoint inhibitory receptors is often considered a marker of T-
741 cell exhaustion, although more recently PD-1 signaling has also been linked to
742 improved effector T-cell priming and enhanced clearance of acute viral infections⁴⁹.
743 Similarly, the induction of regulatory CD4 T-cells, and the inhibitory influence they
744 are likely to exert, may be considered counterproductive to vaccine induced
745 immunity. However, the increased frequency of T-regs observed following FIV
746 vaccination is likely to reflect the immune response typically induced by
747 coronaviruses in this species, as similar increases were also seen in the
748 unvaccinated animals early after SARS-CoV2 infection, and thus may help to explain

749 the relatively mild disease that develops in this species and the improved outcome
750 observed in FIV vaccine primed macaques^{50 51}.

751 There are limitations to the current study, the principal of which is the small number
752 of ferrets in the early culled group. The transient nature of the pathology meant that
753 these differences resolved by the second necropsy time point.

754 Some insight into the weak anti-S neutralising response induced by the FIV was
755 gained using a capture ELISA which preserved the conformation of the S trimer on
756 the solid phase, unlike direct coating onto the ELISA plate which modified
757 antigenicity (data not shown), as has been observed for soluble forms of the HIV-1
758 envelope glycoprotein trimer⁵². Formaldehyde treatment of S trimer in this format
759 was the same as that used to inactivate the vaccine, allowing extrapolation of ligand
760 binding to the ELISA-captured formaldehyde-treated S trimer to that on the virus.
761 Formaldehyde cross-linking resulted in a 2-fold reduction in binding of ACE2-Fc and
762 two RBD-specific MAbs (CR3022 and EY6A) to formaldehyde-treated compared to
763 untreated S trimer. By contrast, formaldehyde treatment of recombinant RBD did not
764 affect binding of ACE2-Fc or these MAbs, implying that formaldehyde treatment
765 cross-linked a proportion of S trimer into a non-ligand binding conformation. These
766 results are consistent with the location of lysine and arginine residues, and suggest
767 that RBD exposure may be limited by cross-linking, reducing exposure of
768 neutralising antibody epitopes on the RBD. This result may be of more general
769 interest, since other viral envelope glycoproteins, such as those of HIV-1, are
770 metastable and sample different conformational states, some of which are more
771 relevant to neutralisation than others⁵³. Cross-linking may trap these different
772 conformational states, modifying exposure of neutralising antibody epitopes to B cell
773 recognition⁵⁴.

774 Formaldehyde inactivation has been widely used to prepare inactivated viral
775 vaccines⁵⁵ and as a toxoiding agent for bacterial toxin vaccines. Some of the
776 considerations for inactivated SARS-CoV-2 vaccines are discussed in a recent
777 commentary⁵⁶. The study reported here has confirmed that caution should be used if
778 formaldehyde is the inactivation reagent for COVID-19 vaccines. Several vaccines
779 are in development that use β -propiolactone as inactivation agent. One such vaccine
780 has been shown to be protective in rhesus macaques following SARS-CoV-2
781 challenge without induction of VED⁷. A preliminary report of phase 1 and 2 studies
782 with another β -propiolactone vaccine indicated that it is tolerated, safe and produced
783 neutralising antibodies in phase 1 and 2 studies⁴⁸. In addition, the authors mention in
784 the discussion that enhancement of disease was not observed in primates following
785 SARS-CoV-2 challenge but no pathology results are presented.

786 In conclusion, we have prepared an experimental SARS-CoV-2 vaccine based on
787 previous inactivated virus studies that induced VED. We showed no evidence of
788 enhanced disease at later time points in ferrets or at any time in a more in-depth
789 analysis in rhesus macaques which included CT imaging. However, we did observe
790 increased pathology scores, early in the infection of FIV-vaccinated ferrets which
791 resolved by the later necropsy time point. It is reassuring that, even with a vaccine
792 deliberately designed to induce enhanced disease, no enhancement was seen apart
793 from at 7 days post infection in ferrets. Future studies to investigate the potential of
794 SARS-CoV-2 vaccines to cause enhanced disease should examine lung pathology
795 at multiple time points including soon after challenge. Formalin-inactivated virus can
796 be used as a suboptimal comparator to determine the potential of SARS-CoV-2
797 vaccine candidates to induce VED so that unsuitable vaccines are identified at an
798 early stage of development before significant clinical studies commence.

799

800

801 **References**

- 802 1. [https://www.who.int/docs/default-source/coronaviruse/situation-](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200824-weekly-epi-update.pdf?sfvrsn=806986d1_4)
803 [reports/20200824-weekly-epi-update.pdf?sfvrsn=806986d1_4](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200824-weekly-epi-update.pdf?sfvrsn=806986d1_4).
- 804 2. Lurie, N., Saville, M., Hatchett, R. & Halton, J. Developing covid-19 vaccines at
805 pandemic speed. *New England Journal of Medicine* vol. 382 1969–1973
806 (2020).
- 807 3. Krammer, F. SARS-CoV-2 vaccines in development. *Nature* vol. 586 516–527
808 (2020).
- 809 4. Jackson, L. A. *et al.* An mRNA Vaccine against SARS-CoV-2 — Preliminary
810 Report. *N. Engl. J. Med.* (2020) doi:10.1056/nejmoa2022483.
- 811 5. Mulligan, M. J. *et al.* Phase 1/2 study of COVID-19 RNA vaccine BNT162b1 in
812 adults. *Nature* 1–8 (2020) doi:10.1038/s41586-020-2639-4.
- 813 6. Ramasamy, M. N. *et al.* Safety and immunogenicity of ChAdOx1 nCoV-19
814 vaccine administered in a prime-boost regimen in young and old adults
815 (COV002): a single-blind, randomised, controlled, phase 2/3 trial. *Lancet*
816 (2020) doi:10.1016/S0140-6736(20)32466-1.
- 817 7. Wang, H. *et al.* Development of an Inactivated Vaccine Candidate, BBIBP-
818 CorV, with Potent Protection against SARS-CoV-2. *Cell* **182**, 713-721.e9
819 (2020).
- 820 8. Gao, Q. *et al.* Development of an inactivated vaccine candidate for SARS-
821 CoV-2. *Science* **369**, 77–81 (2020).
- 822 9. Xia, S. *et al.* Effect of an Inactivated Vaccine Against SARS-CoV-2 on Safety
823 and Immunogenicity Outcomes: Interim Analysis of 2 Randomized Clinical
824 Trials. *JAMA* (2020) doi:10.1001/jama.2020.15543.
- 825 10. van Doremalen, N. *et al.* ChAdOx1 nCoV-19 vaccine prevents SARS-CoV-2
826 pneumonia in rhesus macaques. *Nature* (2020) doi:10.1038/s41586-020-2608-
827 y.
- 828 11. Corbett, K. S. *et al.* Evaluation of the mRNA-1273 Vaccine against SARS-CoV-
829 2 in Nonhuman Primates. *N. Engl. J. Med.* (2020)
830 doi:10.1056/nejmoa2024671.
- 831 12. Smatti, M. K., Al Thani, A. A. & Yassine, H. M. Viral-induced enhanced disease
832 illness. *Front. Microbiol.* **9**, (2018).
- 833 13. Arvin, A. M. *et al.* A perspective on potential antibody-dependent enhancement
834 of SARS-CoV-2. *Nature* **584**, (2020).
- 835 14. Iwasaki, A. & Yang, Y. The potential danger of suboptimal antibody responses
836 in COVID-19. *Nature Reviews Immunology* vol. 20 339–341 (2020).
- 837 15. Kapikian, A. Z., Mitchell, R. H., Chanock, R. M., Shvedoff, R. A. & Stewart, C.

- 838 E. An epidemiologic study of altered clinical reactivity to Respiratory Syncytial
839 (RS) virus infection in children previously vaccinated with an inactivated RS
840 virus vaccine. *Am. J. Epidemiol.* **89**, 405–421 (1969).
- 841 16. Polack, F. P., Hoffman, S. J., Crujeiras, G. & Griffin, D. E. A role for
842 nonprotective complement-fixing antibodies with low avidity for measles virus
843 in atypical measles. *Nat. Med.* **9**, 1209–1213 (2003).
- 844 17. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue
845 disease in humans. *Science (80-.)*. **358**, 929–932 (2017).
- 846 18. Bolles, M. *et al.* A Double-Inactivated Severe Acute Respiratory Syndrome
847 Coronavirus Vaccine Provides Incomplete Protection in Mice and Induces
848 Increased Eosinophilic Proinflammatory Pulmonary Response upon
849 Challenge. *J. Virol.* **85**, 12201–12215 (2011).
- 850 19. Tseng, C. Te *et al.* Immunization with SARS coronavirus vaccines leads to
851 pulmonary immunopathology on challenge with the SARS virus. *PLoS One* **7**,
852 (2012).
- 853 20. Czub, M., Weingartl, H., Czub, S., He, R. & Cao, J. Evaluation of modified
854 vaccinia virus Ankara based recombinant SARS vaccine in ferrets. in *Vaccine*
855 vol. 23 2273–2279 (Elsevier BV, 2005).
- 856 21. Zhou, J. *et al.* Immunogenicity, safety, and protective efficacy of an inactivated
857 SARS-associated coronavirus vaccine in rhesus monkeys. *Vaccine* **23**, 3202–
858 3209 (2005).
- 859 22. Wang, Q. *et al.* Immunodominant SARS coronavirus epitopes in humans
860 elicited both enhancing and neutralizing effects on infection in non-human
861 primates. *ACS Infect. Dis.* **2**, 361–376 (2016).
- 862 23. Chen, Z. *et al.* Recombinant Modified Vaccinia Virus Ankara Expressing the
863 Spike Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus
864 Induces Protective Neutralizing Antibodies Primarily Targeting the Receptor
865 Binding Region. *J. Virol.* **79**, 2678–2688 (2005).
- 866 24. Ryan, K. A. *et al.* Dose-dependent response to infection with SARS-CoV-2 in
867 the ferret model: evidence of protection to re-challenge. *bioRxiv* **2**,
868 2020.05.29.123810 (2020).
- 869 25. Salguero, F. J. *et al.* Comparison of Rhesus and Cynomolgus macaques as an
870 authentic model for COVID-19. *bioRxiv* 2020.09.17.301093 (2020)
871 doi:10.1101/2020.09.17.301093.
- 872 26. Caly, L. *et al.* Isolation and rapid sharing of the 2019 novel coronavirus (SARS-
873 CoV-2) from the first patient diagnosed with COVID-19 in Australia. *Med. J.*
874 *Aust.* **212**, 459–462 (2020).
- 875 27. van Doremalen, N. *et al.* A single dose of ChAdOx1 MERS provides protective
876 immunity in rhesus macaques. *Sci. Adv.* **6**, (2020).
- 877 28. Amanat, F. *et al.* An In Vitro Microneutralization Assay for SARS-CoV-2
878 Serology and Drug Screening. *Curr. Protoc. Microbiol.* **58**, e108 (2020).
- 879 29. Folegatti, P. M. *et al.* Safety and immunogenicity of the ChAdOx1 nCoV-19

- 880 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind,
881 randomised controlled trial. *Lancet* **396**, (2020).
- 882 30. Sharpe, S. A., Smyth, D., McIntyre, A., Gleeson, F. & Dennis, M. J.
883 Refinement and reduction through application of a quantitative score system
884 for estimation of TB-induced disease burden using computed tomography.
885 *Lab. Anim.* **52**, 599–610 (2018).
- 886 31. Kruse, R. L. Therapeutic strategies in an outbreak scenario to treat the novel
887 coronavirus originating in Wuhan, China. *F1000Research* **9**, 72 (2020).
- 888 32. Yuan, M. *et al.* A highly conserved cryptic epitope in the receptor binding
889 domains of SARS-CoV-2 and SARS-CoV. *Science (80-.)*. **368**, 630–633
890 (2020).
- 891 33. Zhou, D. *et al.* Structural basis for the neutralization of SARS-CoV-2 by an
892 antibody from a convalescent patient. *Nat. Struct. Mol. Biol.* (2020)
893 doi:10.1038/s41594-020-0480-y.
- 894 34. Davidson, A. D. *et al.* Characterisation of the transcriptome and proteome of
895 SARS-CoV-2 reveals a cell passage induced in-frame deletion of the furin-like
896 cleavage site from the spike glycoprotein. *Genome Med.* **12**, 68 (2020).
- 897 35. Walls, A. C. *et al.* Structure, Function, and Antigenicity of the SARS-CoV-2
898 Spike Glycoprotein. *Cell* **181**, 281-292.e6 (2020).
- 899 36. Bonhoeffer, J. *et al.* Guidelines for collection, analysis and presentation of
900 vaccine safety data in pre- and post-licensure clinical studies. *Vaccine* **27**,
901 2282–2288 (2009).
- 902 37. Lambert, P. H. *et al.* Consensus summary report for CEPI/BC March 12–13,
903 2020 meeting: Assessment of risk of disease enhancement with COVID-19
904 vaccines. in *Vaccine* vol. 38 4783–4791 (Elsevier Ltd, 2020).
- 905 38. Graham, B. S. Rapid COVID-19 vaccine development. *Science* vol. 368 945–
906 946 (2020).
- 907 39. Connors, M. *et al.* Enhanced pulmonary histopathology induced by respiratory
908 syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized
909 BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J.*
910 *Virol.* **68**, 5321–5325 (1994).
- 911 40. Moghaddam, A. *et al.* A potential molecular mechanism for hypersensitivity
912 caused by formalin-inactivated vaccines. *Nat. Med.* **12**, 905–907 (2006).
- 913 41. Acosta, P. L., Caballero, M. T. & Polack, F. P. Brief History and
914 Characterization of Enhanced Respiratory Syncytial Virus Disease. *Clinical*
915 *and Vaccine Immunology* vol. 23 189–195 (2016).
- 916 42. Wang, Q. *et al.* Immunodominant SARS coronavirus epitopes in humans
917 elicited both enhancing and neutralizing effects on infection in non-human
918 primates. *ACS Infect. Dis.* **2**, 361–376 (2016).
- 919 43. Shaw, C. A. *et al.* The role of non-viral antigens in the cotton rat model of
920 respiratory syncytial virus vaccine-enhanced disease. *Vaccine* **31**, 306–312
921 (2013).

- 922 44. He, P., Zou, Y. & Hu, Z. Advances in aluminum hydroxide-based adjuvant
923 research and its mechanism. *Human Vaccines and Immunotherapeutics* vol.
924 11 477–488 (2015).
- 925 45. Weingartl, H. *et al.* Immunization with Modified Vaccinia Virus Ankara-Based
926 Recombinant Vaccine against Severe Acute Respiratory Syndrome Is
927 Associated with Enhanced Hepatitis in Ferrets. *J. Virol.* **78**, 12672–12676
928 (2004).
- 929 46. Li, T. C. *et al.* Ferret hepatitis E virus infection induces acute hepatitis and
930 persistent infection in ferrets. *Vet. Microbiol.* **183**, 30–36 (2016).
- 931 47. Dennis, M. J. *et al.* A Flexible Approach to Imaging in ABSL-3 Laboratories.
932 *Appl. Biosaf.* **20**, 89–99 (2015).
- 933 48. Xia, S. *et al.* Effect of an Inactivated Vaccine Against SARS-CoV-2 on Safety
934 and Immunogenicity Outcomes. *JAMA* (2020) doi:10.1001/jama.2020.15543.
- 935 49. Ahn, E. *et al.* Role of PD-1 during effector CD8 T cell differentiation. *Proc. Natl.*
936 *Acad. Sci. U. S. A.* **115**, 4749–4754 (2018).
- 937 50. Cecere, T. E., Todd, S. M. & LeRoith, T. Regulatory T cells in arterivirus and
938 coronavirus infections: Do they protect against disease or enhance it? *Viruses*
939 vol. 4 833–846 (2012).
- 940 51. Meckiff, B. J. *et al.* Imbalance of Regulatory and Cytotoxic SARS-CoV-2-
941 Reactive CD4+ T Cells in COVID-19. *Cell* **183**, 1340-1353.e16 (2020).
- 942 52. Schiffner, T. *et al.* Chemical Cross-Linking Stabilizes Native-Like HIV-1
943 Envelope Glycoprotein Trimer Antigens. *J. Virol.* **90**, 813–828 (2016).
- 944 53. Guttman, M. *et al.* Antibody potency relates to the ability to recognize the
945 closed, pre-fusion form of HIV Env. *Nat. Commun.* **6**, (2015).
- 946 54. Schiffner, T. *et al.* Structural and immunologic correlates of chemically
947 stabilized HIV-1 envelope glycoproteins. *PLoS Pathog.* **14**, (2018).
- 948 55. Delrue, I., Verzele, D., Madder, A. & Nauwynck, H. J. Inactivated virus
949 vaccines from chemistry to prophylaxis: Merits, risks and challenges. *Expert*
950 *Review of Vaccines* vol. 11 695–719 (2012).
- 951 56. Mulligan, M. J. An Inactivated Virus Candidate Vaccine to Prevent COVID-19.
952 *JAMA* (2020) doi:10.1001/jama.2020.15539.

953

954

955 **Acknowledgements**

956 The authors acknowledge the contributions of all staff within the PHE Biological

957 Investigations Group for assistance with the delivery of the *in vivo* study and B

958 Cavell, J Gouriet, V Lucas, D Ngabo, S Thomas and R Watson for assistance with

959 processing of *in vivo* samples. The authors thank S Findlay-Wilson, T Hender, N
960 McLeod and C Turner for their assistance with RNA extraction and PCR, E Penn for
961 assistance with neutralisation assays and T Tipton for preparation of ELISpot
962 peptides. Alexandra Morrison, Adam Mabbut and Dinos Gkolfinos assisted with
963 ELISpot assays. The authors thank M Elmore and M Matheson for assistance with
964 data analysis. The authors also acknowledge the kind donation of the S trimer
965 expression construct by R. Shattock and P. McCay, the ACE2-Fc construct by H.
966 Waldmann, and the RBD-specific monoclonal antibodies CR3022 and EY6A by T.
967 Tan and K-Y. Huang respectively. We thank T. Schiffner for assistance with the
968 molecular modelling and S. Zhang for assistance with S trimer production and the
969 Jenner Institute Viral Vector Core Facility for the production of Ad-GFP. Q.
970 Sattentau, S. Gilbert and T. Lambe are Jenner Institute Investigators. Q. Sattentau is
971 a James Martin Senior Fellow. The work was supported by UKRI Grant
972 MC_PC_19080 and MRC UKRI Grant MC_PC_19055. Viral stock preparation was
973 funded by the Coalition for Epidemic Preparedness Innovations.

974

975 **Author contributions:** Kevin R. Bewley, development of virus growth, plaque and
976 neutralisation assay methods. Design of virus inactivation strategy, vaccine
977 preparation, drafting manuscript and data analysis; Karen Gooch, study plan and
978 management, data analysis and manuscript review; Kelly M. Thomas, study plan and
979 management, vaccine preparation, data collation and analysis, PBMC preparation,
980 manuscript review; Stephanie Longet, ELISA design, assay and analysis; Nathan
981 Wiblin, *in vivo* study supervisor; Laura Hunter, histopathology slide preparation and
982 staining; Kin Chan, SDS-PAGE and western blots; Phillip Brown, ELISpot assay and

983 analysis, processing of in vivo samples; Rebecca A. Russell, performed
984 experimental procedures, analysis and preparation of figures; Catherine Ho,
985 Preparation of in vivo samples and sera; Gillian Slack, PCR data analysis; Holly E.
986 Humphries, PRNT assays and analysis; Leonie Alden, in vivo study management;
987 Lauren Allen, performed PRNT assays; Marilyn Aram, performed PCR assays;
988 Natalie Baker, virus inactivation and confirmation; Emily Brunt, performed PRNT
989 assays; Rebecca Cobb, processing of in vivo samples; Susan Fotheringham, in vivo
990 study management; Debbie Harris, in vivo study management; Chelsea Kennard,
991 histology analysis including RNAscope; Stephanie Leung, performed PRNT assay;
992 Kathryn Ryan, processed in vivo samples; Howard Tolley, performed electron
993 microscopy; Nadina Wand, RNA extraction and PCR assays; Andrew White, Laura
994 Sibley and Charlotte Sarfas designed and performed the macaques ELISpot assays;
995 Xiaochao Xue, prepared and characterised proteins, manuscript drafting; Yper Hall,
996 preparation and characterisation of inactivated virus; Teresa Lambe, in vivo study
997 design and provision of control vaccine; Sue Charlton, in vivo study design and
998 preparation of inactivated vaccine; Simon Funnell, study design and manuscript
999 preparation; Sarah Gilbert, in vivo study design and provision of control vaccine;
1000 Quentin J. Sattentau, designed and performed experiments to characterise the effect
1001 of formaldehyde on SARS-CoV-2 spike protein, drafted manuscript; Francisco J.
1002 Salguero, lead the histopathology analysis and reporting, drafted manuscript; Geoff
1003 Pearson, histopathology analysis; Emma Rayner, histopathology analysis; Sally
1004 Sharpe, CT scanning and analysis, manuscript preparation; Fergus Gleeson,
1005 analysis of CT scans; Andrew Gorringe, scientific direction and lead preparation of
1006 the manuscript; Miles Carroll, established the project, in vivo study design, scientific
1007 direction and manuscript preparation.

1008

1009 **Data availability statement:** The data that support the findings of this study are
1010 available from the corresponding authors upon reasonable request.

1011

1012 **Competing interests:** Sarah Gilbert and Teresa Lambe are named on a patent
1013 application covering a vaccine ChAdOx1 nCoV-19. The remaining authors declare
1014 no competing interests. The funders played no role in the conceptualisation, design,
1015 data collection, analysis, decision to publish, or preparation of the manuscript.

1016

1017 **Tables and Figures**

1018

1019 **Table 1 Experimental animal groups.**

Group	Number of animals	Vaccination - days prior to challenge	Sampling	Euthanasia days post challenge
Ferret – Ad-GFP	4 females	28	Blood for serology prior to vaccination, on day of challenge and at necropsy.	6 days; n=2 13 days; n=1 14 days; n=1
Ferret – FIV	6 females	14	Nasal washes and throat swabs on days 0, 2, 4, 6, 8, 10, 12 post challenge and necropsy	7 days; n=2 14 days; n=4
Rhesus macaque – no vaccine	3 males 3 females	14	Blood serology prior to vaccination, on day of challenge and day 7 post challenge.	7 days; n=6
Rhesus macaque - FIV	3 males 3 females	-	Nasal wash and throat swab on days 1, 3, 5, and 7 post challenge. BAL at necropsy	7 days; n=6

1020

1021

1022

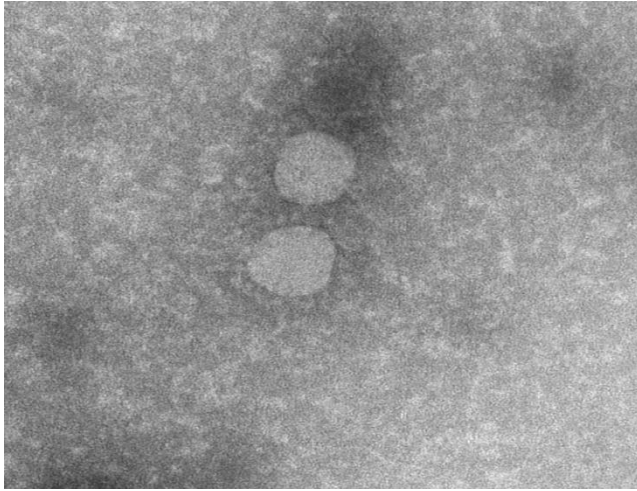
1023

1024

1025

1026

A



B

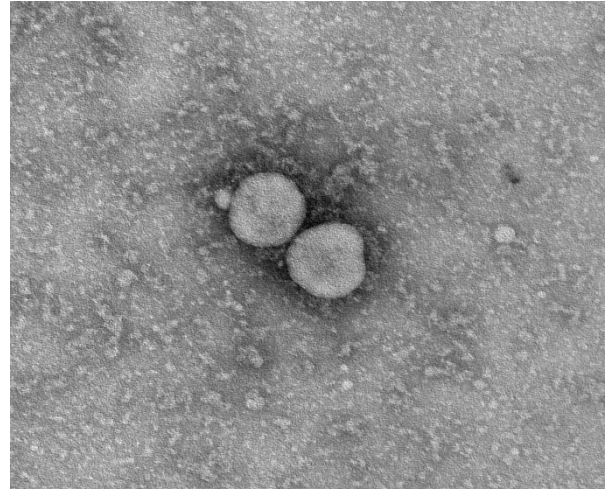


Fig. 1. Representative transmission electron microscopy images of (A) the initial SARS-CoV-2 virus preparation and (B) following formaldehyde inactivation and washing to remove medium constituents.

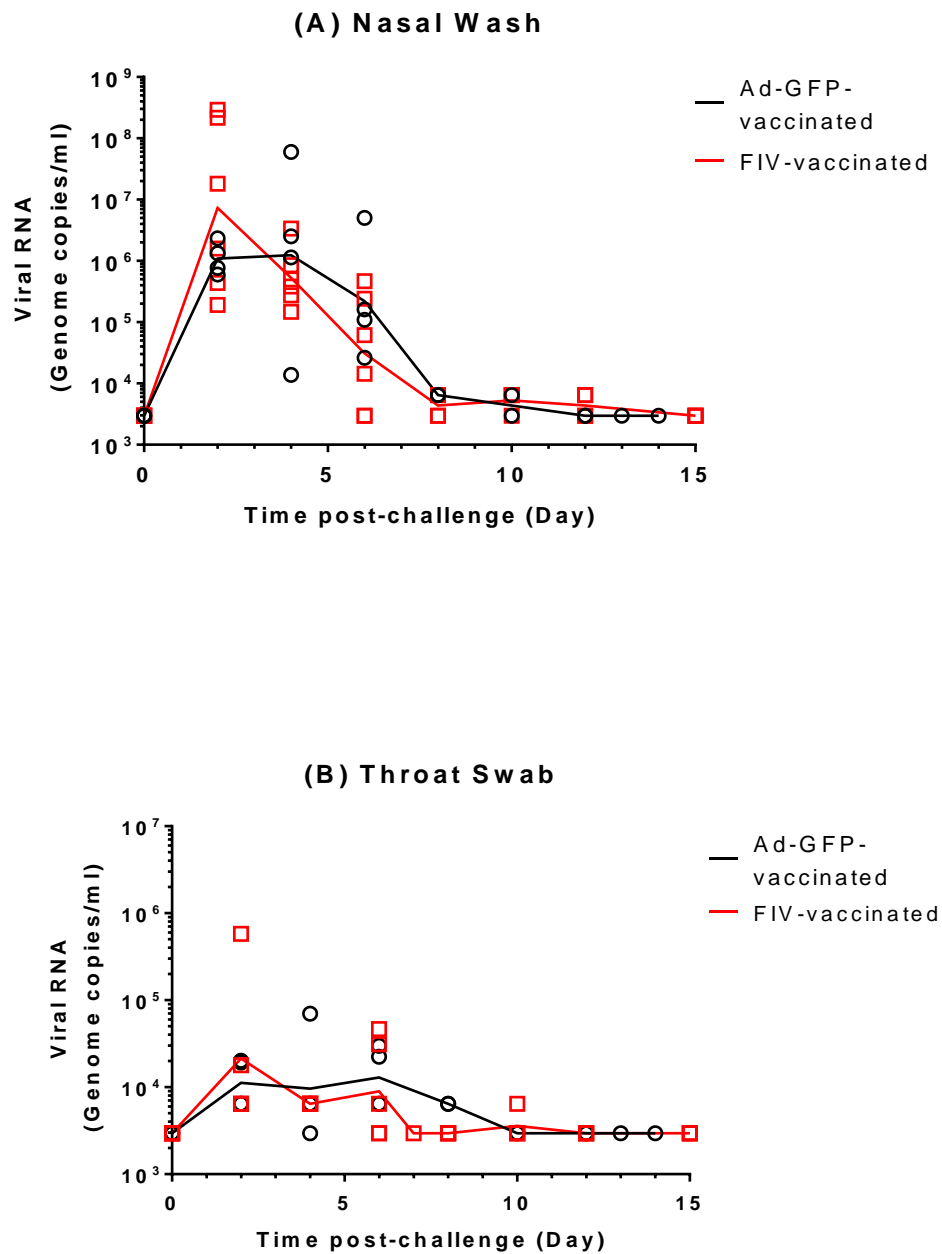
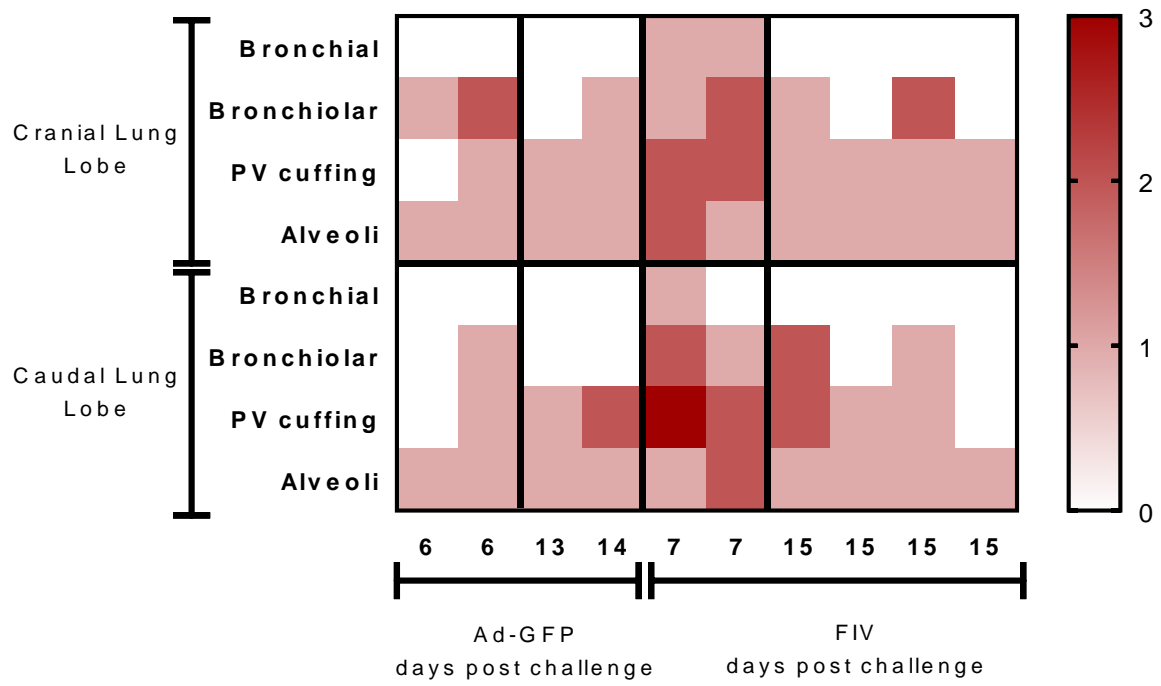


Fig. 2. Detection of SARS-CoV-2 RNA in ferret respiratory samples. Viral RNA in Ad-GFP and FIV-vaccinated ferrets was quantified by RT-PCR in (A) nasal washes and (B) throat swabs. Lines plotted are the geometric mean genome copies per mL.

A



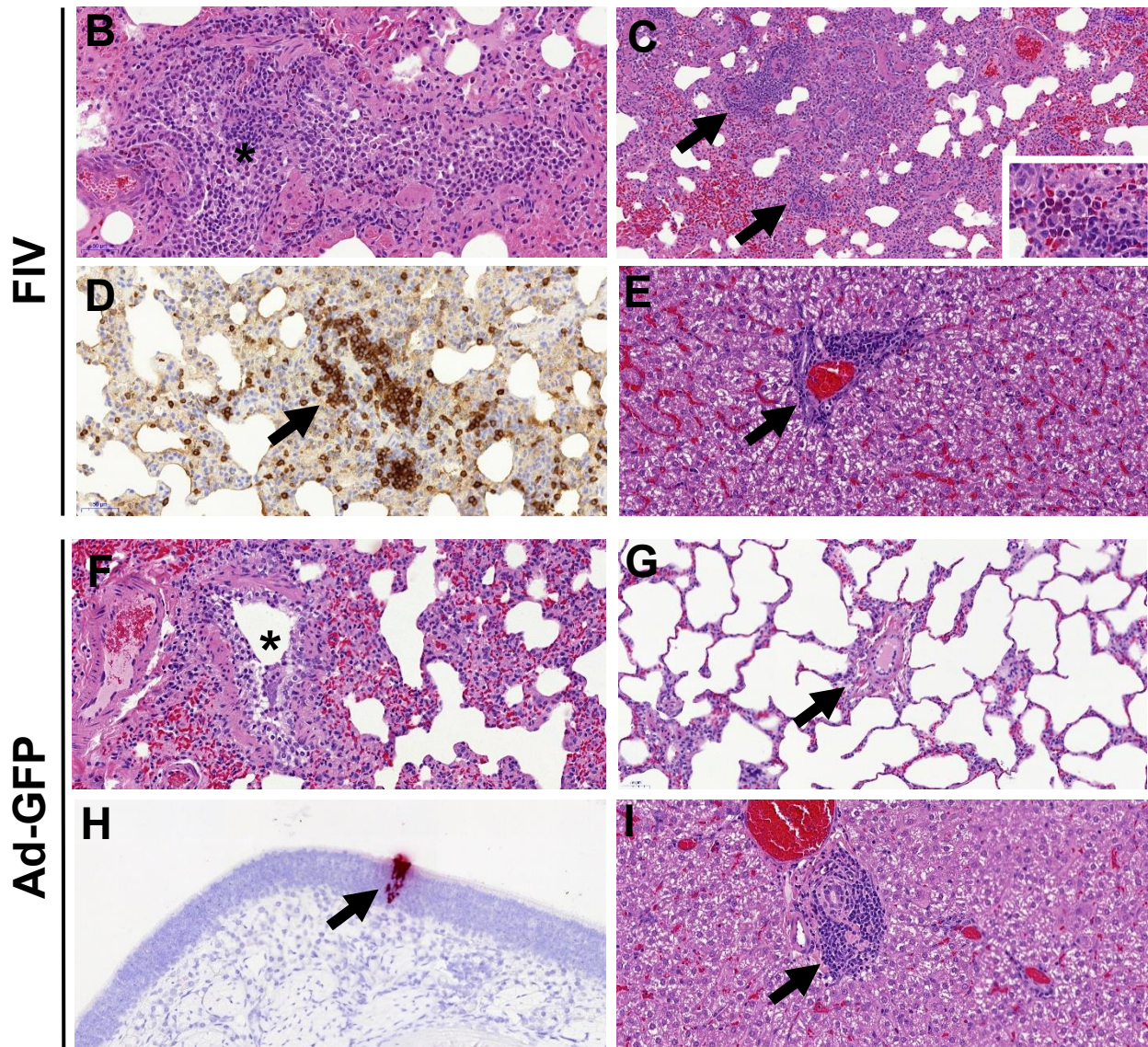


Fig. 3. A: Heatmap showing the individual lung histopathology scores for each ferret and parameter following FIV- or Ad-GFP-vaccination, and challenged with SARS-CoV-2 and culled at 6/7 days and 13/15 days pc. Histopathology of FIV- (B-E) and Ad-GFP (F-I) -vaccinated ferrets. **B.** Inflammatory infiltrates within a bronchiole (*), with abundant mononuclear cells but also some neutrophils and eosinophils. H&E, 200x. **C.** Multiple inflammatory infiltrates surrounding blood vessels (perivascular cuffing, arrows). H&E, 100x. The infiltrates are composed mostly of macrophages and lymphocytes, but abundant eosinophils can also be

observed in some areas within the infiltrates (insert; H&E, 400x). **D.** A perivascular cuff (arrow) with abundant mononuclear cells, many of them identified as CD3⁺ T lymphocytes. IHC, 200x. **E.** Periportal mononuclear inflammatory infiltrate in the liver (mild multifocal hepatitis). H&E, 400x. **F.** Mild inflammatory infiltrate within a bronchiole (*). H&E, 200x. **G.** Blood vessel (arrow) within the lung parenchyma not showing any perivascular cuffing. H&E, 200x. **H.** ISH detection of SARS-CoV-2 RNA in a small focus of epithelial and sustentacular cells within the nasal cavity. ISH, 200x. **I.** Periportal mononuclear inflammatory infiltrate in the liver (mild multifocal hepatitis). H&E, 400x.

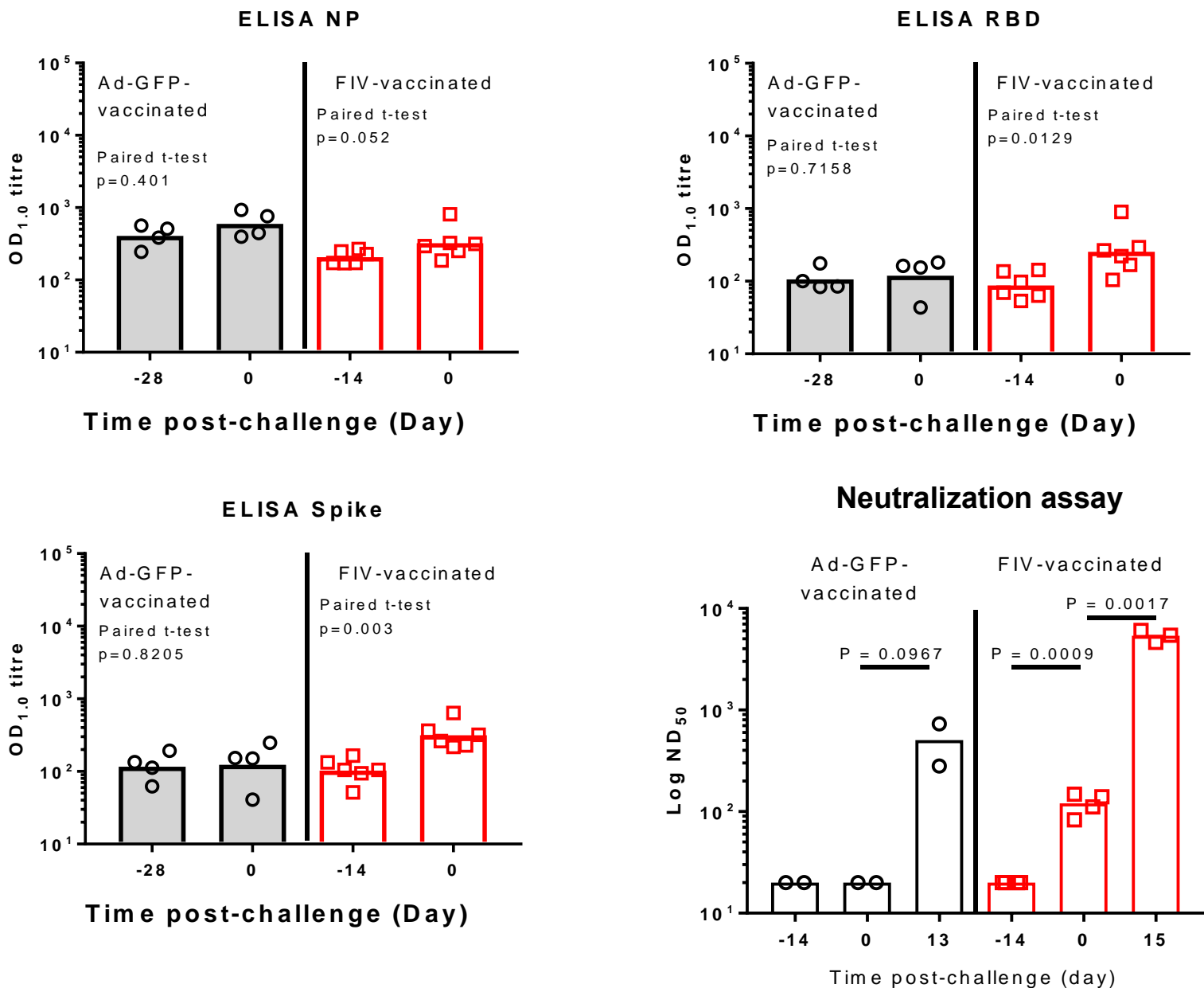


Fig. 4. Serological response to Ad-GFP and FIV in ferrets. IgG was quantified by ELISA to recombinant nucleocapsid protein (NP), receptor binding domain (RBD) and full-length trimeric and stabilised spike protein (Spike). Bars are geometric mean titre. The significance of any difference from pre- to post-vaccination is shown, determined by a paired t-test. The plaque reduction neutralisation 50% titre

(PRNT₅₀) is also shown with samples obtained pre- and post-vaccination and following SARS-CoV-2 challenge. Bars are geometric mean PRNT₅₀ titre.

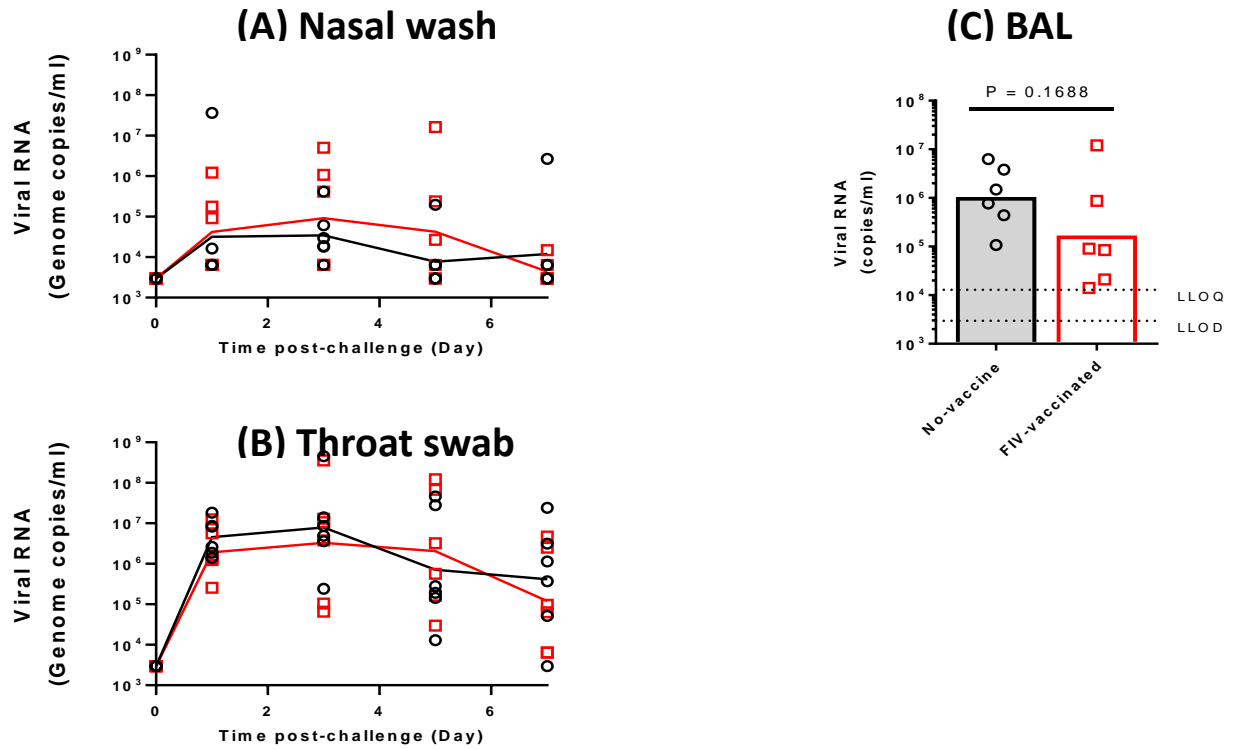


Fig. 5. Detection of SARS-CoV-2 RNA in macaque respiratory samples. Viral RNA in unvaccinated and FIV-vaccinated macaques was quantified by RT-PCR in (A) nasal washes, (B) throat swabs and (C) bronchiolar lavage. Lines plotted are the geometric mean genome copies per mL.

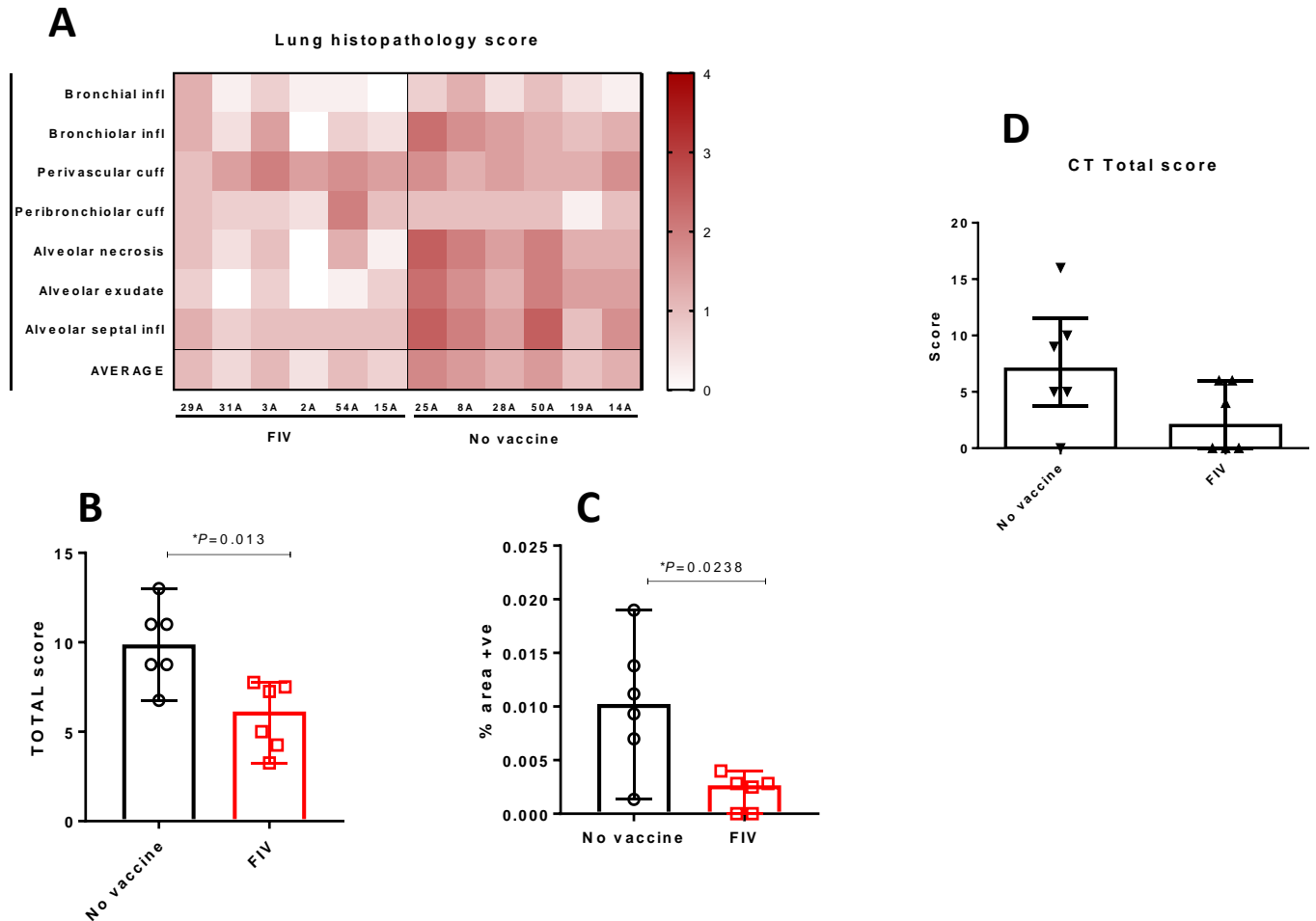


Fig. 6 Histopathology and CT scan analysis in rhesus macaques. A) Heatmap showing the individual lung histopathology scores for each animal and parameter, and the AVERAGE severity for each animal. B) Total histopathology scores in non vaccinates and FIV animals, showing a significant reduction in severity in the FIV group ($P=0.013$, Mann-Whitney U-tests; Boxes and whiskers show median +/- 95% C.I.). C) Percentage of area positively stained with ISH RNAScope (viral RNA) in non vaccinates and FIV animals, showing a significant reduction in the FIV group ($P=0.0238$, Mann-Whitney U-tests; Boxes and whiskers show median +/- 95% C.I.).

D) Plot shows the total CT scores in non-vaccinated and FIV-vaccinated animals showing a non-significant trend for reduction in severity in the FIV group ($p = 0.1364$ Mann-Whitney U-test); Box plots show the experimental group median with +/- IQR indicated by box whiskers, symbols show scores measured in individual animals.

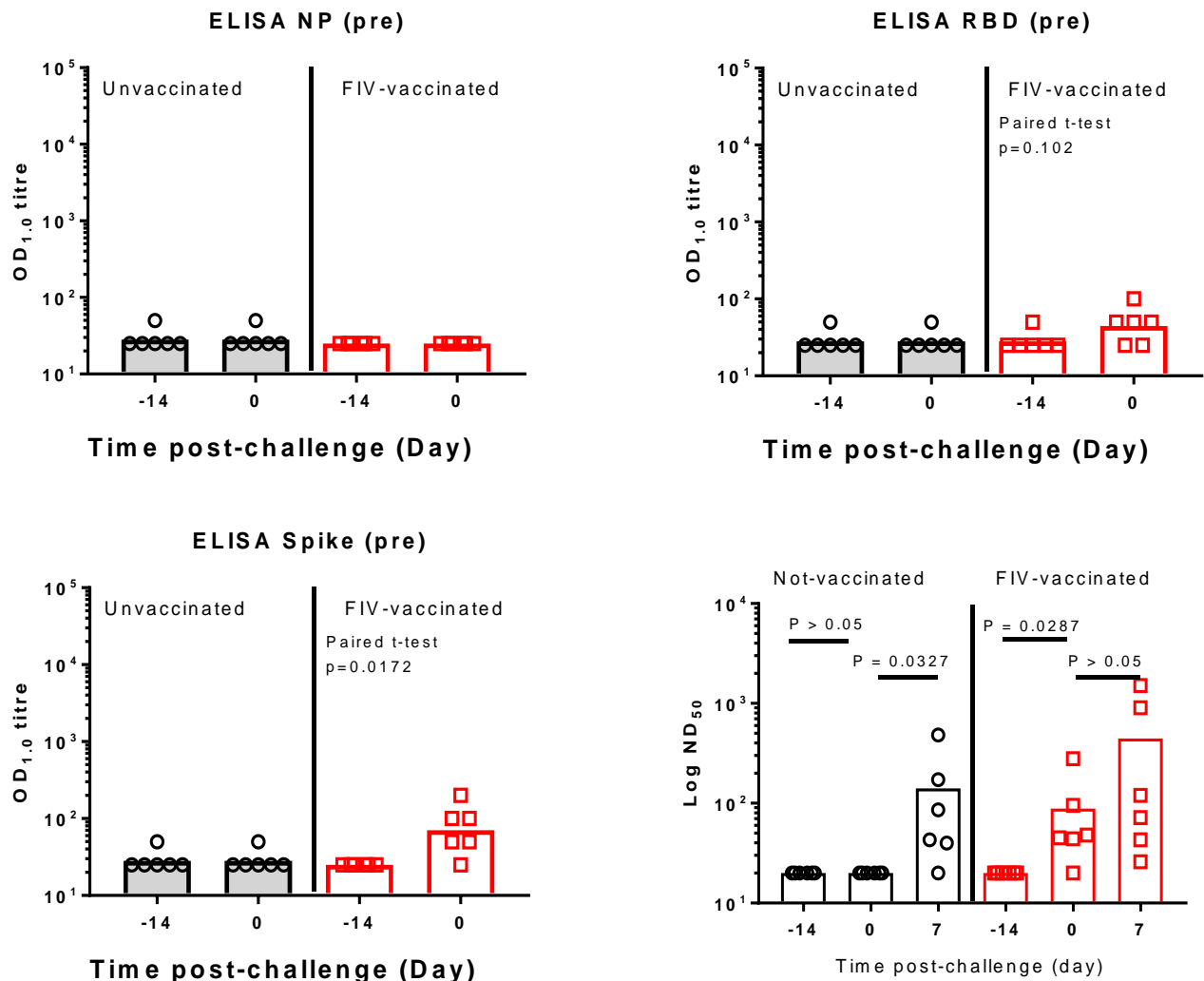


Fig. 7. Serological response in unvaccinated and FIV-vaccinated macaques.

IgG was quantified by ELISA to recombinant nucleocapsid protein (NP), receptor binding domain (RBD) and full-length trimeric and stabilised spike protein (Spike).

Bars are geometric mean titre. The significance of any difference from pre- to post-

vaccination is shown, determined by a paired t-test. The micronutralisation 50% titre

(ND₅₀) is also shown with samples obtained pre- and post-vaccination and following

SARS-CoV-2 challenge. Bars are geometric mean ND₅₀ titre.

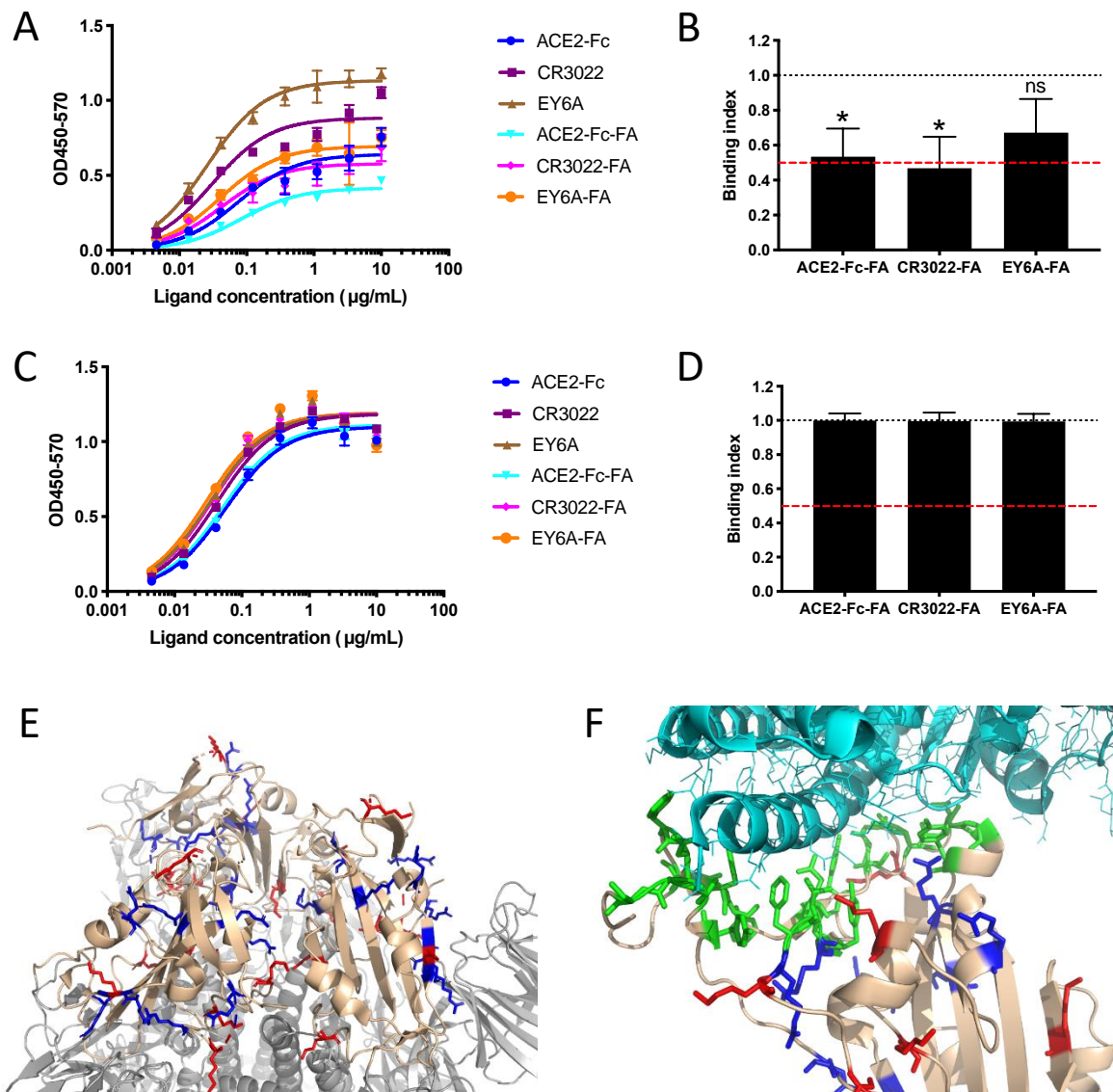


Fig. 8. Formaldehyde treatment reduces S trimer binding to ACE2.

A) Representative ELISA with S trimer captured onto the ELISA plate using anti-Myc mAb 9E10, untreated or treated with formaldehyde (FA) for 72 h at room temperature prior to addition of ligands at the concentrations shown and ligand detection using anti-human Fc-HRP. B) Area Under the Curve (AUC) analysis was used to determine the binding index, where 1=equivalent binding between untreated and FA-treated S trimer, and 0=zero binding to formaldehyde-treated trimer. The red line represents 50% binding. n=means of pooled data from 3 independent ELISAs,

each performed in triplicate. * $p < 0.05$ compared to unmodified condition, Student's t-test after normality testing, ns=not significant. C) Representative ELISA with RBD-Fc captured onto ELISA plate with anti-human-Fc, untreated or treated with FA as for the S trimer. D) AUC analysis was used to determine the binding index as for S trimer above, n=means of pooled data from 3 independent ELISAs, each performed in triplicate. Student's t-test after normality testing revealed no significant differences. E) Model prepared from PBD6LZG with 3 RBD 'down'. RBD rendered in beige, S trimer in grey, lysines in red, arginines in blue. Close up of RBD-ACE2 interface with RBD in beige, ACE2 in light cyan, binding site in green, lysines in red, arginines in blue.