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1 **Design, immunogenicity, and efficacy of a pan-sarbecovirus dendritic-cell targeting**
2 **vaccine**

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39

40 **Abstract (211 words)**

41 **Background:** There is an urgent need of a new generation of vaccine that are able to
42 enhance protection against SARS-CoV-2 and related variants of concern (VOC) and
43 emerging coronaviruses.

44 **Methods:** We identified conserved T- and B-cell epitopes from Spike (S) and Nucleocapsid
45 (N) highly homologous to 38 sarbecoviruses, including SARS-CoV-2 VOCs, to design a
46 protein subunit vaccine targeting antigens to Dendritic Cells (DC) via CD40 surface receptor
47 (CD40.CoV2).

48 **Findings:** CD40.CoV2 immunization elicited high levels of cross-neutralizing antibodies
49 against SARS-CoV-2, VOCs, and SARS-CoV-1 in K18-hACE2 transgenic mice, associated
50 with improved viral control and survival after SARS-CoV-2 challenge. A direct comparison of
51 CD40.CoV2 with the mRNA BNT162b2 vaccine showed that the two vaccines were equally
52 immunogenic in mice. We demonstrated the potency of CD40.CoV2 to recall in vitro human
53 multi-epitope, functional, and cytotoxic SARS-CoV-2 S- and N-specific T-cell responses that
54 are unaffected by VOC mutations and cross-reactive with SARS-CoV-1 and, to a lesser
55 extent, MERS epitopes.

56 **Interpretation:** We report the immunogenicity and antiviral efficacy of the CD40.CoV2
57 vaccine in a preclinical model providing a framework for a pan-sarbecovirus vaccine.

58 **Fundings:** This work was supported by INSERM and the Investissements d’Avenir program,
59 Vaccine Research Institute (VRI), managed by the ANR and the CARE project funded from
60 the Innovative Medicines Initiative 2 Joint Undertaking (JU).

61

62 **Keywords:** COVID-19, SARS-CoV-2, vaccine, pre-clinical model, sarbecoviruses

63

64 **Word count manuscript:** 9173 words

65

66

67 **Research in context**

68 ***Evidence before this study***

69 Since the advent of effective vaccines against SARS-CoV-2 in late 2020, more than 900
70 publications are referenced in pubmed. In Europe, 5 vaccines are authorized: two mRNAs
71 (Comirnaty, Spikevax), one adjuvanted protein (Nuvaxovid), two recombinant adenoviruses
72 (Janssen, Vaxzevria). These vaccines are well tolerated, confer a protection against severe
73 disease and hospitalization. However, all these vaccines trigger immune responses to the
74 Spike (S) protein of SARS-CoV-2 with the aim to elicit neutralizing antibodies. Recent results
75 from large vaccination campaigns show a waning of neutralizing antibody levels and a
76 reduced efficacy against emergent variants of concern (VOC) characterized by the
77 accumulation of mutations in S and Region Binding Domain (RBD) leading to escape the
78 vaccine responses. As it has been shown that cellular immunity is of importance for long
79 term protection and less impacted by mutations as compare to the humoral immunity, there
80 is a global consensus for the development of a new generation of vaccines against SARS-
81 CoV-2 and related variants and more globally against other members of the sarbecovirus
82 family in the context of the preparedness to the next pandemic.

83 **Added value of this study**

84 We have developed an *in silico* process to identify sequences from S, RBD and
85 Nucleocapsid (N) from SARS-CoV-2 highly conserved across 38 sarbecoviruses including
86 SARS-CoV-2, related VOCs, SARS-CoV-1 and other viruses described in bats and at high
87 risk for potential new zoonosis. We show that targeting these conserved sequences,
88 containing a large stretch of T and B cell epitopes, to Dendritic Cells (DC) through the CD40
89 receptor (CD40.CoV2 vaccine) induces cross neutralizing antibodies against SARS-CoV-2,
90 VOCs, SARS-CoV-1 and protect K18-hACE2 transgenic mice from SARS-CoV-2 challenge.
91 We demonstrate *in vitro* the potency of this polyepitope-based vaccine, which contained
92 nucleocapsid (N) antigen, to elicit polyfunctional and strong cross-reactive T cells responses
93 unaffected by VOC mutations and to a lesser extent conserved against SARS-CoV-1. We
94 also show the potency of this vaccine to recall cytotoxic memory T cell responses.

95 **Implications of all the available evidence**

96 We provide here the framework for a polyepitope-based vaccine platform. This vaccine will
97 enrich the current *portfolio* of vaccines as a boost of preexisting immunity with the aim to
98 extend the breadth of immune responses against current and emerging VOCs. In addition,
99 this innovative vaccine represents an important step in preparing for future pandemics.

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105 **Introduction**

106 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in late
107 2019 in the Hubei province of China, has caused devastating human and economic losses
108 worldwide. Unprecedented mobilization of the scientific community has led to the
109 implementation of viral diagnostics, immunological monitoring tools, and the rapid
110 development of protective vaccines.

111 The current landscape of COVID-19 vaccines is based on the delivery of SARS-CoV-2 Spike
112 (S) through various vaccine platforms that elicit neutralizing-antibody responses against the
113 S protein, including the receptor binding domain (RBD). Most of these vaccines induce Th1
114 responses restricted to S epitopes, depending on the type of platform and variations in the S
115 protein¹⁻⁹, but vary in their capacity to elicit CD8⁺ T-cell responses, known to be an important
116 element for control of the infection.¹⁰ Although neutralizing antibodies are a key component
117 of a broadly protective vaccine, several recent studies have suggested that the induction of
118 broad virus-specific CD4⁺ and CD8⁺ T cells could greatly augment antibody-based protection
119 and the long-term durability of vaccine responses.¹¹

120 Despite current progress, control of the ongoing SARS-CoV-2 pandemic is endangered by
121 the emergence of viral variants, called variants of concern (VOC). Among them, the B.1.1.7
122 Alpha,¹² B.1.351 Beta,¹³ P.1 Gamma,¹⁴ B.1.617.2 Delta,¹⁵ and recently emerged B.1.1.529
123 Omicron variants¹⁶ exhibit several specific or shared mutations within the S sequences,
124 raising substantial new concerns due to their increased transmissibility¹⁷ and ability to
125 escape convalescent and vaccine-induced antibody responses.¹⁸⁻²² Recent studies showing
126 a decrease in the effectiveness of mRNA vaccines against the new VOCs²³ report of
127 breakthrough infections,²⁴ and concerns of reduced efficacy of vaccination in older patients²⁵
128 or immune-compromised individuals²⁶ highlight the need to develop a new and
129 complementary generation of vaccines as prophylaxis or boosters that include T- and B-cell
130 selected antigens that are potentially less affected by the mutations of VOCs.

131 Within the last 20 years, SARS-CoV-2 is the third major human infectious disease outbreak
132 caused by zoonotic coronaviruses after SARS-CoV-1 in 2002-2003 and Middle East
133 respiratory syndrome coronavirus (MERS-CoV) in 2012. The first available sequence of
134 SARS-CoV-2 identified this novel human pathogen as a member of the *Sarbecovirus*
135 subgenus of *Coronaviridae*²⁷, the same subgenus as SARS-CoV-1. The high prevalence and
136 diversity of viruses in bats and the fact that all zoonotic sarbecoviruses identified to date use
137 hACE2 as their entry receptor raise major concerns about a future epidemic²⁸⁻³². These
138 additional concerns underscore the urgent need for new vaccine candidates that are able to
139 enhance protection against VOCs and emerging coronaviruses.

140 Dendritic cells (DCs) are immune system controllers that can deliver differential signals to
141 other immune cells through intercellular interactions and soluble factors, resulting in a variety
142 of host immune responses of varying quality. Targeting vaccine antigens to DCs via surface
143 receptors represents an appealing strategy to improve subunit-vaccine efficacy while
144 reducing the amount of required antigen. This strategy, which allows the delivery of designed
145 and selected antigens, in addition to an activation signal, may also evoke a danger signal
146 that stimulates an immune response, with or without the need of additional immune
147 stimulants, such as adjuvants. Among the various DC receptors tested, including lectins and
148 scavenger receptors, we previously reported the superiority of vaccines targeting diverse
149 viral antigens to CD40-expressing antigen-presenting cells to evoke strong antigen-specific
150 T- and B-cell responses.³³⁻⁴⁰

151 We have recently reported results on the efficacy of a new generation of subunit vaccines
152 targeting the RBD of the SARS-CoV-2 spike protein to the CD40 receptor (aCD40.RBD).⁴¹
153 We demonstrated that a single dose of the aCD40.RBD vaccine, injected without adjuvant, is
154 sufficient to boost a rapid increase in neutralizing antibodies in convalescent non-human
155 primates (NHPs) infected six months previously with SARS-CoV-2. Interestingly, the
156 aCD40.RBD vaccine-elicited antibodies cross-neutralized D614G SARS-CoV-2 and the
157 VOCs Alpha (B1.1.7) and, to a lesser extent, Beta (B1.351). This vaccination significantly

158 improved protection against a new high-dose virulent challenge versus that in non-
159 vaccinated convalescent animals.⁴¹

160 Drawing from this knowledge, we used *in silico* approaches to design a next-generation
161 CD40-targeting vaccine, CD40.CoV2, including T- and B-cell epitopes spanning sequences
162 from S and nucleocapsid (N) proteins from SARS-CoV-2 and highly homologous to 38
163 sarbecoviruses, including SARS-CoV-2 VOCs. We report here the immunogenicity and
164 antiviral efficacy of this vaccine in a preclinical model.

165

166 **Methods**

167 **Ethics**

168 Animal housing and experimental procedures were conducted according to the French and
169 European Regulations (*Parlement Européen et du Conseil du 22 septembre 2010, Décret n°*
170 *2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins*
171 *scientifiques*) and the National Research Council Guide for the Care and Use of Laboratory
172 Animals (*National Research Council (U.S.), Institute for Laboratory Animal Research (U.S.),*
173 *and National Academies Press (U.S.), Eds., Guide for the care and use of laboratory*
174 *animals, 8th ed. Washington, D.C: National Academies Press, 2011*). The animal BSL3
175 facility is authorized by the French authorities (Agreement N° B 13 014 07). All animal
176 procedures (including surgery, anesthesia, and euthanasia, as applicable) used in the
177 current study were submitted to the Institutional Animal Care and Use Committee either of
178 the CIPHE or Anses/ENVA/UPEC (CEEA-016) depending on the experiments and approved
179 by the French authorities (CETEA DSV – APAFIS#26484-2020062213431976 v6 or
180 APAFIS#25329-2020051119073072 v4, respectively). All CIPHE BSL3 facility operations are
181 overseen by a biosecurity/biosafety officer and accredited by the Agence Nationale de
182 Sécurité du Médicament (ANSM).

183 For human samples, we enrolled a subgroup of COVID-19 patients of the prospective French
184 COVID cohort (registered at clinicaltrials.gov NCT04262921). Ethics approval was given on
185 February 5, 2020, by the French Ethics Committee CPP-Ile-de-France VI (ID RCB: 2020-
186 A00256-33). The study was conducted with the understanding and consent of each
187 participant or their surrogate covering the sampling, storage, and use of biological samples.

188 **Animals**

189 Heterozygous K18-hACE C57BL/6J mice (strain: 2B6.Cg-Tg (K18-ACE2)2PrImn/J) were
190 obtained from The Jackson Laboratory. The hCD40-OST transgenic mice expressed a
191 human *Cd40* gene in place of the mouse *Cd40* gene. They were derived at CIPHE under
192 CIPHE-Sanofi Research Collaborative program n° 171137A10 and kindly provided by Sanofi

193 under the agreement MTA #209012. All breeding, genotyping, and production of hCD40/K18-
194 hACE2 was performed at the CIPHE. The sample size was based on previous articles
195 reporting the use of K18-hACE2 mice in SARS-CoV2 challenge experiments with 4-5
196 experimental units per group. Animals were housed in groups within cages and fed standard
197 chow diets.

198 ***COVID-19 convalescent patients***

199 Eligible patients were those who were hospitalized between March and November 2020 with
200 virologically confirmed COVID-19. Convalescent follow-up visits were performed between
201 one, three, and six months after infection. Patients were not vaccinated against COVID-19 at
202 this period.

203 ***Cloning and production of the CD40.CoV2 vaccine***

204 The vaccine was produced using the expression plasmids described in materials availability
205 via transient transfection (TransIT-PRO® Transfection Kit, Mirus) into mammalian CHO-S
206 cells (ThermoFisher) followed by Protein A-affinity purification.^{35,42} The eluted product (2.2
207 mg/ml, 0.3 ng LPS/mg) was stored at -80°C in PBS with 125 mM hydroxypropyl β -
208 cyclodextrin (Cavitron W7 HP5). CD40 binding was validated by ELISA as previously
209 described.³⁶

210 ***Wuhan/D614 SARS-CoV-2 virus production***

211 Vero E6 cells (CRL-1586; American Type Culture Collection) were cultured at 37°C in
212 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum
213 (FBS), 10 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 1X non-essential amino acids, and
214 100 U/ mL penicillin–streptomycin. The strain BetaCoV/France/IDF0372/2020 was supplied
215 by the National Reference Centre for Respiratory Viruses hosted by the Institut Pasteur
216 (Paris, France). The human sample from which strain BetaCoV/France/IDF0372/2020 was
217 isolated was provided by the Bichat Hospital, Paris, France. Infectious stocks were grown by
218 inoculating Vero E6 cells and collecting supernatants upon observation of the cytopathic

219 effect. Debris was removed by centrifugation and passage through a 0.22- μ m filter.
220 Supernatants were stored at -80°C.

221

222 ***Vaccination and infection of hCD40/K18-hACE2 transgenic mice***

223 Mice of 8 to 12 weeks of age of both sexes received two intraperitoneal injections of the
224 CD40.CoV2 vaccine (10 μ g) plus polyinosinic-polycytidylic acid (Poly-IC; Oncovir) (50 μ g) or
225 poly(IC) alone three weeks apart. Mice were further infected with Wuhan/D614 SARS-CoV-2
226 at week 4. Vaccinated and mock-vaccinated mice were administered 2.5×10^4 PFU of
227 SARS-CoV-2 via intranasal administration. Mice were monitored daily for morbidity (body
228 weight) and mortality (survival). During the monitoring period, mice were scored for clinical
229 symptoms (weight loss, eye closure, appearance of the fur, posture, and respiration). Mice
230 obtaining a clinical score defined as reaching the experimental end-point were humanely
231 euthanized. Blood was collected on day -2 (before vaccination), day 28 (before viral
232 infection), and day 40 post-vaccination. Only the experimenters who injected the vaccine or
233 the adjuvant alone were aware of the group allocation during the conduct of the experiment.
234 The group allocation was determined according to data analysis and statistical test
235 relevance. Two independent experiments of CD40.CoV2 vaccination followed by SARS-CoV-
236 2 inoculation were performed (for a total of 4-5 experimental units) to minimize confounders.
237 No animals were excluded from the study.

238 The clinical and immunological monitoring of Experiment 1 are reported in the manuscript
239 and principal figures. The antibody responses monitored in Experiment 2 to confirm the main
240 results are presented in Supplemental Figure S3.

241 In an independent set of experiments, the immunogenicity of the CD40.CoV2 and mRNA
242 BNT162b2 vaccines was assessed. huCD40/K18-hACE2 and huCD40 transgenic mice
243 received two injections of CD40.CoV2 (10 μ g corresponding to 1.33 μ g of antigen + poly (IC),
244 50 μ g) or mRNA BNT162b2 (1 μ g, intramuscularly) three weeks apart, respectively. Sera

245 were collected before immunization and one week after the second injection. No animals
246 were excluded from the study.

247

248 ***Measurement of SARS-CoV-2 viral load by RT-qPCR and TCID50 (50% of tissue-culture***
249 ***infective dose)***

250 For viral titration by RT-qPCR, tissues were homogenized with ceramic beads in a tissue
251 homogenizer (Precellys – Bertin Instruments) in 0.5 mL RLT buffer. RNA was extracted using
252 the RNeasy Mini Kit (QIAGEN) and reverse transcribed using the High-Capacity cDNA
253 Reverse Transcription Kit (Thermo Fisher Scientific). Amplification was carried out using
254 OneGreen Fast qPCR Premix (OZYME) according to the manufacturer’s recommendations.
255 The number of copies of the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) gene in
256 samples was determined using the following primers: forward primer – catgtgtggcggttcactat,
257 reverse primer – gttgtggcatctcctgatga. This region was included in a cDNA standard to allow
258 the copy number determination down to ~100 copies per reaction. The copies of SARS-CoV-
259 2 were compared and quantified using a standard curve and normalized to total RNA levels.
260 An external control (mock-infected wildtype animal, nondetectable in the assay) and a
261 positive control (SARS-CoV-2 cDNA containing the targeted region of the RdRp gene at a
262 concentration of 10^4 copies/ μ l [1.94×10^4 copies/ μ l detected in the assay]) were used in the
263 RT-qPCR analysis to validate the assay. The median tissue-culture infectious dose (TCID50)
264 represents the dilution of a virus-containing sample at which half of the inoculated cells show
265 signs of infection. To perform the assay, lung tissue was weighed and homogenized using
266 ceramic beads in a tissue homogenizer (Precellys – Bertin Instruments) in 0.5 ml RPMI
267 media supplemented with 2% FCS and 25 mM HEPES. Tissue homogenates were then
268 clarified by centrifugation and stored at -80°C until use. Forty-thousand cells per well were
269 seeded in 96-well plates containing 200 μ l DMEM + 4% FCS and incubated for 24 h at 37°C .
270 Tissue homogenates were serially diluted (1:10) in RPMI media and 50 μ l of each dilution
271 was transferred to the plate in six replicates for titration at five-days post-inoculation. Plates

272 were read for the CPE (cytopathology effect) using microscopy reader and the data were
273 recorded. Viral titers were then calculated using the Spearman-Karber formula and
274 expressed as TCID₅₀/mg of tissue.

275 ***Antibody measurement***

276 Three multiplexed MesoScale Discovery immunoassays (V-PLEX Coronavirus Panel 3, V-
277 PLEX SARS-CoV-2 Panel 11 and V-PLEX SARS-CoV-2 Panel 22 [IgG] Kits, MesoScale
278 Discovery, Rockville, MD, USA) were used on all available plasma samples to measure
279 plasma IgG antibodies to SARS-CoV-2, SARS-CoV, MERS-CoV, and HCoVs. Coronavirus
280 Panel 3 plates are MULTI-SPOT 96-well, 10 Spot, coated with three SARS-CoV-2 antigens
281 (spike, receptor binding domain [RBD], and nucleocapsid), and spike proteins from SARS-
282 CoV, MERS-CoV, and seasonal HCoVs OC43, HKU1, 229E, and NL63. SARS-CoV-2 Panel
283 11 plates are MULTI-SPOT 96-well, 10 Spot, coated with RBD proteins from various SARS-
284 CoV-2 lineages: Wuhan; Alpha; Beta and Botswana; Gamma; Delta sub-lineages and
285 Vietnam; Epsilon, California, and New York; Eta, Iota, India, Zeta, and Kentucky; New York;
286 U.K. and Philippines; Kappa and India. SARS-CoV-2 Panel 22 plates are MULTI-SPOT 96-
287 well, 10 Spot, coated with RBD proteins from various SARS-CoV-2 lineages: Wuhan; Alpha;
288 Beta and Botswana; Gamma; Delta sub-lineages and Vietnam; Omicron sub-lineages.
289 Assays were performed according to the manufacturer's instructions, with samples diluted
290 1:50 000. The electro-chemiluminescence (ECL) signal was recorded and the results are
291 expressed as arbitrary units (AU).

292 Three alternative immunoassays (V-PLEX Coronavirus Panel 3, V-PLEX SARS-CoV-2 Panel
293 11 and V-PLEX SARS-CoV-2 Panel 22 [ACE-2] Kits, MesoScale Discovery) were used to
294 measure the ability of mouse plasma samples to inhibit angiotensin-converting enzyme 2
295 (ACE2) binding to the Spike protein of different coronaviruses and different variants of
296 SARS-CoV-2 RBD proteins. The assays were performed according to the manufacturer's
297 instructions with samples diluted 1:33 to 1:3333. Antibody concentrations were quantified
298 using a reference standard (ACE2 Calibration Reagent) and are expressed as units/mL (one

299 unit per mL concentration of ACE2 Calibration Reagent corresponds to neutralizing activity of
300 1 µg/mL monoclonal antibody to SARS-CoV-2 Spike protein) except for V-PLEX SARS-CoV-
301 2 Panel 22 kit for which samples were diluted 1:200 and results were expressed in
302 percentage of inhibition as no reference standard was included in this kit. For V-PLEX
303 Coronavirus Panel 3, the lower limit of quantification (LLOQ) was calculated to be between
304 0.68 and 0.82 unit/mL for Spike CoV-2 and between 3.10 and 3.24 units/mL for Spike CoV-1.
305 For V-PLEX SARS-CoV-2 Panel 11, the LLOQ was calculated to be between 2.83 and 2.94
306 units/mL for RBD Wuhan, 2.43 and 3.20 units/mL for RBD Alpha, 2.09 and 2.87 units/mL for
307 RBD Beta, 2.56 and 2.99 units/mL for RBD Gamma, 2.01 and 3.02 units/mL for RBD Delta,
308 and between 2.51 and 3.12 units/mL for RBD Kappa. Values under the LLOQ were imputed
309 at the LLOQ. Thirty plasma samples from unvaccinated mice were used to determine the
310 threshold for positivity, defined as the whole units/mL value immediately above the
311 concentration of the highest sample for RBD (i.e., 8 units/mL) and Spike (i.e., 4 units/mL)
312 proteins.

313 ***Specific antigens***

314 Various peptide pools from reference strain Human 2019-nCoV HKU-SZ-005b, from JPT
315 Peptide Technologies (Berlin, Germany) or BEI Resources, were used, as mentioned, for the
316 various assays. A set of four pools of 15-mer peptides, overlapping by 11 amino acids,
317 covering the four regions of S and N sequences of SARS-CoV-2 included in the CD40.CoV2
318 vaccine: vS1 (29 peptides), vRBD (54 peptides), vS2 (37 peptides), and vN2 (32 peptides).
319 In certain experiments, vS1, vRBD, vS2, and vN2 were pooled to provide a combination of all
320 sequences included in the CD40.CoV2 vaccine (vOLPmix). A pool of 54 peptides (15-mers
321 overlapping by 11 amino acids) encompassing the three RBD mutations K417N (four
322 peptides), E484K (three peptides), and N501Y (four peptides): RBD SARS-CoV-2
323 beta/gamma. Three PepMix pools of RBD SARS-CoV-2 delta, RBD SARS-CoV-2 kappa and
324 RBD SARS-CoV-2 Omicron (15-mer peptides, overlapping by 11 amino acids). A set of two
325 pools of 15/20-mer peptides, overlapping by 10 amino acids, covering the two regions of

326 RBD and N sequences of SARS-CoV-1 corresponding to the CD40.CoV2 vaccine
327 sequences: vRBD-CoV-1 (30 peptides), vN2-CoV-1 (18 peptides). A pool of 15-mer peptides,
328 overlapping by 11 amino acids, covering the S1 region sequence of SARS-CoV-1: S1-CoV-1
329 (156 peptides). A pool of 15-mer peptides, overlapping by 11 amino acids, covering the S1
330 region sequence of MERS: S1-MERS (168 peptides). As controls (cont.OLP), we used a set
331 of two pools of 15-mer peptides, overlapping by 11 amino acids, of N and M sequences of
332 SARS-CoV-2 not included in the vaccine: N1-N2 (34 peptides) and M (53 peptides) or an
333 irrelevant pool of overlapping 15-mer peptides (11-amino acid overlaps) from the Ebola virus
334 Mayinga variant glycoprotein (Gpz: 77 peptides).

335 ***Quantification of culture supernatant analytes***

336 We quantified 25 analytes in supernatants from convalescent COVID-19 PBMCs on day 2
337 after CD40.CoV2 vaccine (1 nM), vOLP (equimolar concentration) or a control CD40 fused
338 to the HIV Env glycoprotein 140 (strain ZM96) (CD40.Gp140z) (1nM) stimulation using the
339 Human XL Cytokine Luminex® Performance Panel Premixed Kit: CCL2/MCP-1, CCL3/MIP-
340 1 α , CCL4/MIP-1 β , CCL5/RANTES, CD40 Ligand/TNFSF5, CXCL1/GRO α , CXCL10/IP-10,
341 GCSF, Granzyme B, IFN- α , IFN- β , IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-
342 12 p70, IL-13, IL-17/IL-17A, PD-L1/B7-H1, TNF, and TRAIL/TNFSF10 (R&D Systems/Bio-
343 Techne), according to the manufacturers' instructions.

344 ***Characterization of SARS-COV-2-specific immune responses in convalescent COVID- 345 19 patients***

346 Cellular responses to CD40.CoV2 vaccine were assessed using the Activation Induced
347 Marker assay (AIM) and EpiMax technology.⁴³ For the AIM assay, PBMCs were stimulated *in*
348 *vitro* with various concentrations of the CD40.CoV2 vaccine or an equimolar combination of
349 15-mer overlapping peptide pools covering the full-length sequence of vaccine antigens
350 (vS1+vS2+ vRBD+vN2) referred to as vOLPmix. PBMCs (1×10^6) were incubated in 300 μ l
351 RPMI supplemented with 10% human serum AB (SAB) for 24 h at 37°C in 5% CO₂. T-cell
352 activation was assessed by detection of the extracellular activation markers CD69 and

353 CD137, in addition to a viability marker and CD3, CD4, and CD8 to determine the T-cell
354 lineage. For the EpiMax technology, PBMCs were stimulated *in vitro* with 1 nM CD40.CoV2
355 vaccine on D0 and restimulated on D8 with 1 µg/ml of various vOLPs (vS1, vRBD, vS2, or
356 vN2). Cell functionality was assessed by intracellular cytokine staining (ICS), with Boolean
357 gating. The flow cytometry panel included a viability marker, CD3, CD4, and CD8 to
358 determine the T-cell lineage, and IFN-γ, TNF, and IL-2 antibodies. Distributions were plotted
359 using SPICE version 5.22, downloaded from <http://exon.niaid.nih.gov/spice>.⁴⁴ T-cell
360 proliferation was evaluated using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) as
361 previously described.⁴⁵ PBMCs were stimulated *in vitro* with 1 nM CD40.CoV2 vaccine or an
362 equimolar amount of vOLPmix or 1nM CD40.Gp140z for seven days without IL-2. The
363 medium (RPMI 10% SAB) was changed 48 h after stimulation.

364 **Cytotoxicity assay**

365 PBMCs from convalescent COVID-19 patients exhibiting CD8⁺ T cell responses in Epimax
366 assay were stimulated with 1 nM CD40.CoV2 vaccine (effector cells) or 2.5 µg/mL
367 phytohemagglutinin-L (PHA-L) (ThermoFischer Scientific) (target-Blast cells) in RPMI 10%
368 SAB medium replenished every 2 to 3 days with fresh medium supplemented with IL-2 (100
369 U/mL) (Myltenyi Biotec). After seven days, effector-CD8⁺ T cells were isolated using a CD8⁺
370 T-cell isolation kit from Myltenyi Biotec following the manufacturers' instructions and target-
371 Blast cells were pulsed for 1 h at 37°C in 5% CO₂, either with 0.4% DMSO (control) or 2
372 µg/mL vOLP. After two washes, cells were labeled using various combinations of
373 carboxyfluorescein succinimidyl ester (CFSE) (0.1 µM), Cell Trace Violet (CTV) (0.5 µM),
374 and/or Cell Trace Far Red (CTFR) (0.02 µM) (Thermofisher Scientific) for 15 min at 37°C.
375 Target-Blast specific populations were then mixed at a 1:1 ratio and co-cultured with effector-
376 CD8⁺ T-cells at various ratios in triplicate. To measure basal apoptosis, three wells were
377 seeded with target-Blast cells alone. After a 24 h-incubation, cells were stained with
378 LIVE/DEAD Fixable Near-IR stain (Thermofisher Scientific) and analyzed using an LSR II-3
379 laser flow cytometer (405, 488, and 640 nm) (Becton Dickinson). The percentage of specific

380 cytotoxicity among live cells was calculated as follows: specific lysis (%) = 100*[(average
381 [count vOLP pulsed/count DMSO pulsed] target-Blast cells alone - [count vOLP
382 pulsed/count.DMSO pulsed]) target-Blast cells + CD8⁺ effector T cells / (average [count
383 vOLP pulsed/count DMSO pulsed] target-Blast cells alone)].

384 **Statistics**

385 Graphpad Prism software version 8 was used for nonparametric statistics and plots, as
386 described in the figure legends. Heatmaps were generated using the heatmap function from
387 package NMF in R software, version 4.0.0. R: A language and environment for statistical
388 computing. R Foundation for Statistical Computing, Vienna, Austria. URL: [https://www.R-](https://www.R-project.org)
389 [project.org](https://www.R-project.org). Statistical differences in the expression of standardized biomarkers were
390 determined using the nonparametric Wilcoxon test, adjusting for multiple testing using the
391 Benjamini & Hochberg correction.

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398 **Results**

399 ***In silico* down-selection of T- and B-cell polyepitope regions from SARS-CoV-2 for an** 400 **improved dendritic cell-targeting vaccine platform**

401 We first screened three structural proteins (S, N, and M) of SARS-CoV-2 for the identification
402 of T-cell epitopes using NetMHC 4.0⁴⁶ and NetMHCII 2.3⁴⁷ software, which predict peptides
403 that bind to a large panel of class-I and -II HLAs, respectively. Linear B-cell epitopes were
404 predicted using BepiPred 2.0.⁴⁸ We mapped a set of 9-mer epitopes binding to 80 HLA-class
405 I molecules and 15-mer epitopes binding to 54 HLA-class II molecules, as well as all linear B-

406 cell epitopes. We evaluated amino-acid (aa) regions encompassing both the highest number
407 of predicted epitopes and the largest HLA coverage. Selected regions were further screened
408 for their sequence homology with other β coronaviruses, including SARS (designed
409 thereafter as SARS-CoV-1), focusing on described T-cell epitopes and B-cell epitopes that
410 generate neutralizing antibodies. Down-selected regions containing epitopes were also
411 compared to early predicted and described SARS-CoV-2 T- and B-cell epitopes. Thus,
412 following all ahead mentioned criteria, four designated T-and B-cell “epitope-enriched
413 regions” were selected as vaccine (v) regions, one from N: vN2 (aa 276-411) and three from
414 S: vS1 (aa 125-250), vRBD (aa 318-541), and vS2 (aa 1056-1209) (Figure 1a-b). These
415 epitope-enriched regions contain a total of 640 aa with 2,313 predicted CD8⁺ T-cell epitopes
416 covering 100% of HLA-Class I haplotypes, 2,985 predicted CD4⁺ T-cell epitopes covering
417 100% of HLA-Class II haplotypes, and 17 predicted SARS-CoV-2 linear B-cell epitopes
418 (Figure 1 a-b, Table S1). Then, we examined whether selected vaccine sequences
419 significantly matched so far described SARS-CoV-2 T-cell epitopes reviewed by Grifoni et al.
420 ⁴⁹ We found the vaccine sequences to contain 71/171 (42%) and 21/44 (48%) described
421 CD8⁺ T-cell epitopes for S and N, respectively (Table S2). These values were 57/123 (46%)
422 and 21/53 (40%) for described CD4⁺ T-cell epitopes (Table S3).

423 We then used Cobalt (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) to perform the
424 alignment of sequences from SARS-CoV-2, four SARS-CoV-2 VOCs (α , β , γ , δ), SARS-CoV-
425 1, and 32 recently described SARS-CoV-related coronaviruses ^{50–52}, which include 30 viruses
426 of bat origin and two of pangolin origin (all from the *Sarbecovirus* subgenus). Globally, the
427 mean [min-max] percentage of homology between these 38 sarbecoviruses for vS1, vRBD,
428 vS2, and vN2 vaccine sequences was 53.5 [36.5 to 99.2], 73.6 [63.8 to 99.6], 94.3 [86.4 to
429 100], and 93.5 [89.7 to 100] %, respectively (Table S4). As expected, homology between
430 vaccine sequences and members of *Embecovirus*, *Merbecovirus*, *Setracovirus*, and
431 *Duvinacovirus* subgenus coronaviruses was lower and varied from 6 to 38%. Beyond
432 sequence homology across sarbecoviruses, we observed the vaccine T-cell epitopes to be

433 highly conserved between SARS-CoV-2 and SARS-CoV-1 and the 32 sarbecoviruses,
434 reaching 75 to 100% homology (Tables S2 and S3). More in-depth analysis showed that
435 among all CD8⁺ T-cell epitopes, 62% (n = 57) differed between SARS-CoV-2 and CoV-1 by
436 at least one mutation, but these mutations did not affect HLA-Class I binding for a large
437 majority of them (81%), as predicted by NetMHC4.0 (Table S2). Moreover, two CD4⁺ T-cell
438 epitopes included in the vN2 sequence (N301-315, WPQIAQFAPSASAFF and N306-320,
439 QFAPSASAFFGMSRI) and nine CD8⁺ T-cell epitopes from the vS2 and vN2 sequences
440 (S1056-1063, APHGVVFL; S1089-1096, FPREGVFV; S1137-1145, VYDPLQPEL) and vN2
441 (N305-314, AQFAPSASAF; N306-315, QFAPSASAF; N307-315, FAPSASAFF; 308-317,
442 APSASAFFGM; 310-319, SASAFFGMSR; 311-319, ASAFFGMSR) were 100% homologous
443 across all sarbecoviruses (Table S5). These results confirm that vaccine sequences,
444 particularly vS2 and vN2, are theoretically suitable for the design of a pan-sarbecovirus
445 vaccine aimed at eliciting broad cross-reactive specific T-cell responses.

446 Next, we engineered plasmids expressing the vaccine sequence vRBD fused to the C-
447 terminus of the Heavy (H) chain of anti-human CD40 humanized 12E12 IgG4 antibody,
448 whereas the vN2, vS1, and vS2, sequences were fused sequentially to the Light (L) chain C-
449 terminus to generate the CD40.CoV2 vaccine (Figure 1c). We have previously shown that
450 12E12 anti-CD40 fused to various viral antigens enhances CD40-mediated internalization
451 and antigen-presentation by mononuclear cells and *ex-vivo* generated monocyte-derived
452 DCs.^{36,39}

453 **The CD40.CoV2 vaccine induces protection after viral challenge and cross-neutralizing** 454 **antibody responses in the hCD40/K18-hACE2 transgenic mouse model**

455 Transgenic mice expressing both the human (h) ACE2 receptor, the receptor of SARS-CoV-
456 2,⁵³ and hCD40 receptor (hCD40/K18-hACE2) were vaccinated with two intraperitoneal
457 injections of CD40.CoV2 vaccine (10 µg) supplemented with polyinosinic-polycytidylic acid
458 (Poly(IC) (50 µg) three weeks apart and challenged with Wuhan/D614G SARS-CoV-2
459 (Figure 2a). Poly(IC) was selected as an adjuvant due to its ability to increase antigen-

460 presenting cell maturation.⁵⁴ In contrast to the vaccinated animals, controls exhibited
461 significant weight loss from day 5 post-infection (pi), lasting until day 12 pi (Figure 2b). This
462 was associated with the development of clinical symptoms in the controls from day 5 pi
463 (Figure S1), leading to death of 67% of the animals by day 12 pi, whereas the vaccinated
464 animals showed no symptoms and none died (Figures 2b and S1). Accordingly, the SARS-
465 CoV-2 viral replication (genome equivalent/ μ g RNA) and viral infectious particles (PFU/mg of
466 tissue) were lower in the lungs of the vaccinated mice than the controls or, indeed,
467 undetectable (Figure 2c). We next assessed the antibody responses elicited *in vivo* by the
468 CD40.CoV2 vaccine. One week after the booster injection (d28 post-vaccination [dpv]), the
469 SARS-CoV-2 RBD- and S-specific IgG (Wuhan strain) binding levels were significantly higher
470 in the vaccinated than mock-vaccinated mice ($P = 0.0004$ for both, Mann Whitney U test)
471 (Figure 2d-2e). The CD40.CoV2 vaccine was also able to elicit RBD-specific IgG with cross-
472 reactivity against VOCs ($\alpha, \beta, \gamma, \delta$) or the variant of interest (VOI) κ ($P = 0.0004$ for all
473 comparisons between vaccinated and mock-vaccinated animals, Wilcoxon U test) (Figure
474 2d). Moreover, vaccine-elicited IgG highly cross-reacted with the SARS-CoV-1 spike protein
475 (Figure 2e), but not the S protein of MERS or common cold coronaviruses, of which the
476 sequences show less homology (Figure S2). By day 12 pi (corresponding to 40 dpv), cross-
477 reactive IgG levels had increased in the control animals, but the response remained
478 significantly lower than in the vaccinated animals ($P = 0.0238$ between vaccinated and mock-
479 vaccinated animals, Wilcoxon U test) (Figure 2d-2e). Overall, the CD40.CoV2 vaccine
480 elicited cross-neutralizing antibody responses against RBD from SARS-CoV-2 Wuhan and
481 VOCs (Figure 2f) and S from both SARS-CoV-2 and SARS-CoV-1 (Figure 2g). These results
482 were confirmed in a second replicate animal experiment (Figure S3).

483 In addition, the comparison of antibody responses in animals immunized either with the
484 CD40.CoV2 and poly(IC) or mRNA BNT162b2 vaccine showed comparable levels of RBD-
485 binding IgG (Figure 3a) and neutralizing activity (Figure 3b) against the ancestral strain
486 (Wuhan) and $\alpha, \beta, \gamma, \delta$ VOCs. A retained activity against Omicron was observed, but with

487 7.2- and 9.4-fold lower binding for those raised by the CD40.CoV2 and BNT162b2 vaccines
488 respectively, and 3.1- and 3.3-fold lower neutralizing activity (Figure 3).

489 In conclusion, the vaccine-elicited immune responses provided protection against SARS-
490 CoV-2 challenge, with 100% survival, no clinical symptoms, and significant viral load control
491 *in vivo*. These results significantly add to our previous vaccine studies in various animal
492 models (Hu-mice, or NHP), in which aCD40 targeting vaccines were able to induce potent
493 humoral and cellular immune responses against Influenza virus, HIV, and, more recently,
494 SARS-CoV-2 RBD.^{37,38,41,55}

495 **The CD40.CoV2 vaccine recalls cross-reactive functional SARS-CoV-2 T-cell** 496 **responses *in vitro***

497 We next investigated the potency of the CD40.CoV2 vaccine in recalling T-cell responses *in*
498 *vitro* using PBMCs collected from individuals who had experienced a viral infection, as
499 previously demonstrated. Peripheral blood mononuclear cells (PBMCs) from 39 convalescent
500 COVID-19 patients (between one to six months following infection) from the French COVID
501 cohort⁵⁶ were collected. The median [IQR] age of the patients was 56 [47-64], of whom 67%
502 were male. First, we evaluated the frequency of CD4⁺- and CD8⁺-specific T cells, as
503 assessed by the expression of the activation markers CD69⁺ and CD137⁺ (Figure 4a-b).⁵⁷
504 PBMCs (n = 5 donors) were stimulated with various doses of CD40.CoV2 vaccine, ranging
505 from 10 to 10⁻⁴ nM, or an equimolar concentration of a combination of overlapping peptides
506 (OLP) spanning the full-length sequence of the vaccine antigens (vS1+vS2+vRBD+vN2),
507 referred as vOLPmix. The effective range of vaccine potency eliciting SARS-CoV-2-specific
508 CD4⁺ T cells was between 1 to 10 nM, with maximal activity at 1 nM. At these concentrations,
509 the recall of specific CD4⁺ T cells was 10-fold higher than with vOLPmix stimulation (Figure
510 4b) (P = 0.0062, Wilcoxon U test). We observed a similar activation profile for CD8⁺ T cells,
511 with the highest potency at 1 nM. Next, we confirmed the functionality of these cells, showing
512 that the CD40.CoV2 vaccine induced robust and significantly higher proliferation of specific
513 CD4⁺ and CD8⁺ T cells and CD19⁺ B cells than cells stimulated with vOLPmix or a control

514 CD40 vaccine fused to the HIV Env glycoprotein 140 (strain ZM96) (CD40.Gp140z) (Figure
515 4c and 4d).

516 These responses were likely favored by the targeting of vaccine epitopes through the anti-
517 CD40 vehicle, as demonstrated by the broad and high levels of secretion of soluble factors
518 produced by PBMCs from convalescent COVID-19 patients (n = 15) stimulated for two days
519 with either the CD40.CoV2 vaccine (1 nM) or vOLPmix (Figure 5 and Figure S4). Vaccine
520 stimulation induced the production of chemokines involved in monocyte, macrophage, and
521 DC chemotaxis (MCP-1, IP-10), as well as those associated with T-cell (CCL5) and
522 neutrophil (IL-8) recruitment. Moreover, the level of cytokines produced by activated
523 monocytes/macrophages and DCs (TNF, MIP-1 α , MIP-1 β , and IL-12p70) and those specific
524 to cytotoxic activity (Granzyme B) also increased. Interestingly, Th1 (IFN- γ , IL-2), Th2 (IL-4,
525 IL-13), and Th17 (IL-17A) cytokines were also detected. By contrast, stimulation with the
526 matched vOLPmix only significantly induced the production of IL-2 (Figure 5). Moreover,
527 coculture of PBMCs with CD40.Gp140z control vaccine did not stimulate the production of
528 cytokines excluding bystander and “non-specific” activation through CD40 receptor (Figure
529 S5). Overall, these results demonstrate the potency of minute amounts of CD40.CoV2
530 vaccine to promote the recall of functional specific memory T and B cells.

531 **The CD40.CoV2 vaccine elicits multiepitope and cross-reactive specific T-cell**
532 **responses against SARS-related sequences.**

533 We next confirmed the potency of the CD40.CoV2 vaccine construct to elicit cross-reactive
534 functional memory T cells against individual vaccine regions from SARS-CoV-2 Wuhan or
535 those harboring VOC/VOI mutations within these regions and SARS-CoV-1 S1/RBD/N2
536 epitopes.

537 First, PBMCs from convalescent COVID-19 patients (M1-M6 post-infection, n = 14) were
538 stimulated with the CD40.CoV2 vaccine (1 nM) and restimulated on day 8 either with one of
539 the vOLPs (vRBD, vS1, vS2, or vN2) or control OLPs (cont.OLP), defined as SARS-CoV-2

540 regions either not contained in the CD40.CoV2 vaccine (SARS-CoV-2 Nucleocapsid [N1-N2]
541 or SARS-CoV-2 Matrix [M]) or irrelevant peptides, such as Ebola glycoprotein (Gpz). The
542 CD40.CoV2 vaccine recalled polyfunctional SARS-CoV-2-specific CD4⁺ T cells
543 simultaneously producing up to three cytokines (IFN- γ \pm IL-2 \pm TNF) (Figure 6a-c and Figure
544 S6). Specific CD4⁺ and, to a lesser extent, CD8⁺ T cells produced IFN- γ against all vaccine
545 antigens but not against control antigens (non-significant P value for control antigens vs the
546 unstimulated condition, Wilcoxon U test). The CD40.CoV2 vaccine recalled polyepitope IFN-
547 γ ⁺ responses ranked from vS1 > vRBD > vN2 > vS2 for CD4⁺ T cells and vN2 > vRBD > vS1
548 for CD8⁺ T cells (Figure 6c). We also observed significant specific TNF⁺ and IL-2⁺ CD4⁺ T-
549 cell responses against various vaccine antigens, whereas only the IL-2⁺ CD8⁺ T-cell
550 response was significant after stimulation with vRBD (Figure S6). Interestingly, the strongest
551 CD8⁺ T-cell response was directed against vN2, known to be important for long-term
552 immunity⁵⁸ (Figure 6c). Finally, vaccine-expanded specific memory CD8⁺ T cells from five
553 different patients showed cytotoxic activity against autologous cells pulsed with different
554 vOLPs (vN2 or vRBD), whereas there was no cytotoxic activity when target cells were pulsed
555 with cont.OLP (N1-N2 or M) (Figure 6d).

556 We also observed that CD40.CoV2 vaccine-expanded *in-vitro* CD4⁺ and CD8⁺ T-cell
557 responses were not affected when the cells were stimulated with RBD OLPs containing
558 common mutations of β/γ , δ (VOCs) and κ (VOI) (Figure 7a). Similar results were obtained
559 when the cells were stimulated with RBD OLPs from Omicron VOC (Figure 7b). The cross-
560 reacting CD8⁺ and CD4⁺ T-cell responses were polyfunctional, simultaneously producing up
561 to 2 or 3 cytokines, respectively, with no major differences between VOCs except for
562 Omicron where we observed a decreased polyfunctionality (Figure 7c).

563 Finally, we re-stimulated CD40.CoV2-stimulated PBMCs from convalescent COVID-19
564 patients with OLPs covering the S1 (S1-CoV-1), vRBD (vRBD-CoV-1), and vN2 (vN2-CoV-1)
565 regions from SARS-CoV-1 and S1 from MERS. The vaccine elicited a high frequency of
566 cross-reactive SARS-CoV-1 CD4⁺ and CD8⁺ T cells and, to a lower extent, cross-reactive

567 MERS-S1 CD8⁺ T cells (Figure 8). Interestingly, the CD40.CoV2 vaccine-induced SARS-
568 CoV-1- and SARS-CoV-2-specific T-cell responses were highly correlated for all
569 corresponding antigen sequences (Figure S7). Overall, we confirm that the breadth of recall
570 responses induced by the CD40.CoV2 vaccine is not affected by RBD mutations from SARS-
571 CoV-2 VOCs and fully recognizes SARS-CoV-1 epitopes.

572

573

574 **Discussion**

575 Despite the rapid development of several effective vaccines against SARS-CoV-2, recent
576 observations from vaccine campaigns in the general population have shown that the
577 antibody response is waning, with reduced efficacy against VOCs. Most are characterized by
578 mutations found in areas that are likely targeted by neutralizing antibodies, leading to vaccine
579 escape and compromising the first line of immunological defense against SARS-CoV-2.
580 Moreover, whether the current first-generation vaccines based on the original virus strain
581 would still protect against emerging VOCs or pre-emergent coronaviruses, which may be
582 responsible for future pandemics, is unknown. Thus, the development of new-generation
583 vaccines that can induce B- and T-cell responses to a broad range of epitopes, less prone to
584 variation, are warranted.

585 Here, we demonstrate the immunogenicity and anti-viral efficacy of a protein vaccine
586 composed of three regions from S (aa 125-250, 318-541, 1056-1209) and one from N (aa
587 276-411) of SARS-CoV-2, accumulating a large set of predicted CD4⁺ and CD8⁺ T- and B-
588 cell epitopes that are highly homologous to those of SARS-CoV-1 and 32 recently described
589 SARS-CoV-2-related coronaviruses. The CD40.CoV2 vaccine elicited potent SARS-CoV-2-
590 specific cross-reactive and neutralizing antibodies associated with anti-viral and protective
591 activity against SARS-CoV-2 challenge in the hCD40/K18-hACE2 mouse model.
592 Furthermore, vaccinated mice developed high neutralizing antibody levels against the RBD
593 region from not only SARS-CoV-2 Wuhan but also several SARS-CoV-2 VOCs/VOI (α , β , γ , δ
594 and κ) and S from SARS-CoV-1. These results confirm and extended our previous
595 observations of the antiviral efficacy of the CD40.RBD vaccine in convalescent macaques.⁴¹

596 Our vaccine design was also driven by the need to include conserved epitopes to generate
597 robust memory CD4⁺ or CD8⁺ T cells to provide early control of acute infection with a novel
598 SARS-CoV-2 VOC or closely related virus in the absence of pre-existing cross-protective
599 antibodies.⁹ The *in-silico* definition of vaccine sequences was supported by several
600 observations. First, various CD40.CoV2 vaccine epitopes have already been shown by

601 others, through structure-based network analysis and assessment of HLA class-I peptide
602 stability, to be structurally constrained, thus limiting genetic variation across SARS-CoV-2,
603 SARS-CoV-1, and sarbecoviruses.⁵⁹ For example, the CD40.CoV-2 vaccine contains 6/28 S
604 (S319-329, RVQPTEIVRF; S321-329, QPTEIVRF; S386-395, KLNDLCFTNV; S386-396,
605 KLNDLCFTNVY; S515-524, FELLHAPATV; S1093-1102, GVFVSNNGTHW) and 6/11 N
606 (N276-286, RRGPEQTQGNF; N305-313, AQFAPSASA; N306-314, QFAPSASAF; N306-
607 315, QFAPSASAFF; N308-315, APSASAFF; N308-317, APSASAFFGM) of highly networked
608 constrained regions with stabilizing CD8⁺ T-cell epitopes with global HLA coverage.⁵⁹
609 Second, we showed the reactivity of CD4⁺ and CD8⁺ T cells to nanomolar concentrations of
610 the CD40.CoV2 vaccine using an approach combining the expression of activation markers,
611 cytokine production, T-cell proliferation, and cytotoxic function. These results demonstrate
612 the immunogenicity of vaccine epitopes and are consistent with those of prior studies
613 describing a number of them in several patient cohorts (Tables S3 and S4).^{49,60-62} Third,
614 based on the recent review of all SARS-CoV-2 CD4⁺ and CD8⁺ T-cell epitopes reported in 25
615 studies,⁴⁹ it appears that the CD40.CoV2 vaccine contains 42% of the described
616 immunodominant CD8⁺ T-cell epitopes for S and 39% for N. The respective values are 54%
617 (S) and 35% (N) for the immunodominant CD4⁺ T-cell epitopes. Moreover, all vaccine regions
618 contain dominant epitopes. For example, the vS1 and vRBD regions of the CD40.CoV2
619 vaccine closely fit or overlap the two immunodominant S regions for CD4⁺ T cells (S154-254,
620 S296-370). Similarly, the vN2 sequence overlaps the described CD4⁺ and CD8⁺ T-cell
621 immunodominant region of the nucleocapsid (201-371).⁴⁹

622 A crucial question for the development of vaccines to counteract the escape of the virus from
623 neutralizing antibodies is whether SARS-CoV-2 VOCs can evade T-cell immunity. However,
624 even if SARS-CoV-2 does mutate, analysis of mutations associated with the various VOCs
625 shows the vast majority of CD40.CoV2 vaccine epitopes to be conserved in SARS-CoV-2
626 variants (Tables S2 and S3). Accordingly, we found that *in-vitro* stimulation with the
627 CD40.CoV-2 vaccine elicited specific cross-reactive polyfunctional CD4⁺ and CD8⁺ T-cell

628 responses against RBD from VOCs/VOI. Similarly, we found that the breadth of vaccine-
629 elicited CD4⁺ and CD8⁺ T-cell responses extended to S1, RBD, and N sequences from
630 SARS-CoV-1. Indeed, expanded CD8⁺ T cells were even cross reactive to S1 peptides from
631 MERS, despite low homology with SARS-CoV-2 (15%).

632 While it is critical to determine to what extent VOCs may or may not be susceptible to
633 evading existing humoral responses, T-cell associated immunity is, in general, significantly
634 more difficult for viruses to overcome, due to the broad and adaptable response generated in
635 a given individual and because of the variety of HLA haplotypes. In this regard, the new
636 SARS-CoV-2 B.1.1.529 (Omicron) VOC, which emerged in November 2021, is characterized
637 by the presence of 32 mutations in Spike, located mostly in the N-terminal domain (NTD) and
638 the RBD. Recent results have shown that this VOC significantly escapes from neutralizing
639 antibodies, either therapeutic or those from convalescent or vaccinated individuals at various
640 levels.⁶³⁻⁶⁵ However, preliminary studies have shown minimal cross-over between mutations
641 associated with the Omicron variant of SARS-CoV-2 and CD8⁺ T-cell epitopes identified in
642 convalescent COVID-19 individuals.⁶⁶ Of note, the vN2 vaccine sequence is 100%
643 homologous with that of most VOCs, including the Omicron variant. Overall, concerning the
644 objective to develop a vaccine with a broader range of protection, our results confirm
645 previous observations that single amino-acid substitutions or deletions across large
646 peptidomes do not significantly affect polyclonal memory T-cell responses.⁶⁷

647 The design of the CD40.CoV-2 vaccine benefited from the high number of genetically
648 conserved SARS-CoV-2 S and N sequences across VOCs, as well as those of SARS-CoV-
649 related viruses, with the goal of inducing broad immune cross reactivity, a key component for
650 the development of a next-generation pan-sarbecovirus vaccine. We show that the vaccine
651 T-cell epitopes are highly conserved with those of SARS-CoV-2 VOCs, SARS-CoV-1, and,
652 more generally, all 38 sarbecoviruses tested, with up to 80-100% homology for the most
653 highly conserved T-cell epitopes (Tables S2 and S3). Moreover, we show that nine CD8⁺ T-
654 cell epitopes, from S and N, contained in the vaccine are 100% homologous among all

655 sarbecoviruses (Table S5). Globally, these results confirm that vaccine sequences,
656 particularly vS2 and vN2, are theoretically suitable for the design of a pan-sarbecovirus
657 vaccine aiming to elicit broad cross-reactive T-cell responses.

658 We further propose in this vaccination strategy to deliver the antigens through a DC-targeting
659 platform, as the antigens were fused to a humanized anti-CD40 monoclonal antibody. This
660 platform has already been tested *in vitro*, in various preclinical animal models, and is
661 currently in phase I/II clinical development for a prophylactic HIV vaccine (NCT04842682).
662 Thus, by targeting epitopes of the S and N proteins, the CD40.CoV2 vaccine may represent
663 an excellent booster of pre-existing immunity, induced either by previous priming with
664 available vaccines or by natural infection, as we recently demonstrated that a single dose of
665 the CD40.RBD vaccine, injected without adjuvant, is sufficient to elicit neutralizing antibodies
666 that protect macaques from a new viral challenge.⁴¹

667 Because SARS-CoV-2 humoral responses decline rapidly over time, repeated vaccinations
668 at short time intervals are required to maintain high neutralizing responses, at least with the
669 currently available vaccines, which all target the S protein.⁶⁸ However, aside from antibody
670 responses, early induction of functional SARS-CoV-2-specific T cells is observed in patients
671 with mild disease and rapid viral clearance.⁶⁹ In addition, recent studies have highlighted the
672 significant cross-protective advantage of a heterologous boost, even if the vaccine antigens
673 do not fully match the viral challenge⁷⁰ and the interest to target also conserved regions of
674 the spike protein, outside the RBD domain, for induction of cross-neutralizing antibodies.⁷¹
675 Thus, a significant advantage of our vaccine may be to extend the breadth of the responses
676 of current vaccines.

677 Our study had several limitations. Due to the limited availability of hCD40/K18-hACE2 mice,
678 we did not evaluate the potency of the CD40.CoV2 vaccine against various SARS-CoV-2
679 related VOCs or other sarbecovirus strains in vaccinated mice. However, we showed that the
680 CD40.CoV2 vaccine induced binding and neutralizing IgG responses very similar or
681 equivalent to those induced by the mRNA BNT162b2 vaccine. One potential advantage of

682 our vaccine is its capacity to elicit functional cross-reactive T-cell responses. We favored the
683 analysis of T cell responses using samples from recovered individuals instead of *in vivo*
684 preclinical models. Taking account that these responses may be dependent on the “clinical
685 history” of patients and their HLA haplotypes, they are less biased than those that would be
686 observed in an animal model. Our results show that the *in-vitro* vaccine responses are
687 directed against all vaccine proteins, which confirmed the broad HLA coverage of the vaccine
688 sequences. Although, we did not test responses against N sequences from VOC or other
689 sarbecoviruses, because of the high homology (100%) between vaccine sequences and
690 VOCs including Omicron.

691 In conclusion, it is becoming urgent to develop a “pan-sarbecovirus vaccine”. The
692 development of a new protein-based vaccine with expected improved tolerability suitable for
693 people with specific vulnerabilities and children would extend the portfolio of current vaccines
694 and be instrumental in controlling the circulation of the virus and the emergence of new
695 variants. By selecting a narrow range of immunodominant epitopes, presented by a wide
696 variety of HLA alleles and less prone to genetic variations across sarbecoviruses, we provide
697 a rationale for the development of a global T cell-based vaccine to counteract emerging
698 SARS-CoV-2 variants and future SARS-like coronaviruses.

699

700 **Contributors**

701 Y.L, V.G, and S.C conceived and designed the study. S.Co, A.W, M.S, C.L, A.Z, B.M, Q.B,
702 M.F, S.H, S.C, G.Z, S.Z, V.G, M.P, R.T and Y.L analyzed and interpreted the data. S.Co,
703 M.S, L.D, F.P, A.S, C.P, Q.B, M.F, S.S, MEH and P.H performed the experiments. S.Z, Z.W,
704 J.E, S.C, C.L, M.S., and Y.L. designed and produced the CD40.CoV2 vaccine. A.Z, B.M, and
705 V.G supervised the animal studies. J.G and M.C.G participated in sample and clinical data
706 collection. M.C administered the project. Y.L, V.G, S.Co, AW, M.S, A.Z, S.Z, and G.Z drafted
707 the first version and wrote the original version of the manuscript. S.Co, A.W, V.G, C.L and
708 Y.L verified the underlying data of the final submitted version. All authors approved the final
709 version.

710 **Declaration of interests**

711 The authors S.Z., G.Z., V.G., M.C., S.C., C.L., M.S., and Y.L., are named inventors on patent
712 applications based on this work held by Inserm Transfert. The remaining authors declare no
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739 **Data sharing statement**

740 The CD40.CoV2 vaccine generated in this study was deposited in GenBank: anti-human
741 CD40 12E12 antibody IgG4 H chain (GenBank ID: AJD85779.1 residues 20-467) fused to
742 SARS_CoV_2RBD (GenBank ID: UEP92470.1 residues 17-240) followed by EPEA (C-tag)
743 and the anti-human CD40 12E12 antibody kappa L chain (GenBank ID: AJD85780.1
744 residues 21-236) fused sequentially to a linker (GenBank ID: AJD85777.1 residues 699-725),
745 nucleocapsid phosphoprotein, partial [Severe acute respiratory syndrome coronavirus 2]
746 (GenBank ID: QWE63393.1 residues 95-230), linker residues AR, Chain A, Spike protein S1
747 [Severe acute respiratory syndrome coronavirus 2] (GenBank ID: 7M8J_A residues 113-
748 237), linker residues TR, and Sequence 12 from patent US 8518410 (Genbank ID:
749 AGU17682.1 residues 3-27), surface glycoprotein, partial [Severe acute respiratory
750 syndrome coronavirus 2] (GenBank ID: UET03776.1 residues 195-348). The authors declare
751 that other data supporting the findings of this study are available from the corresponding
752 author upon request.

753

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939

940 **Figure legends**

941 **Figure 1. Selection of SARS-CoV-2 T- and B-cell polyepitope regions for an improved dendritic cell-**
942 **targeting vaccine platform.** (a) Mapping of selected SARS-CoV-2 epitope-enriched regions. (1) Four selected
943 vaccine regions. (2) Predicted SARS-CoV-2 CD8⁺ T-cell epitopes (NetMHC 4.0). (3) Described SARS-CoV-2
944 CD8⁺ T-cell epitopes at the time of vaccine region selection. (4) Predicted SARS-CoV-2 CD4⁺ T-cell epitopes
945 (NetMHCII 2.3). (5) Described SARS-CoV-2 CD4⁺ T-cell epitopes at the time of vaccine region selection. (6)
946 Predicted linear B-cell epitopes (BepiPred 2.0). (7) Described SARS-CoV-2 IgM, IgA, and IgG epitopes at the
947 time of vaccine region selection. (b) Vaccine (v) regions (vS1, vRBD, vS2 and vN2) and control region not
948 included in the vaccine (N1-N2) and (c) CD40.CoV2 vaccine construct.
949

950 **Figure 2. CD40.CoV2-vaccinated animals survive SARS-CoV-2 infection and show neutralizing and cross-**
951 **reactive antibody responses.** (a) Design of the CD40.CoV2 vaccination strategy before SARS-CoV-2 infection.
952 (b) Relative weight and survival of mock-vaccinated (grey) and vaccinated (blue) hCD40/K18-hACE2
953 transgenic mice after SARS-CoV-2 inoculation. Both parameters were recorded from days 0 to 12 post infection
954 (pi). The mean \pm SD is presented. A Mann-Whitney U test was conducted to compare differences in weight
955 between the two groups on day 12 (n = 9-12 animals per group) (**P < 0.01). Kaplan-Meier survival curves
956 were generated (n = 6-9 animals per group) and the P value was calculated using the [log-rank (Mantel-Cox)
957 test] (*P < 0.05). (c) Viral load (genome equivalent/ μ g RNA) and viral infectious particle units (PFU/mg of
958 tissue) in the lungs of mock-vaccinated (grey) and vaccinated (blue) hCD40/K18-hACE2 transgenic mice (n = 3
959 animals per group) on day 5 pi with the median plotted as a line. (d) Levels of IgG antibodies (AU) binding to
960 Wuhan and VOCs SARS-CoV-2 RBD proteins before vaccination (baseline, -2 days post-vaccination (dpv), n =
961 9-12 animals per group), after the completion of the vaccination schedule (28 dpv, n = 9-12 animals per group),
962 and 40 dpv (i.e., day 12 pi time point, n = 3-5 animals per group). (e) Levels of IgG antibodies (AU) binding to
963 SARS-CoV-2 (circles) and SARS-CoV-1 (triangles) S proteins in mock-vaccinated (grey) and vaccinated (blue)
964 animals at -2, 28, and 40 dpv. Medians [Min-Max] are shown. The grey dashed lines represent prime and boost
965 vaccines. The red dashed line represents SARS-CoV-2 inoculation. Neutralizing activity of (f) anti-RBD
966 antibodies (units/mL) and (g) anti-S antibodies (units/mL) in mock-vaccinated (grey) and vaccinated (blue)
967 animals post-vaccination (open circles) and post-infection (solid circles). Medians \pm Interquartile ranges (IQRs)
968 are shown. Thirty plasma samples from unvaccinated mice were used to determine the threshold for positivity,
969 defined as the whole units/mL value immediately above the concentration of the highest sample for RBD (i.e., 8
970 units/mL) and Spike (i.e., 4 units/mL) proteins. These results were reproduced in a second independent
971 experiment (Figure S3).

972 **Figure 3. CD40.CoV2 and mRNA BNT162b2 vaccines elicit similar cross-reactive and neutralizing**
973 **antibody responses.** Levels of IgG antibodies (AU) binding to Wuhan and VOCs SARS-CoV-2 RBD proteins
974 (α , β , γ , δ , Omicron) (a) and neutralizing activity of anti-RBD antibodies (percentage of inhibition relative to the
975 Wuhan RBD protein) (b) one week after completion of the vaccination schedule in BNT162b2 mRNA (red) or
976 CD40.CoV2 vaccinated animals (blue) (n=5 and 20 animals, respectively). Medians \pm Interquartile ranges
977 (IQRs) are shown.

978 **Figure 4. Determination of the optimal immunogenic CD40.CoV2 vaccine concentration and proliferation**
979 **of specific T cells induced by the CD40.CoV2 vaccine.** (a) Gating strategy for specific T cells that upregulate
980 activation-induced markers (AIM). (b) Antigen specific activation of CD4⁺ (blue) and CD8⁺ (green) T cells from
981 COVID-19 convalescent patients (n = 5) stimulated with various concentrations of CD40.CoV2 vaccine or a
982 combination of OLPs covering the full-length sequence of the vaccine antigens (vOLPmix). Activation of
983 SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells is shown as the percentage of CD69⁺ CD137⁺ cells within the
984 CD4⁺ or CD8⁺ subset after background subtraction. Median values (solid line) \pm interquartile ranges (IQRs)
985 (dashed lines) are shown. (c) Gating strategy for assessing the proliferation of specific T and B cells after seven
986 days of CD40.CoV2 stimulation. (d) Proliferation of CD4⁺ T-cells, CD8⁺ T-cells, and B-cells from COVID-19
987 convalescent patients (n = 10) induced by the CD40.CoV2 vaccine, an irrelevant vaccine (CD40 Gp140z [1
988 nM]), or an equimolar concentration of vOLPmix. Data are expressed as a proliferation index obtained by
989 dividing the frequency of proliferating cells after specific stimulation over background. Median values \pm IQRs
990 are shown. [Friedman and Dunn's multiple comparison tests] were used for statistical analysis (**P < 0.01, ***P
991 < 0.001).
992

993 **Figure 5. Heatmap of standardized biomarker expression in culture supernatants induced by the**
994 **CD40.CoV2 vaccine.** Supernatants from convalescent COVID-19 patient PBMCs collected on day 2 after
995 stimulation with the CD40.CoV2 vaccine (1 nM) or an equimolar concentration of vOLPmix (n = 15). The

996 colors represent standardized expression values centered around the mean, with variance equal to 1. Biomarker
997 hierarchical clustering was computed using the Euclidean distance and Ward's method.⁷²
998

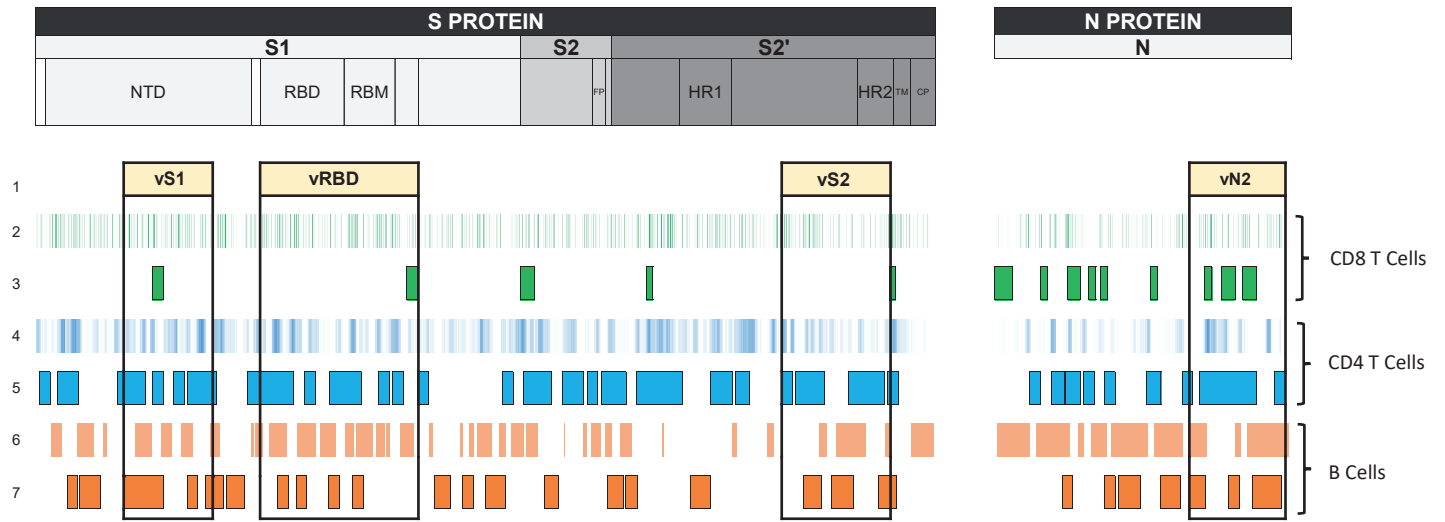
999 **Figure 6. Polyfunctional and cytotoxic specific T-cell responses of convalescent COVID-19 patients after**
1000 ***in-vitro* stimulation with the CD40.CoV2 vaccine.** (a) Representative dot plots of SARS-CoV-2-specific CD4⁺
1001 and CD8⁺ T-cell responses after *in vitro* stimulation of patient PBMCs with the CD40.CoV2 vaccine (1 nM) on
1002 D0 and re-stimulation with various vOLPs (vRBD, vS1, vS2 or vN2) (1 µg/ml) on D8. (b) Functional
1003 composition of SARS-CoV-2-specific CD4⁺ and CD8⁺ T-cell responses induced by the CD40.CoV2 vaccine and
1004 various vOLPs (vRBD, vS1, vS2 or vN2) (1 µg/ml). Responses are color coded according to the combination of
1005 cytokines produced. The arcs identify cytokine-producing subsets (IFN-γ, IL-2, and TNF) within the CD4⁺ or
1006 CD8⁺ T cell populations. (c) Frequency and radar charts of the merged median of IFN-γ⁺ SARS-CoV-2-specific
1007 CD4⁺ (blue) or CD8⁺ (green) T cells from convalescent COVID-19 patients (n = 14) stimulated or not with the
1008 CD40.CoV2 vaccine (1 nM) on D0 and re-stimulated with various vOLPs (vRBD, vS1, vS2 or vN2), cont.OLP
1009 (SARS-CoV-2 N1-N2, or Ebola Gpz) (grey) on D8 (1 µg/mL). Median values ± IQRs are shown. [Friedman and
1010 Dunn's multiple comparison tests] were used for statistical analysis (*P < 0.05, **P < 0.01, ***P < 0.001, ****P
1011 < 0.0001, ns: not significant). (d) Specific lysis of CD8⁺ T cells stimulated with the CD40.CoV2 vaccine (1 nM)
1012 against autologous PHA-blasted PBMCs from five different convalescent COVID-19 patients, pulsed with either
1013 vRBD (light green), vN2 (dark green), or cont.OLP (SARS-CoV-2 N1-N2 or M) (grey). The means of triplicate
1014 values ± the standard deviation (SD) are shown. Each symbol represents a different patient.

1015 **Figure 7. Polyfunctional specific T-cell responses against SARS-CoV-2 VOCs after stimulation with the**
1016 **CD40.CoV2 vaccine.** Frequency of total cytokines (IFN-γ ± IL-2 ± TNF) produced by specific CD4⁺ (blue) or
1017 CD8⁺ (green) T cells from convalescent COVID-9 patients (n = 18) after *in-vitro* stimulation with the
1018 CD40.CoV2 vaccine (1 nM) on D0 and re-stimulation with RBD OLP from various VOCs/VOI (a) or RBD
1019 OLP from Omicron VOC (n = 13) (1 µg/mL) (b). Functional composition of SARS-CoV-2-specific CD4⁺ and
1020 CD8⁺ T-cell responses induced by the CD40.CoV2 vaccine against VOCs/VOI. Responses are color coded
1021 according to the combination of cytokines produced. The arcs identify cytokine-producing subsets (IFN-γ, IL-2,
1022 and TNF) within the CD4⁺ and CD8⁺ T-cell populations (c). Median values ± IQRs are shown. [Friedman's test]
1023 and [Wilcoxon U test] were used for comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

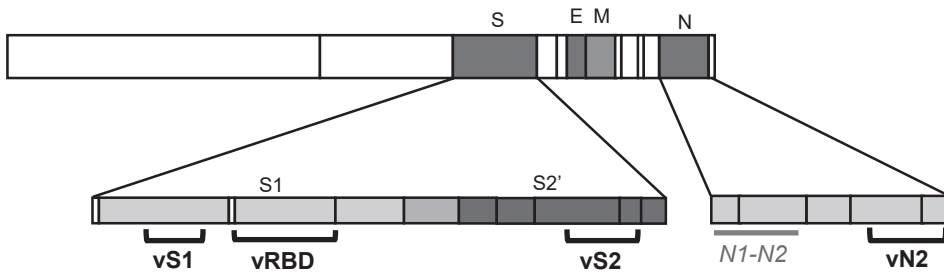
1024 **Figure 8. Cross-reactive specific T-cell responses against SARS-CoV-1 and MERS of convalescent**
1025 **COVID-19 patients after *in-vitro* stimulation with the CD40.CoV2 vaccine.** Frequency of total cytokines
1026 (IFN-γ ± IL-2 ± TNF) produced by specific CD4⁺ (blue) or CD8⁺ (green) T cells after *in-vitro* stimulation with
1027 the CD40.CoV2 vaccine (1 nM) on D0 and re-stimulation with OLPs representing the sequences of S1, vRBD,
1028 and vN2 from (a) SARS-CoV-1 and (b) S1 from MERS (1 µg/mL). Median values ± IQRs are shown. The
1029 [Wilcoxon U test] was used for comparisons (**P < 0.01, ***P < 0.001, ns: not significant).

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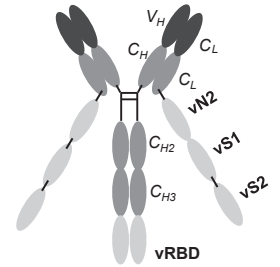
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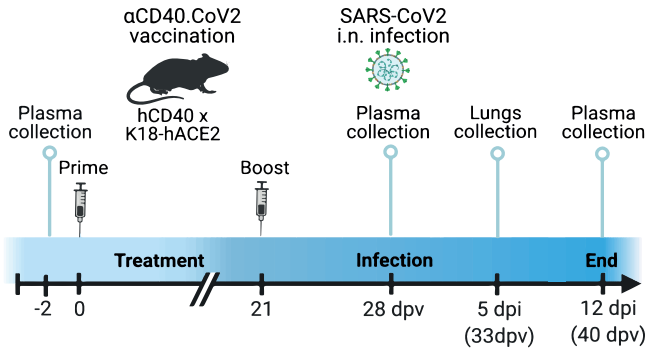
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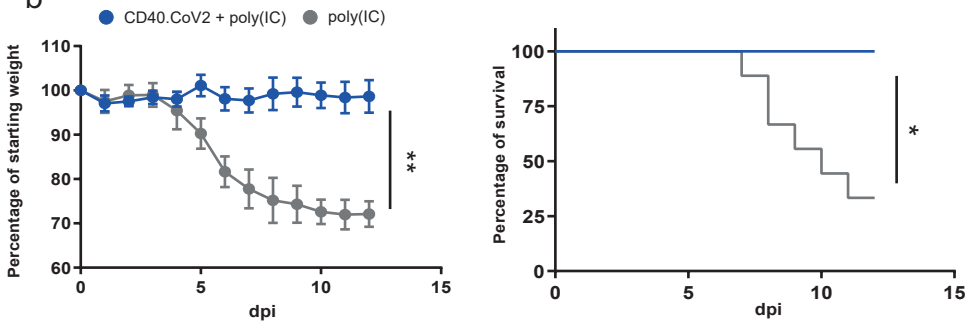
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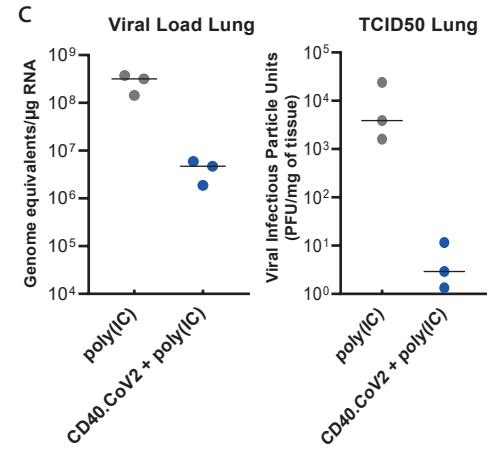
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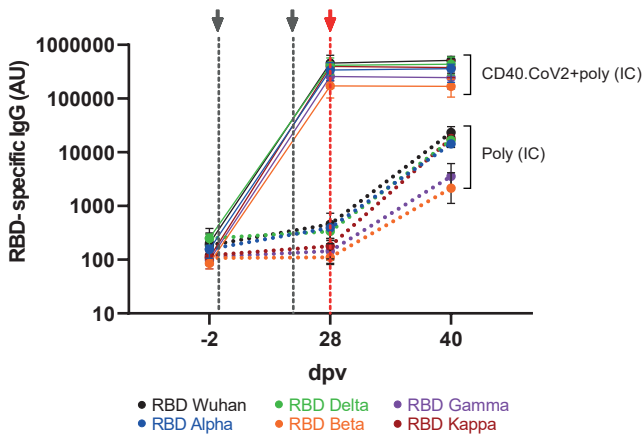
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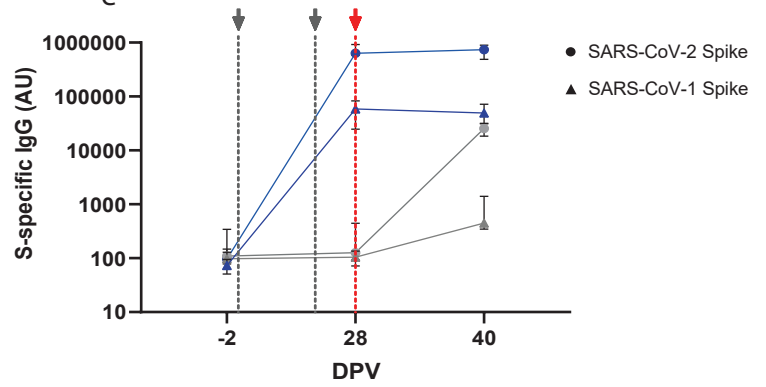
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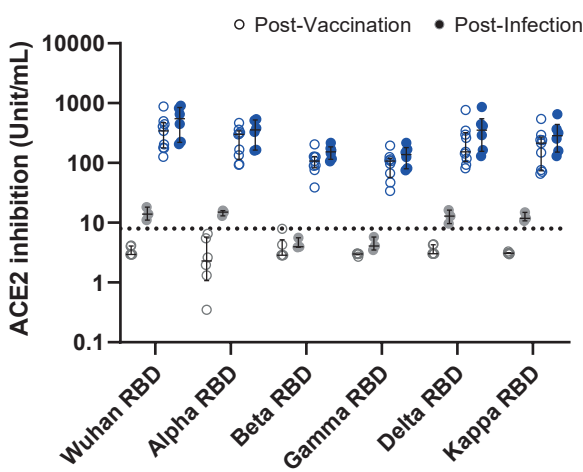
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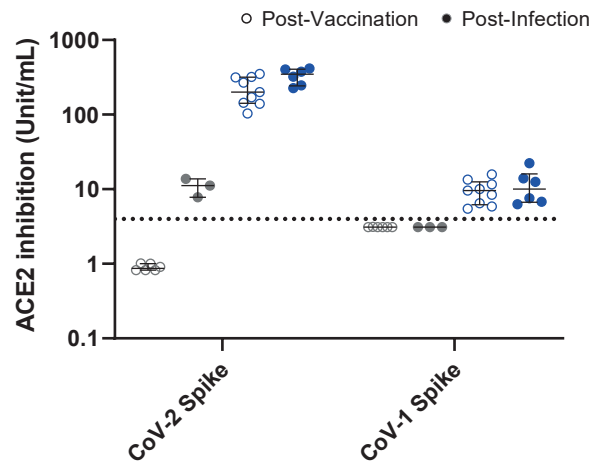


Figure 3

