

1 **Title:**

2
3 **Nasal prevention of SARS-CoV-2 infection by intranasal influenza-based boost vaccination**

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30

31 **Abstract**

32 *Background:* Vaccines in emergency use are efficacious against COVID-19, yet vaccine-induced
33 prevention against nasal SARS-CoV-2 infection remains suboptimal.

34 *Methods:* Since mucosal immunity is critical for nasal prevention, we investigated an
35 intramuscular PD1-based receptor-binding domain (RBD) DNA vaccine (PD1-RBD-DNA) and
36 intranasal live attenuated influenza-based vaccines (LAIV-CA4-RBD and LAIV-HK68-RBD)
37 against SARS-CoV-2.

38 *Findings:* Substantially higher systemic and mucosal immune responses, including
39 bronchoalveolar lavage IgA/IgG and lung polyfunctional memory CD8 T cells, were induced by
40 the heterologous PD1-RBD-DNA/LAIV-HK68-RBD as compared with other regimens. When
41 vaccinated animals were challenged at the memory phase, prevention of robust SARS-CoV-2
42 infection in nasal turbinate was achieved primarily by the heterologous regimen besides
43 consistent protection in lungs. The regimen-induced antibodies cross-neutralized variants of
44 concerns. Furthermore, LAIV-CA4-RBD could boost the BioNTech vaccine for improved
45 mucosal immunity.

46 *Interpretation:* Our results demonstrated that intranasal influenza-based boost vaccination is
47 required for inducing mucosal and systemic immunity for effective SARS-CoV-2 prevention in
48 both upper and lower respiratory systems.

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58 **Keywords:** SARS-CoV-2; Receptor binding domain; Mucosal immunity; Nasal prevention;
59 PD1-based DNA vaccine; Live-attenuated influenza-based vaccine.

60

61

62 **1. Introduction**

63 Since the discovery of coronavirus disease 2019 (COVID-19) in human clusters in December
64 2019 (1, 2), the causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-
65 2), has led to over 100 million infections globally with nearly 2.1 million deaths after just one
66 year. While the COVID-19 pandemic continues to evolve, hundreds of vaccine candidates have
67 been brought into preclinical and clinical trials at an expedited speed using various platforms of
68 technology (3-5). Encouragingly, several vaccines have now acquired regulatory approvals for
69 emergency use in various countries, yet few have been evaluated for inducing mucosal
70 protection, especially for preventing robust SARS-CoV-2 infection in nasal turbinate (NT) (6, 7).
71 NT in the upper respiratory tract (URT) is one of the most important portals of SARS-CoV-2
72 entry into humans. Ciliated nasal epithelial cells in NT have the highest expression of
73 angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2),
74 supporting rapid and robust SARS-CoV-2 infection (8). Unlike SARS patients who have peak
75 viral load in URT at day 10 after symptom onset (9), COVID-19 patients exhibit the highest viral
76 loads in URT at or soon after the clinical presentation (1, 10, 11). Multiple SARS-CoV-2
77 variants can be transmitted simultaneously without a genetic bottleneck (12). Transmitted viruses
78 then quickly impair host innate and adaptive immune responses (12), allowing for robust viral
79 replication and asymptomatic viral spread. These findings indicated that the prevention of
80 SARS-CoV-2 infection in NT is critical for pandemic control.

81 Current systemic vaccination and passive immunization are less effective or suboptimal for
82 preventing SARS-CoV-2 in NT. Among vaccines authorized for emergency use, the Pfizer-
83 BioNTech BNT162B2 mRNA, the Moderna mRNA-1273, the Oxford-ChAdOx1 and Novavax's
84 NVX-CoV2373 have released phase III results showing the efficacy of 95%, 94.1%, 70.4% and
85 89.3%, respectively, in preventing COVID-19 (13-15). The efficacy of the Oxford-ChAdOx1
86 vaccine against asymptomatic SARS-CoV-2 infection was only 3.8% among human vaccinees
87 who received two standard doses (15). For passive immunotherapy, a receptor-binding domain
88 (RBD)-specific human neutralizing antibody (NAb) LY-CoV555 has been evaluated in a phase 2
89 trial. Of three doses of 700 mg, 2800 mg and 7000 mg tested for monotherapy, only the medium
90 dose of LY-CoV555 appeared to accelerate the natural decline of viral loads in nasopharyngeal
91 swabs by day 11 (16). These results indicated that vaccine-induced or passive NAb are
92 suboptimal to prevent SARS-CoV-2 infection in human NT. Using the animal model, we

93 reported that robust SARS-CoV-2 infection in NT may outcompete passive or vaccine-induced
94 systemic NAbs, revealing a possible mechanism underlying the subprotection against
95 asymptomatic infection (17). Considering that vaccine-induced subprotection may drive immune
96 escape virus variants of concern and allow re-infection (18-20), we hypothesized that an
97 effective vaccine should induce mucosal immunity for nasal protection to prevent SARS-CoV-2
98 asymptomatic infection and silent spread. Recently, thousands of vaccine-breakthrough
99 infections in the United States have further indicated the need of priority research on nasal
100 prevention of SARS-CoV-2 infection (21).

101 **2. Methods**

102 **2.1 Mice**

103
104 Male and female BALB/c and K18-hACE2 mice (aged 6–10 weeks) were obtained from the
105 HKU Laboratory Animal Unit (LAU). The animals were kept in Biosafety Level-2 housing and
106 given access to standard pellet feed and water *ad libitum* following LAU's standard operational
107 procedures (SOPs). The viral challenge experiments were then conducted in our Biosafety Level-
108 3 animal facility following SOPs strictly.

109 110 **2.2 Cell lines**

111 HEK 293T-hACE2 and Vero E6 cells (RRID:CVCL_0574) were maintained in Dulbecco's
112 Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) containing 10% fetal bovine
113 serum, 2 mM L-glutamine and 100 U/mL penicillin and were incubated at 37°C in 5% CO₂
114 setting (22).

115 116 **2.3 Viruses**

117 Confluent Vero-E6 cells were infected at 0.01 MOI with live SARS-CoV-2 HKU-13 strain
118 (GenBank accession number MT835140). After 3 days incubation, virus supernatant was
119 collected for titration by plaque assay using Vero-E6 cells.

120 121 **2.4 Construction and Generation of PD1-based DNA and live attenuated influenza virus** 122 **(LAIV)-based vaccine**

123 Codon-optimized SARS-CoV-2 RBD gene was in fusion to a human soluble PD1 domain (PD1-
124 RBD) using the pVAX plasmid as the backbone. To maintain functional domains of the fusion

125 protein, a linker (GGGGS)₃ was applied between the PD1 and RBD gene (23). The expression
126 construct contained a human tissue plasminogen activator (tPA) secretory signal sequence to
127 promote antigen secretion. The plasmid DNA transfection into HEK 293T cells was performed
128 using polyethylenimine (PEI), and protein expression was detected by Western blot. The
129 pHW2000-DelNS1-RBD plasmid was constructed by inserting the tPA-linked RBD between the
130 noncoding region (NCR) and autoproteolytic cleavage site (2A) in the pHW2000-DelNS1
131 plasmid. The V5 tag was added to the C terminal of RBD for better detection of RBD. To rescue
132 the virus, eight pHW2000 plasmids containing the DelNS1-RBD and the other 7 influenza virus
133 genomic segments, together with an NS1 expression plasmid, were transfected into 293T cells
134 using Transit-LT1 (Mirus) according to the manufacturer protocol. After overnight incubation at
135 33 °C, DNA mix was removed, and MEM supplemented with 1 µg/ml N-tosyl-L-phenylalanine
136 chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added. Virus supernatant was collected
137 72 hours later and designated passage 0 (p0) virus and was further passaged in chicken
138 embryonated eggs for 48 hours at 33 °C. Viruses were aliquoted and titrated by plaque assay
139 using MDCK cells. Two intranasal recombinant LAIV DelNS1-RBD vaccine strains, namely
140 LAIV-CA4-RBD and LAIV-HK68-RBD, were generated using A/CA/04/2009 (H1N1) and
141 A/Hong Kong/1/68 (H3N2) surface proteins (HA and NA) in the A/CA/04/2009 (H1N1) DelNS1
142 backbone by a reverse genetic procedure (24).

143

144 **2.5 Animal immunization and SARS-CoV-2 challenges**

145 All animal experiments were approved by the Committee on the Use of Live Animals in
146 Teaching and Research of the University of Hong Kong (HKU). For vaccine immunization, 6-8-
147 weeks old BALB/c or K18-ACE2 transgenic mice received DNA immunization by
148 intramuscular/electroporation (i.m./EP) with 50 µg PD1-RBD-DNA. The voltage of EP was pre-
149 set 60 V in the TERESA DNA Delivery Device (Shanghai Teresa Healthcare Sci-Tech Co., Ltd).
150 Mice intramuscularly received 1/5 clinical doses of Pfizer/BioNTech or Sinovac vaccine. Mice
151 received LAIV-CA4-RBD or LAIV-HK68-RBD immunization by intranasal inoculation of 10⁶
152 PFU per mouse at a 3-week interval under anesthesia. Blood sera was collected for anti-RBD
153 IgG and neutralization detection. Mice were sacrificed and cells from lungs and spleen were
154 harvested and subjected to intracellular cytokine staining (ICS) assay. For the SARS-CoV-2
155 challenge, BALB/c mice were anesthetized and transduced intranasally with 4×10⁸ FFU of Ad5-
156 hACE2, kindly provided by Dr. Jincun Zhao (25), in 70 µl DMEM. The transduced BALB/c

157 mice or K18-ACE2 transgenic mice were intranasally infected with live wild type SARS-CoV-2
158 (HKU clone 13) at a dose of 1×10^4 PFU. Infected animals were sacrificed for endpoint analysis
159 at day 4 post infection (4 dpi). All animal experiments related to SARS-CoV-2 were performed
160 in a biosafety level 3 laboratory in HKU.

161

162 **2.6 Enzyme-linked immunosorbent assay (ELISA)**

163 ELISA was performed to detect SARS-CoV-2 RBD-specific IgG, as previously described (26).
164 In brief, 96-well plates (Costar) were coated with recombinant SARS-CoV-2 RBD antigen (25
165 ng/well, Sino Biological) at 4°C overnight. After washing with PBST (0.05% Tween-20 in PBS),
166 the plates were blocked with 4% skim milk in PBS for 1 hour at 37°C and incubated with serially
167 diluted patient plasma for 1 hour at 37°C. After washing with PBST, goat anti-mouse IgG or
168 anti-mouse IgA conjugated with HRP (Invitrogen) was added, and the whole solution was
169 incubated for 1 hour, followed by washing and the addition of 50 μ l HRP chromogenic substrate
170 3,3',5,5'-TMB (Sigma). Optical density (OD) values were measured at 450 nm using the
171 VARIOSKANTM LUX multimode microplate reader (Thermo Fisher Scientific). Area under the
172 curve (AUC) was measured using GraphPad Prism v8, setting the baseline with the defined
173 endpoint (average of negative control wells + 10 standard deviation) and taking the total peak
174 area as previous described (27).

175

176 **2.7 Pseudotyped viral neutralization assay**

177 To determine the neutralizing activity of mouse sera and BAL, specimens were inactivated at 56°C
178 for 30 min before the pseudotype viral entry assay as previously described (22, 28). The results
179 of this assay correlated strongly with that of neutralization assay using replication-competent
180 SARS-CoV or SARS-CoV-2 (8, 29). The plasmids encoding for D614G, Alpha, Beta and Delta
181 variants were kindly provided by Dr. David D. Ho. In brief, different SARS-CoV-2 pseudotype
182 viruses were generated through co-transfection of 293T cells with 2 plasmids, pSARS-CoV-2 S
183 and pNL4-3Luc_Env_Vpr, carrying the optimized SARS-CoV-2 S gene and a human
184 immunodeficiency virus type 1 backbone, respectively. At 48 hours post-transfection, viral
185 supernatant was collected and frozen at -80°C. Serially diluted serum samples were incubated
186 with 200 TCID50 of pseudovirus at 37°C for 1 hour. The serum-virus mixtures were then added
187 into pre-seeded HEK 293T-hACE2 cells. After 48 hours, infected cells were lysed, and luciferase
188 activity was measured using Luciferase Assay System kits (Promega) in a Victor3-1420

189 Multilabel Counter (PerkinElmer). The 50% inhibitory concentrations (IC₅₀) of each specimen
190 were calculated using non-linear regression in GraphPad Prism v8 to reflect anti-SARS-CoV-2
191 potency.

192

193 **2.8 Surface and intracellular cytokine staining (ICS)**

194 The lung cells of mice were washed one time with staining buffer (PBS contained 2% FBS)
195 followed by staining with anti-mouse antibodies for 30 min at 4 °C, including dead cell dye
196 (Zombie Aqua, Biolegend Cat# 423102), CD19-FITC (Biolegend Cat# 152404, RRID:
197 AB_2629813), CD11b-PerCP/Cy5.5 (Biolegend Cat# 101228, RRID: AB_893232), CD11c-PE-
198 Cy7 (Biolegend Cat# 117318, RRID: AB_493568), Ly6c-APC-Fire750 (BioLegend Cat#
199 128046, RRID: AB_2616731), F4/80-BV421 (Biolegend Cat# 123137, RRID: AB_2563102),
200 Ly6G-PE (BioLegend Cat# 127608, RRID: AB_1186099), CD103-BV785 (Biolegend Cat#
201 121439, RRID: AB_2800588) and I-A/I-E-BV605 (Biolegend Cat# 107639, RRID:
202 AB_2565894). To measure antigen-specific T cell response, lymphocytes from mouse lung and
203 spleen were stimulated with 1 µg/mL SARS-CoV-2 RBD peptide pool (15-mer overlapping by
204 11, spanning the whole RBD sequence at Spike₃₀₆₋₅₄₃). Cells were incubated at 37°C overnight,
205 and BFA was added at 2 hours post-incubation. PMA/ionomycin stimulation was included as the
206 positive control. After overnight incubation, cells were washed with staining buffer (PBS
207 containing 2% FBS) and surface stained with anti-mouse-CD4-PerCP/Cy5.5 (Biolegend Cat#
208 116012, RRID: AB_2563023), anti-mouse-CD8-BV785 (Biolegend Cat# 100750, RRID:
209 AB_2562610), anti-mouse CD69-BV711 (Biolegend Cat# 104537, RRID: AB_2566120) and
210 anti-mouse CD103-BV421 (Biolegend Cat# 121422, RRID: AB_2562901). Zombie aqua
211 staining was used to exclude dead cells. For intracellular staining, cells were fixed and
212 permeabilized with BD Cytofix/Cytoperm (BD Biosciences) prior to staining with anti-mouse-
213 IFN-γ-APC (Biolegend Cat# 505810, RRID: AB_315404), anti-mouse-TNF-α-PE (Biolegend
214 Cat# 506306, RRID: AB_315427) and anti-mouse-IL-2-PE-Cy7 (Biolegend Cat# 503832, RRID:
215 AB_2561750). Stained cells were acquired by FACSARIAIII Flow Cytometer (BD Biosciences)
216 and analyzed with FlowJo software (v10.6) (BD Bioscience).

217

218 **2.9 Viral RNA quantification**

219 Half nasal turbinates and lung tissues were homogenized and subjected to viral load
220 determination by quantitative SARS-CoV-2-specific RdRp/Hel reverse-transcription polymerase

221 chain reaction assay (10). Total RNA was extracted using RNAeasy mini kit (Qiagen) and
222 followed by reverse transcription (PrimeScript II 1st Strand cDNA Synthesis Kit). The real-time
223 RT-PCR assay for SARS-CoV-2-RdRp/Hel RNA and NP subgenomic RNA detection was
224 performed using One-Step TB Green PrimeScript RT-PCR Kit II according to the manufacture's
225 instruction. Mouse β -actin was used as normalization.

226

227 **2.10 Plaque assay**

228 Infectious virus titration was determined by plaque assay. Confluent Vero-E6 cells in a 12-well
229 plate were incubated with 10-fold serially diluted tissue homogenates for 1 h. The virus
230 supernatant was discarded, and cells were then overlaid with 1% agarose in DMEM and further
231 incubated for 3 days at 37 °C followed by overnight fixation via 4% PFA. Agarose gels were
232 removed, and plaques were visualized by 1 % crystal violet.

233

234 **2.11 Histopathology and Immunofluorescence (IF) Staining**

235 Tissues collected at necropsy were fixed in zinc formalin and then processed into paraffin-
236 embedded tissue blocks. The tissue sections (4 μ m) were stained with hematoxylin and eosin
237 (H&E) for light microscopy examination. For identification and localization of SARS-CoV-2
238 nucleocapsid protein (NP) in organ tissues, IF staining was performed on deparaffinized and
239 rehydrated tissue sections using rabbit anti-SARS-CoV-2-N protein antibody together with
240 AF568-conjugated goat anti-rabbit IgG (Invitrogen). Briefly, the tissue sections were first treated
241 with antigen unmasking solution (Vector Laboratories) in a pressure cooker. After blocking with
242 0.1% Sudan black B for 15 min and 1% bovine serum albumin (BSA)/PBS at RT for 30 min, the
243 primary antibody rabbit anti-SARS-CoV-2-N antibody (1:4000 dilution with 1% BSA/PBS) was
244 incubated at 4°C overnight. This step was followed by AF568-conjugated goat anti-rabbit IgG
245 for 30 min and then mounted with 4',6-diamidino-2-phenylindole (DAPI). All tissue sections
246 were examined, the images were captured with a Carl Zeiss LSM780 confocal microscope, and
247 the mean fluorescence intensity (MFI) was further measured by ImageJ v1.53c.

248

249 **2.12 Statistical analysis**

250 Statistical analysis was performed with the GraphPad Prism 8 Software. Data represent mean
251 values or mean values with SEM. Significant differences between the means of multiple groups
252 were tested using a one-way analysis of variance (ANOVA) followed by Tukey's multiple

253 comparisons test. Significant differences between the two groups were performed using the 2-
254 tailed Student's t-test. $P < 0.05$ was considered statistically significant.

255

256 **2.13 Animal study approval**

257 All experimental procedures were approved by the Committee on the Use of Live Animals in
258 Teaching and Research (CULATR 5350-20) of the University of Hong Kong.

259

260 **2.14 Role of funding source**

261 The funders of this study had no role in study design, data collection, data analyses,
262 interpretation, or writing of the report.

263

264 **3. Results**

265 **3.1 Construction and characterization of PD1-based DNA and influenza-based vaccines**

266 To address the hypothesis, we sought to study an intramuscular program death 1 (PD1)-based
267 RBD DNA vaccine (PD1-RBD-DNA) and two intranasal live attenuated influenza virus (LAIV)-
268 based vaccines (LAIV-HK68-RBD and LAIV-CA4-RBD). Recently, Y Liu et al. demonstrated
269 that antibodies against the N-terminal domain (NTD) could enhance the binding capacity of the
270 spike protein to ACE2 and infectivity of SARS-CoV-2 (30). To avoid the potential full spike (S)-
271 associated side effects (22, 30, 31), we chose RBD as the vaccine immunogen . Since delayed
272 cytotoxic T lymphocyte (CTL) responses are likely associated with COVID-19 severity (12), we
273 constructed the PD1-RBD-DNA vaccine (Fig. S1A) that might elicit enhanced antibody and
274 CD8 T cell responses (23). The expression of soluble PD1-RBD protein (~80 kDa) was readily
275 detected in supernatants of transfected HEK293T cells by Western blot analysis using either anti-
276 SARS-CoV-2 RBD antibody (green lane) or anti-human PD-1 (red lane) antibody (Fig. S1B).
277 The released PD1-RBD was able to bind PD-L (Fig. S1C), which might help antigen targeting to
278 dendritic cells for cross-presentation (23). Meantime, the NS1-deleted (DelNS1) LAIV was
279 engineered to express the same RBD (Fig. S1D), aiming to induce mucosal NAb and T cell
280 immune responses (32). We previously characterized a panel of DelNS1 influenza viruses and
281 evaluated their potential for being used as vaccine vectors (32). Two intranasal recombinant
282 LAIV DelNS1-RBD vaccine strains, namely LAIV-CA4-RBD and LAIV-HK68-RBD, were
283 generated using A/CA/04/2009 (H1N1) and A/Hong Kong/1/68 (H3N2) surface proteins (HA

284 and NA) in the A/CA/04/2009 (H1N1) DeINS1 backbone by a reverse genetic procedure (24).
285 The SARS-CoV-2 RBD and LAIV NP proteins were stably expressed in MDCK cells by
286 Western blot after 5 times of viral passages in MDCK cells (Fig. S1E).

287

288 **3.2 Systemic and mucosal antibody responses of vaccine regimens**

289 We then chose the immune competent SARS-CoV-2/BALB/c mouse model (25), which was
290 based on available antibody reagents to understand the potential correlate of immune protection.
291 As compared with the vector control group (v1) that received 50 μ g intramuscular
292 electroporation (i.m./EP) pVAX plasmid prime plus i.n. 10^6 PFU LAIV-68 vector boost (Fig.
293 1A), we tested groups treated by the homologous 50 μ g i.m./EP PD1-RBD-DNA twice (v2), a
294 heterologous i.n. 10^6 PFU LAIV-CA4-RBD prime plus i.n. 10^6 PFU LAIV-HK68-RBD boost
295 (v3), a heterologous 50 μ g i.m./EP PD1-RBD-DNA prime plus i.n. 10^6 PFU LAIV-68-RBD
296 boost (v4) and a heterologous i.n. 10^6 PFU LAIV-CA4-RBD prime plus 50 μ g i.m./EP PD1-
297 RBD-DNA boost (v5) at 3-week intervals, consistent with COVID-19 vaccines under emergency
298 use. Vaccine-induced antibody responses were determined at day 9 (acute phase) and day 69
299 (memory phase) post the second vaccination. Both peripheral blood and bronchoalveolar lavage
300 (BAL) samples were collected from vaccinated mice for antibody detection by ELISA and
301 pseudovirus neutralizing assays. We found that the PD1-RBD-DNA/LAIV-HK68-RBD regimen
302 (v4) elicited and significantly sustained the highest amounts of RBD-specific IgG (acute: mean
303 5.2, range 4.86-5.45 logs AUC; memory: mean 4.62, range 4.54-4.69 logs AUC) and NAb
304 (acute: mean 4.19, 4.04-4.34 logs IC_{50} ; memory: mean 2.89, 2.4-3.23 logs IC_{50}) in sera during
305 both acute and memory phases as compared with other groups (Fig. 1B-C). The RBD-specific
306 IgG titer and NAb IC_{50} values were correlated positively (Fig. 1D), similar to COVID-19
307 patients' sera (8). Moreover, v4 animals also developed and sustained significantly higher
308 amounts of RBD-specific mucosal IgG and IgA in BAL during both acute and memory phases as
309 compared with other groups (Fig. 1E-F). The amount of BAL NAb in v4 mice were at mean
310 2.80 (range 2.22-2.87) and mean 2.59 (range 2.02-3.15) logs IC_{50} at the acute and memory phase,
311 respectively (Fig. 1G). In contrast, v2 and v5 elicited similar amounts of NAb to v4 at the acute
312 phase, yet these responses did not sustain into the memory phase. Moreover, despite
313 heterologous intranasal immunizations twice, the v3 regimen did not induce equally potent and
314 sustained mucosal IgG and IgA as well as NAb responses, as compared with the v4 group (Fig.

315 1G). Both BAL IgG and IgA titers correlated positively with the BAL NAb values (Fig. 1H)
316 despite of the higher amount and better acute/memory of BAL IgG than BAL IgA. Interestingly,
317 serum NAb IC₅₀ values were correlated positively with the BAL NAb IC₅₀ values at the memory
318 phase, but not correlated at the acute phase (Fig. 1I). These results indicated that the v4 regimen
319 is likely unique for inducing potent and sustained systemic and mucosal memory IgG/IgA NAb
320 responses.

321 322 **3.3 Acute and memory T cell responses of vaccine regimens**

323 Since SARS-CoV-2-specific T cell responses are essential for control and resolution of viral
324 infection (12, 33), we sacrificed five groups of animals to measure vaccine-induced T cell
325 immune responses at day 9 (acute phase) and day 69 (memory phase) post the second
326 vaccination. Lymphocytes were isolated from both lungs (effector site) and spleens (secondary
327 lymph organ) of vaccinated mice for comparison. We found that the v4 regimen elicited and
328 sustained significantly higher frequencies of RBD-specific IFN- γ ⁺ CD8 T cells in lungs and
329 spleens during both acute (Fig. 2A-B, Fig. S2A-B) and memory (Fig. 2D-E, Fig. S2D-E) phases
330 as compared with other groups. Similar trends were found with IFN- γ ⁺ CD4 T cells elicited in
331 the v4 regimen but at lower frequencies. At the acute phase, the v4 regimen elicited the highest
332 mean frequency of RBD-specific IFN- γ ⁺ CD8 T cells (mean 28.83%, range 22-34.8%) in lungs
333 (Fig. 2B), which was even higher than that in spleens (mean 5.54%, range 3.63-7.06%) (Fig.
334 S2B). These cells included the highest frequencies of polyfunctional CTLs with a capacity of
335 releasing two (mean 21.09%, range 13.87-25.31%) or three (mean 2.27%, range 1.02-3%)
336 cytokines (Fig. 2C), which was also higher than those in splenic CD8 T cells releasing two
337 (mean 6.68%, range 4.68-9.17%) or three cytokines (mean 0.92%, range 0.7-1.36%) (Fig. S2C).
338 At the memory phase, the v4 regimen sustained the highest mean frequency of RBD-specific
339 IFN- γ ⁺ CD8 T cells in lungs (mean 6.11%, range 2.05-9.7%) (Fig. 2E) and spleens (mean 2.35%,
340 range 0.7-4.78%) (Fig. S2E) as compared with other groups. These cells included the highest
341 frequencies of polyfunctional CTLs with a capacity of releasing two (mean 8.66%, range 2.61-
342 12.41%) or three (mean 2.2, range 0.66-3.09%) cytokines (Fig. 2F), which was higher than those
343 in splenic CD8 T cells releasing two (mean 3.59%, range 1.28-6.83%) or three cytokines (mean
344 0.69%, range 0.31-1.17%) (Fig. S2F). These results demonstrated that besides Nabs, the v4
345 regimen also induced potent and polyfunctional memory CD8 T cell responses, especially in

346 lungs. Since overall immune responses induced by the heterologous v3 regimen were much
347 weaker than those by the v4 regimen, we also examined T cell responses against influenza
348 immunodominant nucleoprotein (NP) (34). At acute phase (Fig. S3A), the v3 regimen induced
349 the highest frequencies of CD8 T cell response against influenza NP in lungs (mean 19.55%,
350 range 16.2-21.7%) as compared with v1 (mean 8.3%, range 7.77-8.9%) and v5 (mean 3.99%,
351 range 2.55-5.91%). Similar results were observed at the memory phase (Fig. S3B). In contrast,
352 the v4 regimen induced significantly lower influenza NP-specific T cell response at both acute
353 (mean 2.26%, range 1.68-2.58%) and memory (mean 0.77%, range 0.49-1.06%) phases. The
354 heterologous prime using PD1-RBD-DNA instead of LAIV-CA4-RBD, therefore, offered an
355 advantage in promoting the RBD immunodominance likely by avoiding anti-vector immune
356 responses.

357

358 **3.4 Protective efficacy against intranasal SARS-CoV-2 infection**

359 To investigate the efficacy of various vaccine regimens against the live intranasal SARS-CoV-2
360 challenge, we subsequently immunized additional groups of BALB/c mice (n=6 per group) using
361 the same doses and time interval as described above (Fig. 3A). We did not include v5 due to low
362 mucosal immunogenicity and limited space in our animal P3 facility. Sera were collected at day
363 9 and day 28 after the 2nd immunization to monitor anti-RBD IgG (Fig. 3B) and neutralization
364 (Fig. 3C). Consistently, the highest IgG (acute: mean 4.79, range 4.7-4.89 logs AUC; memory:
365 mean 2.6, range 2.03-2.81 logs AUC) and neutralizing (acute: mean 3.9, range 3.69-4.14 logs
366 IC₅₀; memory: mean 2.94, range 2.34-3.33 logs IC₅₀) titers were induced in mice by the v4
367 regimen at both acute and memory phases. Immunized mice were then transduced with Ad5-
368 hACE2 at the memory phase, 29 days post the boost vaccination for expressing human ACE2 in
369 nasal turbinate and lung (Fig. 3D), followed by the intranasal SARS-CoV-2 challenge 6 days
370 later as previously described (25). At day 4 after the viral challenge, mice were sacrificed for
371 analysis. Lung specimen was harvested to quantify infectious viruses by plaque assay, viral load
372 by real-time PCR (RT-PCR) and infected cells by immunofluorescence staining (IF). We found
373 that all vaccinated animals had decreased infectious plaque-forming units (PFU) to the limit of
374 detection (10 PFU/mL) in lungs (Fig. 3E). The v4 regimen, however, resulted in the most
375 significant genomic RdRp (gRdRp) drop in lungs by an average of 2.31 logs compared with 1.81
376 logs in v2 mice and 1.62 logs in v3 mice (Fig. 3F). A similar observation was found in the

377 measurement of nucleocapsid protein (NP) subgenomic RNA (sgNP) (Fig. 3G). These findings
378 demonstrated that immune responses induced by v2, v3 and v4 regimens had achieved
379 significant protection in lungs. To determine viral infection in both upper and lower respiratory
380 systems, we further performed immunofluorescence staining of SARS-CoV-2 NP antigen in both
381 lung (Fig. 3H) and NT (Fig. 3I) tissues. Since murine NT was too small to be sliced for viral load
382 tests, we only used it for the NP staining to maintain the necessary tissue structure. While
383 significantly reduced NP⁺ cells were observed in lungs of v2 and v3 mice, infected cells were
384 barely found in lungs of v4 mice. Furthermore, no significantly reduced NP⁺ cells were found in
385 NT of v2 and v3 mice as compared with v1 mice, but only a few NP⁺ cells were seen in v4 mice.
386 Our results demonstrated that while protection was consistently found in lungs of vaccinated
387 animals, significant prevention of robust SARS-CoV-2 infection in NT was only achieved by the
388 v4 regimen.

389

390 **3.5 Infection-recalled NAb for correlate of protection**

391 Although we were not allowed to bring tissue specimens for measuring T cell immunity outside
392 the animal P3 laboratory after the SARS-CoV-2 challenge, we managed to determine if viral
393 infection recalled vaccine-induced NAbs for viral neutralization and clearance (35). By testing
394 RBD-specific IgG and NAb at day 28 (before challenge) and day 39 (also 4 dpi) after the 2nd
395 vaccination, we found that SARS-CoV-2 infection indeed recalled significantly anti-RBD IgG
396 responses in all v2, v3 and v4 animals (Fig. S4A). Notably, 62.2-fold and 65.7-fold higher
397 amounts of recalled NAb were found by the v4 regimen (v4, mean 4.64, range 3.22-5.03 logs
398 IC₅₀) than the v2 and v3, respectively (Fig. S4B). Furthermore, there were significant negative
399 correlations between viral loads and IgG or NAb IC₅₀ values (Fig. S4C-D), as well as between
400 NP⁺ cells and IgG or NAb IC₅₀ values in lungs (Fig. S4E-F) and in NT (Fig. S4G-H). Moreover,
401 since the T_H1 immune response is likely associated with protective immune responses against
402 COVID-19 (36), we also evaluated the ratios of IgG1 and IgG2a in all vaccinated mice. The v4
403 regimen likely induced a T_H1 bias with a higher IgG2a/IgG1 ratio (mean 59.57, range 1.44-
404 279.2) than those induced by v2 (mean 6.72, range 2.63-15.14) and v3 (mean 7.65, range 4.25-
405 12.72) (Fig. S4I). Our results demonstrated that v4 vaccine-induced high amounts of NAbs
406 correlated with protective efficacy in both NT and lung.

407

408 **3.6 SARS-CoV-2 prevention in both upper and lower respiratory tracts of K18-hACE2** 409 **mice**

410 To further determine the vaccine efficacy, we tested the PD1-RBD-DNA/LAIV-HK68-RBD
411 regimen in K18-hACE2 transgenic mice, one of commonly used animal models for studying
412 COVID-19 (37-39). 8-week-old K18-hACE2 mice were vaccinated with various regimens using
413 the doses and time interval as described above (Fig. 4A). The titer of RBD-specific IgG (Fig. 4B)
414 and neutralization antibody (Fig. 4C) was measured in serum at day-9 and day-33 (acute phase)
415 after the 2nd immunization. Consistently, the PD1-RBD-DNA/LAIV-HK68-RBD regimen (v4)
416 induced and sustained the highest amount of RBD-specific serum IgG (acute: mean 4.38, range
417 3.81-4.84 logs AUC; memory: mean 4.79, range 4.27-5.25 logs AUC) and Nabs (acute: mean
418 3.74, range 3.10-4.30 logs IC₅₀; memory: mean 4.02, range 2.48-4.64 logs IC₅₀) during both
419 acute and memory phases as compared with other groups. At day 38 after the 2nd immunization,
420 mice were intranasally challenged with 10⁴ PFU of SARS-CoV-2 (HKU Clone 13). In lungs, no
421 measurable infectious viruses (detection limit: 10 PFU/mL) were found in all v4 mice and 40%
422 v2 mice, whereas infectious viruses were detected in all v1 and v3 mice (Fig. 4D). The more
423 sensitive RT-PCR further demonstrated that the v4 regimen resulted in the significant gRdRp
424 drop by an average of 3.57 logs as compared with 0.81 logs in v2 mice and 0.25 logs in v3 mice
425 (Fig. 4E) at 4 dpi. A similar observation was found with the measurement of NP subgenomic
426 RNA (Fig. 4F). Importantly, no measurable infectious viruses were detected in nasal turbinate of
427 v4 mice (Fig. 4G) and the v4 regimen significantly suppressed viral genomic/subgenomic RNA
428 (gRdRp and sgNP) (Fig. 4H-I). Further IF staining of NP antigen confirmed that significantly
429 reduced NP⁺ cells were observed in both lungs (Fig. 4K) and nasal turbinates (Fig. 4L) of v4
430 mice as compared with other regimens. Daily monitoring of body weight after challenge showed
431 that SARS-CoV-2 infection caused average 15%, 8% and 6% weight loss in v1, v2 and v3 mice
432 at 4 dpi, respectively. In contrast, no weight loss was found in v4 mice during the experimental
433 period (Fig. 4J). To further characterize the injury of lung and nasal turbinate, we performed
434 pathological analysis on specimens by haematoxylin and eosin (H&E)-staining. Acute lung
435 injury with peribronchiolar infiltration, alveolar space infiltration and exudation were visualized
436 in v1 mice, while v2 and v3 mice showed less alveolar space infiltration. In contrast, only mild
437 peribronchiolar infiltration was observed in v4 mice (Fig. 4M). In nasal turbinate, damage of
438 epithelium with extensive submucosal immune cell infiltration was observed in v1, v2 and v3
439 mice, but not in v4 mice (Fig. 4N). Consistent results were found in the flow cytometry analysis

440 of immune cell compositions in lungs. A significant decrease in frequency of alveolar
441 macrophages as well as reduced CD103⁺ DCs and CD11b⁺ DCs (Fig. S5A-B) was found in the
442 v4 mice as compared with other groups. These results in K18-hACE2 mice, therefore, further
443 demonstrated that the PD1-RBD-DNA/LAIV-HK68-RBD regimen prevents robust SARS-CoV-
444 2 infection not only in lung and but also in NT with minimal infection-associated inflammation
445 and injury.

446

447 **3.7 Both NAb and antigen-specific CD8 T cells associated with virus control in K18-hACE2** 448 **mice**

449 Compare to the serum collected at day 5 before the challenge, the titer of RBD-specific IgG (Fig.
450 5A) and NAb (Fig. 5B) did not increase in sera collected at 4 dpi. We then assessed the T cell
451 responses in lungs by RBD peptide re-stimulation, and a significant increase in IFN- γ producing
452 CD8 T cells (mean 8.45%, range 6.76-10.4%) was observed in the lungs of the mice that
453 received PD1-RBD-DNA prime/LAIV-HK68-RBD boost regimen (Fig. 5C). Tissue-resident
454 memory T cells (TRM) is a well-known antiviral T cell type that persists at mucosal sites (40).
455 We found that a higher frequency of TRM cells (CD69⁺CD103⁺) (mean 30.1, range 17.6-40.9%)
456 was identified in lung CD8 T cells in v4 mice (Fig. 5D). Moreover, a higher frequency of IFN-
457 γ ⁺ TRMs (mean 21.64%, range 17.1-26.3%) was found in v4 mice after RBD peptide re-
458 stimulation (Fig. 5E). Correlation analysis showed negative correlations between the NAb titer
459 and viral load (RdRp RNA/NP⁺ cells) and between the frequency of IFN- γ ⁺ CD8 T cells and
460 viral load in both lungs (Fig. 5F) and nasal turbinate (Fig. 5G), respectively, indicating their
461 involvements in viral control.

462

463 **3.8 LAIV-CA4-RBD boosted the immunogenicity of vaccines in emergency use**

464 While we were studying the PD1-RBD-DNA/LAIV-HK68-RBD regimen, both the BioNTech
465 mRNA vaccine and the Sinovac inactivated vaccine have been approved for emergency use in
466 Hong Kong. Our LAIV-CA4-RBD vaccine is currently undergoing the phase I clinical trial in
467 Hong Kong (ClinicalTrials.gov Identifier: NCT04809389). Since intramuscular administration of
468 mRNA or inactivated vaccine induces mainly systemic IgG and some T cell immune responses
469 without secretory IgA or tissue-resident memory T cells (41, 42), we sought to determine if

470 LAIV-CA4-RBD could boost their mucosal immunogenicity to provide the implication for
471 clinical use. We, therefore, immunized additional groups of BALB/c mice as v7, v9, v8, v10
472 with a homologous *i.m.* BioNTech (1/5 of clinical dose), a homologous *i.m.* Sinovac (1/5 of
473 clinical dose), a heterologous *i.m.* BioNTech plus 10^6 PFU LAIV-CA4-RBD, a heterologous *i.m.*
474 Sinovac plus 10^6 PFU LAIV-CA4-RBD at a 3-week interval, respectively. The selection of the
475 1/5 of clinical dose was based on animal studies described previously (43). The control mice in
476 v6 group were immunized by 50 μ g *i.m.*/EP PD1-RBD DNA plus LAIV-CA4-RBD as before
477 (Fig. 6A). At day 12 after the 2nd vaccination, serum and BAL were collected and subjected to
478 detection of RBD-specific IgG and IgA as well as neutralization activity. Interestingly, v8 and
479 v10 mice did not show increased titers of IgG and Nab in sera (Fig. 6B, E), but had significantly
480 increased titers of IgG (Fig. 6C), IgA (Fig. 6D) and Nab (Fig. 6F) in BAL as compared with v7
481 and v9. We then assessed T cell responses in lungs by RBD peptide re-stimulation. A significant
482 increase of IFN- γ producing CD8 T cells (mean 55.58%, range 44.9-65.1% in v8 and mean
483 2.16%, range 1.5-2.64% in v10) was found in mice that received the heterologous boost of
484 LAIV-HK68-RBD than mice received homologous BioNTech or Sinovac (mean 22.14%, range
485 8.89-30.8% in v7 and mean 0.37, range 0.1-0.66% in v9) (Fig. 6G). Furthermore, more total
486 TRMs and RBD-specific TRMs were induced in v8 and v10 as compared with v7 and v9 (Fig.
487 6G-H). These results demonstrated that the intranasal LAIV-CA4-RBD boost induced stronger
488 humoral and cellular immune responses in mucosal sites than systemic vaccinations alone.

489

490 **3.9 Vaccine-induced NAbS cross-neutralize global SARS-CoV-2 variants of concern**

491 Recently emerged SARS-CoV-2 variants Alpha (B.1.1.7) from UK, Beta (B.1.351) from South
492 Africa and Delta (B.1.167) from India have become challenges for passive immunotherapy and
493 vaccine-induced protection (19, 44). For example, the Novavax vaccine is effective against the
494 wildtype SARS-CoV-2 (95.6%) but provides reduced protection against the variants Alpha
495 (85.6%) and Beta (60%) (45). We, therefore, compared the neutralizing activity of immune sera
496 from PD1-RBD-DNA/BioNTech/Sinovac prime and LAIV-CA4-RBD boosted mice (Fig. 6)
497 against pseudotyped viruses that contain the D614G, the Alpha, the Beta and the Delta variants
498 (Fig. 7A-B) as described previously (19). Compared to the D614G viral strain, v6 sera showed
499 slightly enhanced neutralizing activity against the Alpha variant, while the sera of other groups
500 exhibited reduced neutralization against the Alpha, Beta and Delta variants (Fig. 7B). In line

501 with a recent studies (19), the Beta and Delta variants were more resistant to neutralization by
502 sera from all vaccine regimens with an average fold reduction of 1.5-1.77 (v6), 3.00-3.60 (v7),
503 1.98-2.03 (v8), 1.56-2.45 (v9) and 1.26-1.59 (v10) as compared to the D614G strain, respectively
504 (Fig. 7B). Although animals in the LAIV-CA4-RBD boost regimens (v6, v8 and v10) showed
505 the mean 1.69-fold reduction against Beta or Delta variants, their mean NAb IC₅₀ titer of 1: 3633
506 (v6, range 1:1586-1:8250), 1:4370 (v8, range 1:2305-1:9068) and 1:3929 (v10, range 1:189-
507 14393) remained high, respectively, which was superior or comparable to those of homologous-
508 vaccinated mice as well as to the results of clinical vaccines against the wild type virus in murine
509 models (6, 46, 47). Importantly, the BAL from LAIV-CA4-RBD boost groups (v6, v8 and v10)
510 were still able to neutralize the Beta and Delta variants (v6: mean 453, range 117-762; v8: mean
511 280, range 93-465; v10: mean 281, range 50-760) although they showed the average fold
512 reduction of 2.64-2.90 (v6), 1.7-4.3 (v8) and 1.26-2.28 (v10) as compared to the D614G strain,
513 respectively (Fig. 7C-D). These results demonstrated that the systemic prime/LAIV boost
514 regimen-induced high amounts of systemic and mucosal NAbs may confer cross-protection
515 against the variants of concern before the tailor-made vaccines become available.

516

517 **4. Discussion**

518 To control the ongoing COVID-19 pandemic, vaccine-induced protective immune responses
519 should prevent SARS-CoV-2 nasal infection effectively to eliminate viral transmission between
520 humans (17, 41). Failure of protection against SARS-CoV-2 in NT may allow asymptomatic
521 viral spread and there have been over 10 thousand cases of vaccine breakthrough infections in
522 the United States (45, 48). To date, little is known about the correlate of vaccine-induced
523 mucosal protection for the prevention of intranasal SARS-CoV-2 infection in animal models (15).
524 In this study, we demonstrated that the intranasal LAIV-based vaccination is critical for boosting
525 the systemic PD1-RBD-DNA vaccine prime for effective prevention of SARS-CoV-2 infection
526 in both NT and lungs by inducing high amounts of mucosal IgA/IgG and polyfunctional memory
527 CD8 T cells. Consistent with previous findings that a heterologous prime and boost vaccine
528 regimen may induce stronger immune responses (49, 50), our results on effective prevention of
529 intranasal SARS-CoV-2 infection in NT would warrant the upcoming clinical development of
530 PD1-based and LAIV-based COVID-19 vaccines (ClinicalTrials.gov Identifier: NCT04809389).
531 Our findings may have significant implications to use LAIV-CA4-RBD for boosting the current

532 vaccines under emergency use especially the extensively used nucleic acid-based COVID-19
533 vaccines (e.g. BioNTech mRNA).

534

535 The induction of mucosal immune responses is essential for preventing SARS-CoV-2
536 transmission effectively (39, 41). There are, however, some challenges underlying the prevention
537 of SARS-CoV-2 in NT. First, SARS-CoV-2 exhibited a rapid burst of viral replication in NT (1),
538 which has not been previously found with SARS-CoV (9, 51). Second, after viral entry, ciliated
539 nasal epithelial cells with the highest expression of ACE2 and TMPRSS2 may facilitate more
540 efficient cell-cell transmission (8), which probably makes it difficult for systemic NAb to block.
541 Third, NAb by systemic vaccination or passive immunization are less distributed on the surfaces
542 of NT for the prevention of SARS-CoV-2 (17). Due to these challenges, it is critical to
543 investigate vaccine-induced mucosal NAb and CD8 T cells for SARS-CoV-2 prevention in NT.
544 Thus far, most published vaccines have indicated excellent protective efficacy in the lungs of
545 vaccinated animals without detailed analysis in NT (52-54). Several previous studies had shown
546 no significant viral load drops in nasal swabs or nasal turbinates of vaccinated animals (6, 7, 43,
547 55, 56). One study found that optimal protection was achieved by only the DNA vaccine
548 encoding the full S protein in both the upper and lower respiratory tracts against SARS-CoV-2 in
549 rhesus macaques when vaccinated animals developed pseudovirus neutralizing IC₅₀ titer less
550 than 1:1000 (57). Serum NAb titers, as measured by both pseudovirus and live virus
551 neutralization, served as a significant correlate of protection. Similar protection in both the upper
552 and lower respiratory tracts was achieved by the AD26 vaccine encoding the full S protein
553 against SARS-CoV-2 in rhesus macaques in another study (56). Both studies, however, used the
554 macaque model that requires inoculation of 10⁵ TCID₅₀ SARS-CoV-2 into each nare and
555 intratracheal for effective infection, which is not natural and is in great contrast to the robust
556 nature of NT infection in humans. Using a DNA vaccine encoding the full S immunogen, we
557 recently reported that there was significant protection in the lung but not in NT against SARS-
558 CoV-2 in Syrian hamsters even though the vaccinated animals developed pseudovirus
559 neutralizing IC₅₀ titer larger than 1:1000 (17). In this study, we consistently found that significant
560 prevention of NT was not achieved by two systemic PD1-RBD-DNA vaccinations in both
561 hACE2-transduced mice and K18-hACE-2 mice.

562

563 For direct mucosal vaccination, a single-dose intranasal ChAd vaccine exhibited a significant
564 reduction of viral loads in NT, yet the extent of infected cells in NT was not determined, and its
565 potential for extensive human use remains unclear (39). Here, we showed the highest frequency
566 of RBD-specific tissue-resident memory CD8 T cells in the respiratory system was found by the
567 intranasal boost of LAIV-based vaccine after intramuscularly prime with various systemic
568 vaccines (e.g. PD1-RBD-DNA, Pfizer/BioNTech and Sinovac). Moreover, besides RBD-specific
569 tissue-resident memory CD8 T cells, we also observed the positive correlation between NAb IC₅₀
570 values and BAL NAb IC₅₀ values, and the negative correlation between NAb IC₅₀ values and
571 NP⁺ cells, especially in NT. It is encouraging to develop the LAIV-RBD vaccine as the boost
572 vaccination combined with the prime of vaccines that were authorized for emergency use to
573 achieve mucosal immunity against SARS-CoV-2. Furthermore, the role of the intranasal LAIV-
574 RBD boost route in optimizing the extent and localization of humoral and cellular immune
575 responses in mucosal site combined with systemic prime immunization is not clear yet and will
576 be determined in the future study. For example, whether the intranasal LAIV-RBD boost can
577 potentially promote the antigen-specific T follicular helper cell responses as well as elicit the potent
578 germinal center reaction for neutralizing antibody production in the mucosal site. Secondly, it is
579 crucial to determine how the intranasal LAIV boost develops the antigen-specific tissue resident
580 memory T cells which are not only destroy the infected cells but also recruit the innate and
581 adaptive immune cells into the infected tissues via cytokines and chemokines (58).

582
583 In summary, our finding of prevention of NT against SARS-CoV-2 by the high amount and long-
584 lasting mucosal NAbs and CD8 T cells induced by the heterologous PD1-RBD-DNA/LAIV-
585 HK68-RBD regimen may have significant implication for COVID-19 pandemic control because
586 of the existing mass-production industry for influenza vaccines. Meantime, one COVID-19 DNA
587 vaccine has been approved for emergency use in India with promising results (59). Our PD1-
588 RBD-DNA vaccine may prime stronger mucosal CD8 T cells responses besides benefits of
589 inducing potent NAbs, better stability than mRNA vaccine, fast-tracked development and cost
590 effective GMP production (60). Although intramuscular electroporation delivery has potential
591 limitation for DNA vaccination in large populations, this approach has demonstrated the safety,
592 tolerability and immunogenicity profile for SARS-CoV-2 DNA vaccines in clinical trials (61).
593 Future study, however, is needed to develop non-invasive delivery techniques for DNA
594 vaccination in humans. In addition, simultaneous or sequential co-infection by SARS-CoV-2 and

595 A(H1N1)pdm09 caused more severe disease than infection by either virus (62), our LAIV
596 platform may offer an opportunity of generating a human vaccine to fight both COVID-19 and
597 influenza.

598

599 **Contributors**

600 Z.C. and H.C. supervised two collaborative teams, conceived of and designed the study, and
601 wrote the manuscript. R.Z., P.W., Y.-C.W and H.X. designed the experiments, analysed the data,
602 and prepared the manuscript. R.Z., P.W., Y.-C.W., H.X., S-. Y.L., B. W.-Y.M., K. F. W., H.H.,
603 R.C.-Y.T., and S.D. conducted the immunologic and virologic assays. L.L., A.Z. and D.Z.
604 performed the immunofluorescence staining of animal tissue section. A.J.Z. analysed tissue
605 pathology. Q.P., N.L., B.Z., C.-Y.C and D.Y. conducted the ELISA and neutralization assay,
606 Z.D. and K.-K.A. performed the viral RNA measurement. K.-Y.Y. provided critical comments,
607 supports and materials. Z.C. and R.Z. have accessed and verified the underlying data reported in
608 the manuscript.

609

610 **Declaration of Interests**

611 H.C., Z.C. and KY.Y. are co-inventors of PD1-based and LAIV-based COVID-19 vaccine patent.
612 YC. W. and L.L are co-inventors of PD1-based COVID-19 vaccine, P.W. is co-inventors of
613 LAIV-based COVID-19 vaccine. The other authors declare no competing interests.

614

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620

621 **Data Sharing Statement**

622 The authors declare that the data supporting the findings of this study are available from the
623 corresponding author upon request.

624

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- 791

792 **Figure Legends**

793 **Fig. 1. Vaccine-induced systemic and mucosal antibody responses.** (A) Vaccine
794 immunization schedule and grouping for BALB/c mice (n=8/group). Blood and bronchoalveolar
795 lavage (BAL) were collected and subjected to antibody response analysis at 9 (acute phase,
796 n=4/group) or 69 (memory phase, n=4/group) days post the 2nd immunization, respectively. In
797 sera, (B) RBD-specific IgG titer, (C) NAb IC₅₀ values and (D) positive correlations between
798 RBD-specific IgG titer and NAb IC₅₀ values were analyzed for both acute and memory phases.
799 In BAL, (E) RBD-specific IgG titer, (F) RBD-specific IgA titer, (G) NAb IC₅₀ values and (H)
800 positive correlations between RBD-specific IgG (square) or IgA titers (triangle) and BAL NAb
801 IC₅₀ values were analyzed for both acute and memory phases. (I) Correlate analysis between
802 Peripheral NAb IC₅₀ values and BAL NAb IC₅₀ values at both acute and memory phase. RBD-
803 specific IgG or IgA titers were determined by ELISA at serial dilutions. The area under the curve
804 (AUC) represented the total peak area calculated from ELISA OD values. Neutralization IC₅₀

805 values were determined against SARS-CoV-2-Spike-pseudovirus infection of 293T-huACE2
806 cells. Correlation analysis was performed by linear regression using GraphPad Prism 8.0. Each
807 symbol represents an individual mouse with color-coding for corresponding groups. Error bars
808 indicate the standard error of the mean. Statistics were generated using one-way ANOVA
809 followed by Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$.

810

811 **Fig. 2. Vaccine-induced acute and memory T cell responses in lungs.** The vaccine
812 immunization schedule for BALB/c mice was the same as described in Fig. 1A. Cells from lungs
813 were collected and subjected to SARS-CoV-2 RBD-specific T cell responses by *ex vivo* RBD
814 peptide pool stimulation followed by ICS at both acute (**A-C**) and memory (**D-F**) phase. (**A, D**)
815 Representative dot plots present the gating of IFN- γ^+ , TNF- α^+ or IL-2 $^+$ CD4 T (left) and CD8 T
816 (right) against SARS-CoV-2 RBD. (**B, E**) Quantified results depict the percentage of IFN- γ^+
817 CD4 T (left) and IFN- γ^+ CD8 T (right). Each symbol represents an individual mouse. Error bars
818 indicate the standard error of the mean. (**C, F**) The pie charts indicate the proportion of single or
819 double or triple cytokines produced by CD4 T (left) and CD8 T (right). Statistics were generated
820 using one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$;
821 *** $p < 0.001$.

822

823 **Fig. 3. Protective efficacy of vaccine regimens in huACE2-transduced mice.** (**A**)
824 Experimental schedule and grouping of BALB/c mice (6 mice/group). At day 29 post the 2nd
825 immunization, mice were transduced to express human ACE2 in vivo by inoculating 4×10^8
826 plaque-forming units (PFU) Ad5-hACE2 intranasally. Six days later, mice were challenged
827 intranasally with 10^5 PFU SARS-CoV-2 and sacrificed at day 4 post-infection. (**B, C**) Serum
828 samples were collected for detection of anti-RBD IgG (**B**) and neutralizing antibody (**C**) against
829 pseudovirus, respectively, at day 9 (acute, left) and day 28 (memory, right) post the 2nd
830 immunization. The Area under the curve (AUC) represents the total peak area calculated from
831 ELISA OD values. (**D**) Human ACE2 expression (green) after the Ad5-hACE2 transduction was
832 evaluated in NT and lung by immunofluorescence (IF) staining using the hACE2-specific
833 antibody at day-6 post-transduction. With nuclei counter staining (blue), images were shown at
834 the 10 \times magnification. (**E**) A viral plaque assay was used to quantify infectious viruses in lung
835 homogenates. Log₁₀-transformed plaque-forming units (PFU) per mL of tissue extractions were

836 shown for each group. LOD: limit of detection. (F, G) Sensitive RT PCR was used to quantify
837 SARS-CoV-2 RdRp RNA (F) and NP subgenomic RNA (G) copy numbers (normalized by β -
838 actin) in lung homogenates. Confocal images showed SARS-CoV-2 NP positive (red) cells (20 \times)
839 in lungs (H) and NT (I) in the bright field. Mean fluorescent intensities (MFI) of NP⁺ cells in
840 lung and NT were measured using ImageJ software and plotted with GraphPad prism. Each
841 symbol represents an individual mouse with consistent color-coding. Error bars indicate the
842 standard error of the mean. Statistics were generated using one-way ANOVA followed by
843 Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

844

845 **Fig. 4. PD1-RBD-DNA/LAIV-HK68-RBD regime prevents SARS-CoV-2 infection in the**
846 **upper and lower respiratory tracts of hACE2 transgenic mice.** (A) Experimental schedule
847 and grouping of K18-hACE-2 mice (4 mice in v1 group and 5 mice in other groups). (B, C)
848 Serum samples were collected for detection of anti-RBD IgG (B) and neutralizing antibody (C)
849 against pseudovirus, respectively, at day 9 (acute, left) and day 33 (memory, right) post the 2nd
850 immunization. The Area under the curve (AUC) represents the total peak area calculated from
851 ELISA OD values. A viral plaque assay was used to quantify infectious viruses in lung (D) and
852 NT (G) homogenates. Log₁₀-transformed plaque-forming units (PFU) per mL of tissue
853 extractions were shown for each group. LOD: limit of detection. Sensitive RT PCR was used to
854 quantify SARS-CoV-2 RdRp RNA (E, H) and NP subgenomic RNA (F, I) copy numbers
855 (normalized by β -actin) in lung and NT homogenates. (J) Daily body weight was measured after
856 infection. Differences between groups that were given the different vaccine regime versus PBS
857 were determined using a 2-tailed Student's t test. Confocal images showed SARS-CoV-2 NP
858 positive (red) cells in lungs (10 \times) (K) and NT (20 \times) (L) in the bright field. Mean fluorescent
859 intensities (MFI) of NP⁺ cells in lung and NT were measured using ImageJ software and plotted
860 with GraphPad prism. Representative images of animal lung (M) and NT (N) tissues by H&E
861 (20 \times). Each symbol represents an individual mouse with consistent color-coding. Error bars
862 indicate the standard error of the mean. Statistics were generated using one-way ANOVA
863 followed by Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

864

865 **Fig. 5. Robust NAb and antigen-specific T cells were responsible for virus control in K18-**
866 **hACE2 mice.** Blood samples were collected at 33 and 42 days (also 4 dpi) after the 2nd
867 vaccination for analysis. (A, B) The mean \pm SEM changes of anti-RBD IgG AUC titer (A) and

868 neutralizing IC₅₀ values (**B**) were determined by anti-RBD IgG ELISA and pseudovirus assay,
869 respectively. (**C**) Lung CD8 T cells were assayed for IFN- γ expression by flow cytometry after
870 re-stimulated with the RBD peptide pool. Tissue-resident memory CD8 T cells (TRM) in lungs
871 were phenotyped for expression CD69 and CD103 (**D**) and the IFN- γ producing TRM were
872 measured by re-stimulated with the RBD peptide pool (**E**). (**F**) Viral load (relative gRdRp) in
873 lungs correlated with NAb IC₅₀ titer or percentage of IFN- γ producing CD8 T cells. NP⁺ cells
874 (MFI) in lungs correlated with NAb IC₅₀ titer or percentage of IFN- γ producing CD8 T cells. (**G**)
875 Viral load (relative gRdRp) in NT correlated with NAb IC₅₀ titer or percentage of IFN- γ
876 producing CD8 T cells. NP⁺ cells (MFI) in NT correlated with NAb IC₅₀ titer or percentage of
877 IFN- γ producing CD8 T cells. Correlation analysis was performed by linear regression using
878 GraphPad Prism 8.0. Each color represents a vaccination regimen. Each symbol represents an
879 individual mouse. Statistics were generated using the 2-tailed Student's t test. *p<0.05; **p<0.01;
880 ***p<0.001.

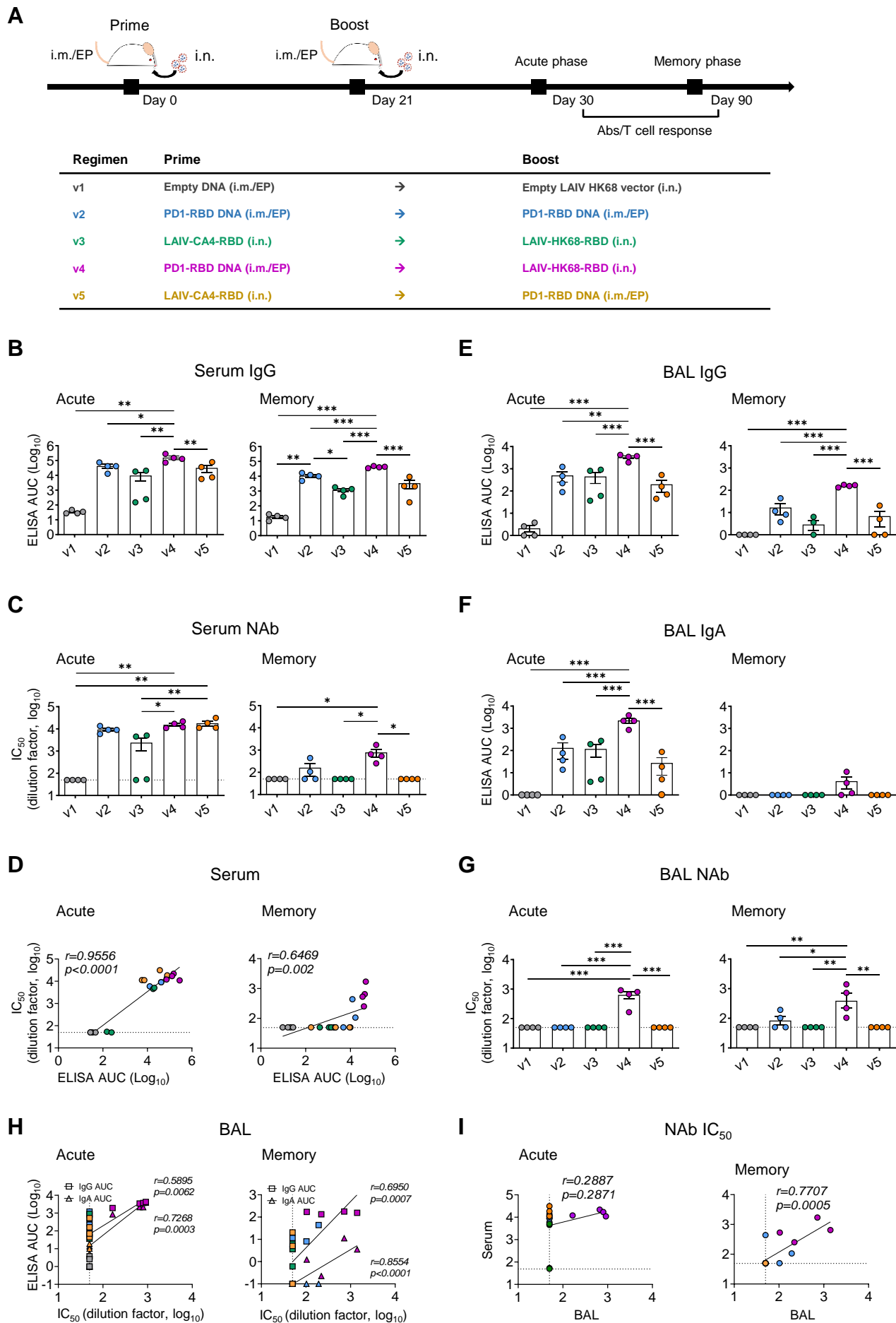
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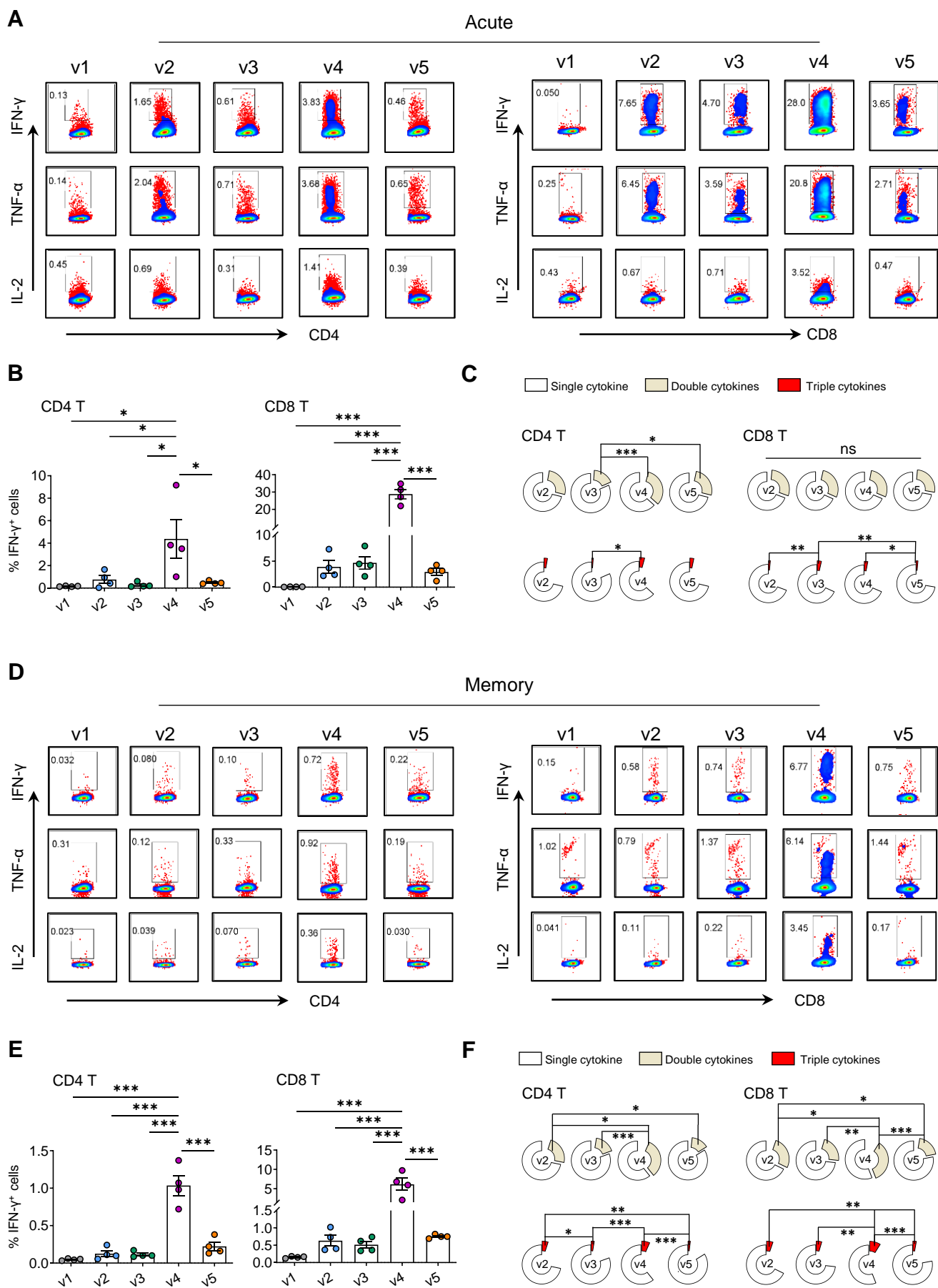
882 **Fig. 6. Combinations of systemic and mucosal immunization induce robust humoral and**
883 **cellular immune responses in the effector site.** Experimental schedule and grouping of
884 BALB/c mice (5 mice per group). Blood (**B**, **E**) and bronchoalveolar lavage (BAL) (**C**, **D**, **F**)
885 were collected and subjected to RBD-specific IgG (**B**, **C**) or IgA (**D**) detection and NAb IC₅₀
886 activity (**E**, **F**). The area under the curve (AUC) represented the total peak area calculated from
887 ELISA OD values. Neutralization IC₅₀ values were determined against SARS-CoV-2-Spike-
888 pseudovirus infection of 293T-huACE2 cells. (**F**) Lung CD8 T cells were assayed for IFN- γ
889 expression by flow cytometry after re-stimulated with the RBD peptide pool. Tissue-resident
890 memory CD8 T cells (TRM) in lungs were phenotyped for expression CD69 and CD103 (**G**) and
891 the IFN- γ producing TRM were measured by re-stimulated with the RBD peptide pool (**H**). Each
892 colour represents a vaccination regimen. Each symbol represents an individual mouse. Statistics
893 were generated using the 2-tailed Student's t test. *p<0.05; **p<0.01; ***p<0.001.

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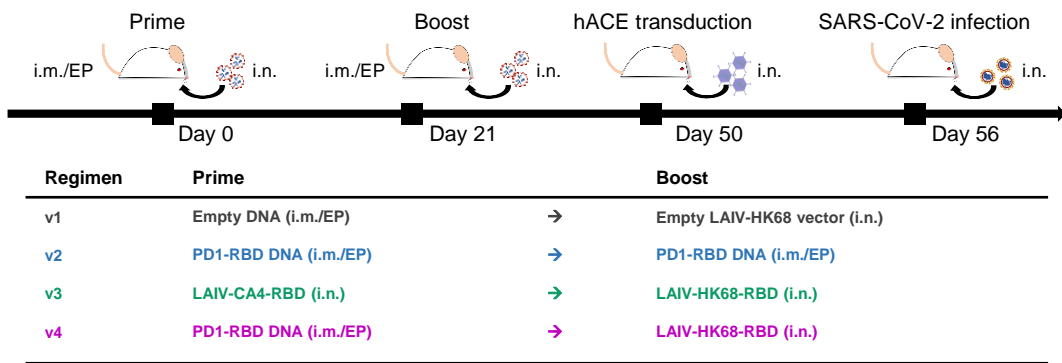
895 **Fig. 7. Cross-neutralization of vaccine-induced systemic and mucosal NAb against SARS-**
896 **CoV-2 variants.** Neutralization activities of immune sera (**A-B**) and BAL (**C-D**) elicited by
897 various vaccine regimens (same as described in Fig. 6) were determined against 3 color-coded
898 pseudoviruses in 293T-ACE2 cells. Data showed mean \pm SEM of 5 mice in each vaccine group.

899 **(B and D)** Comparison of mean IC_{50} values against different SARS-CoV-2 variants (left) and
900 fold-change of mean IC_{50} values in relation to the SARS-CoV-2 D614G strain (right).

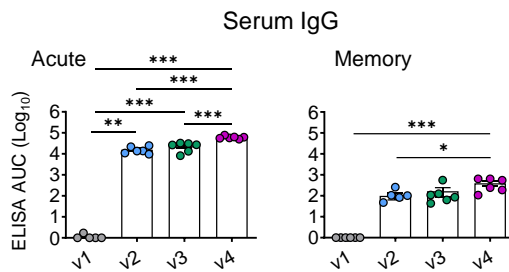




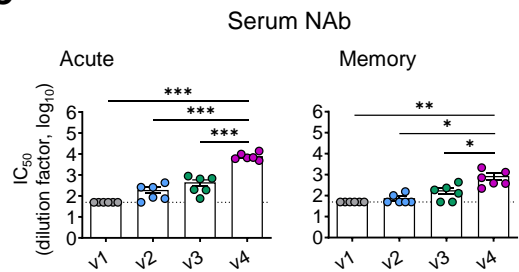
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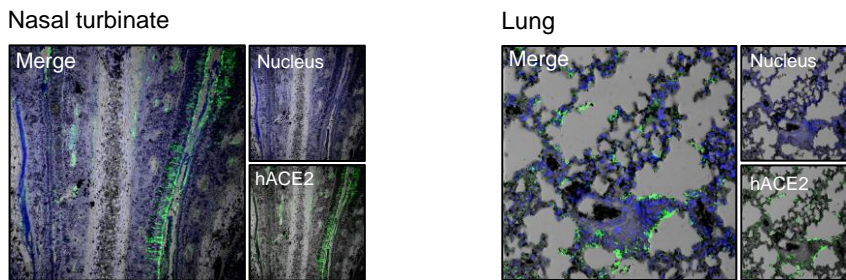
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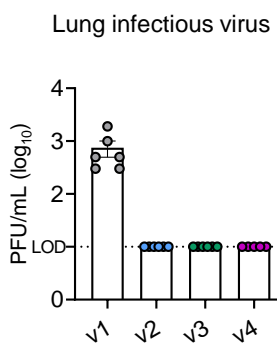
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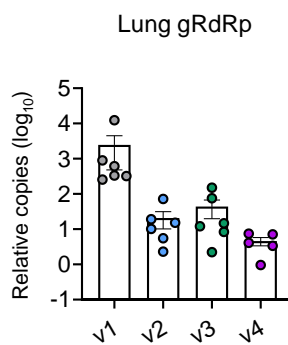
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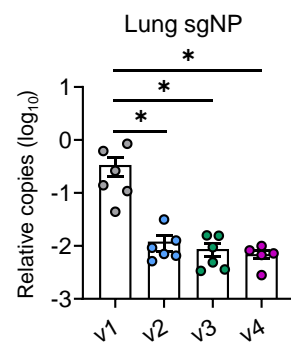
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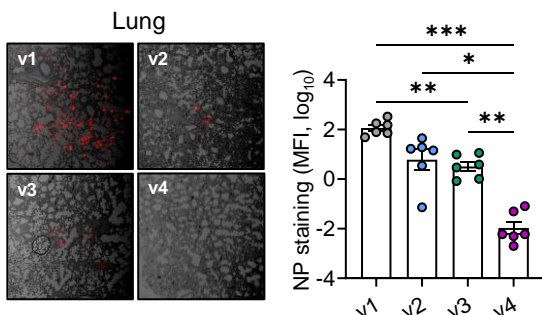
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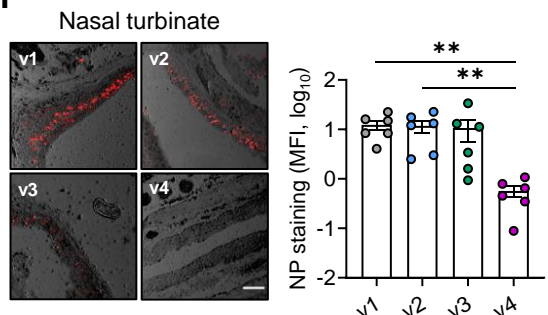
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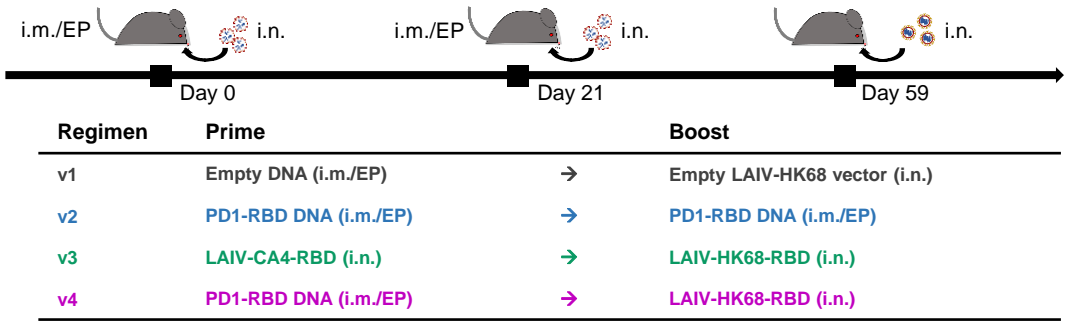
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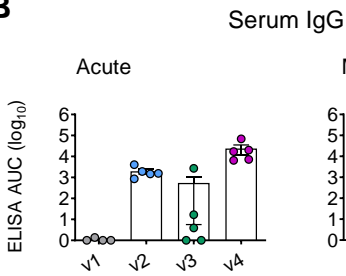
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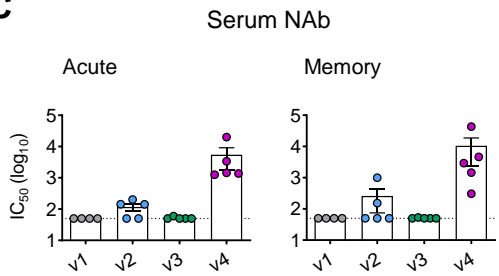
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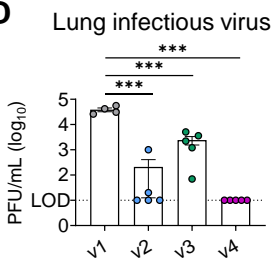
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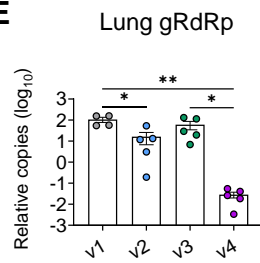
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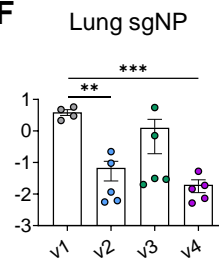
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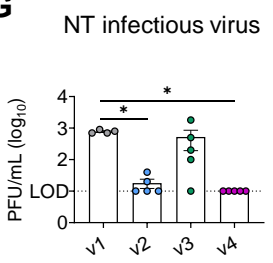
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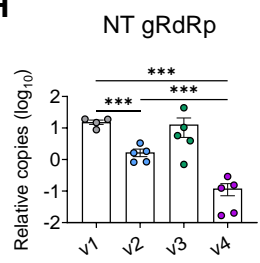
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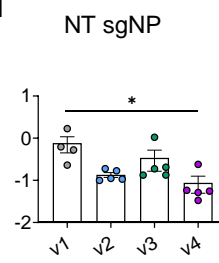
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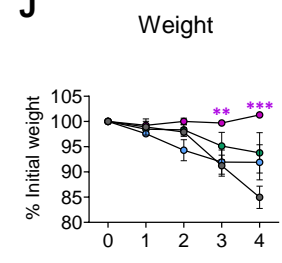
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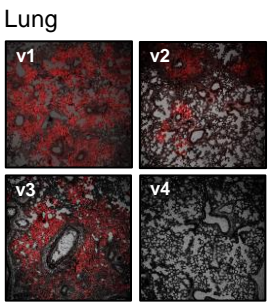
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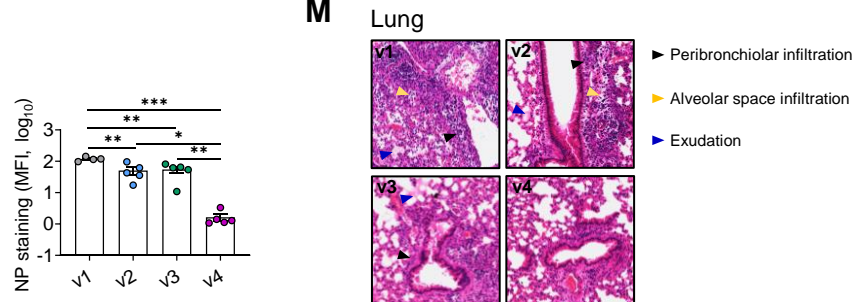
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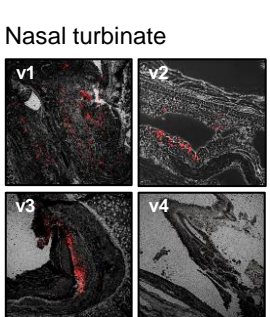
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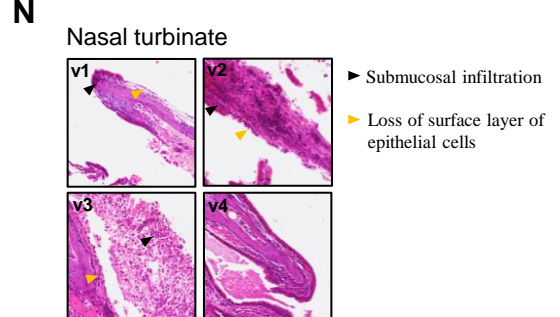
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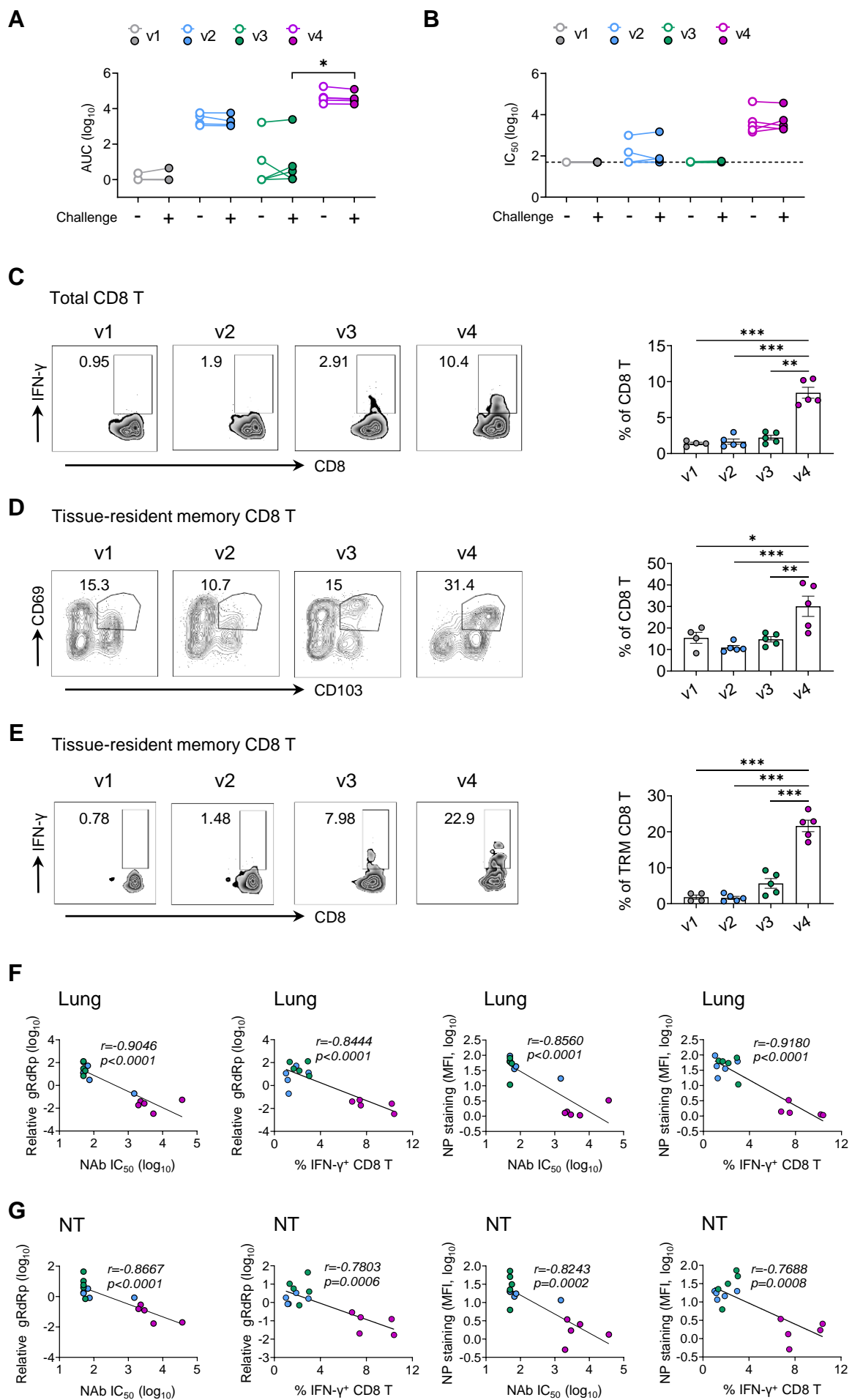


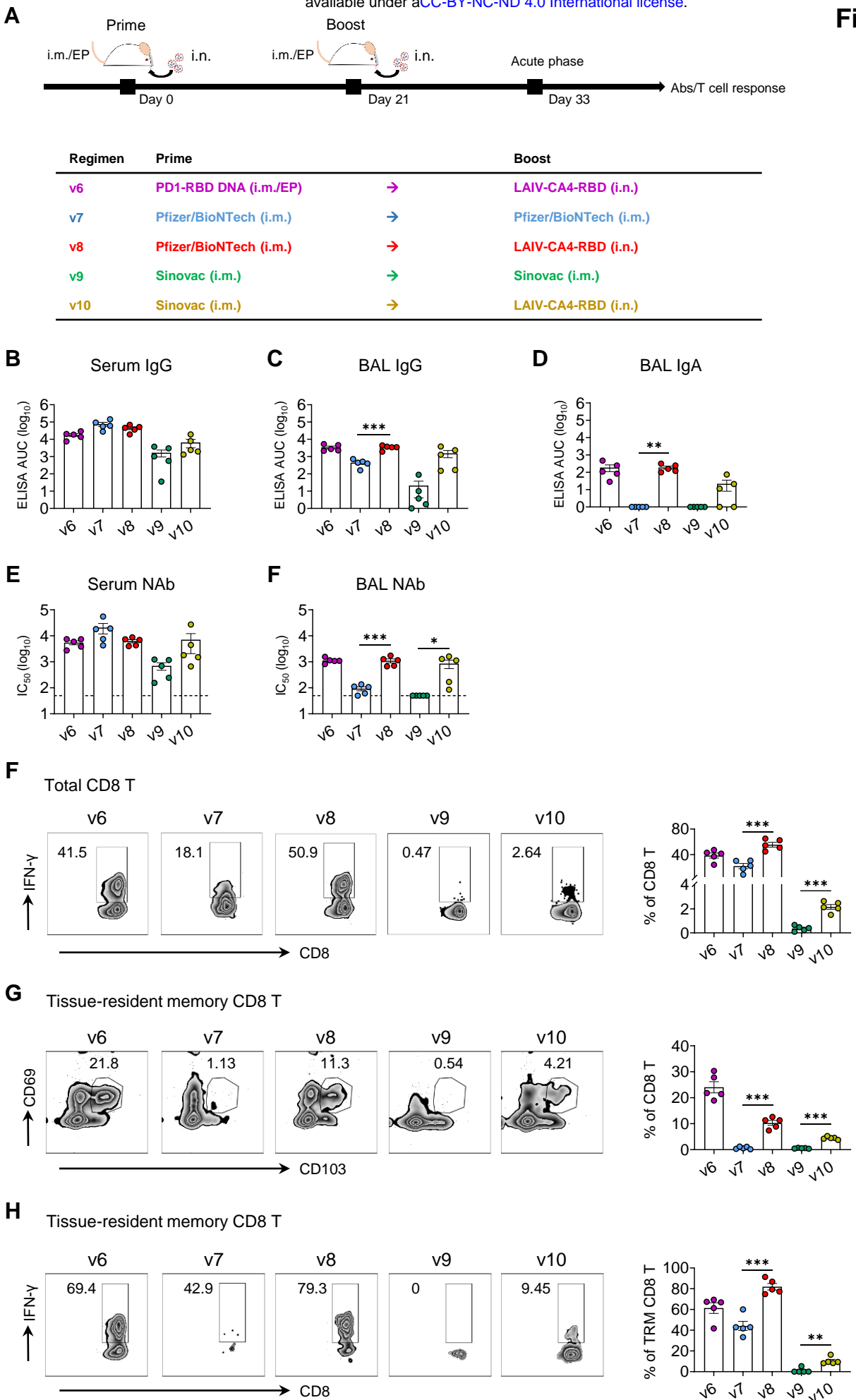
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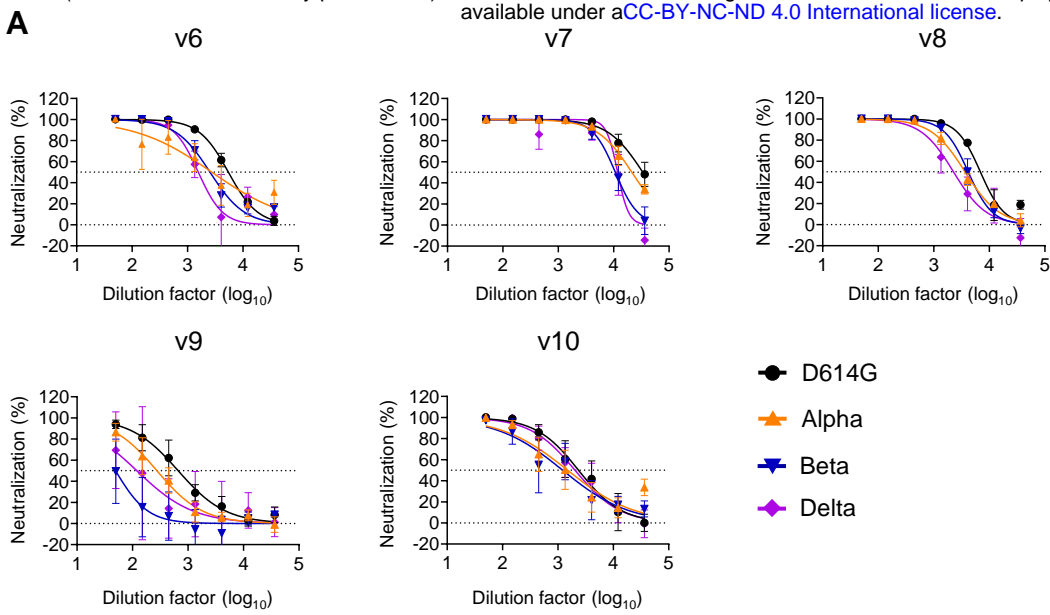


N









B

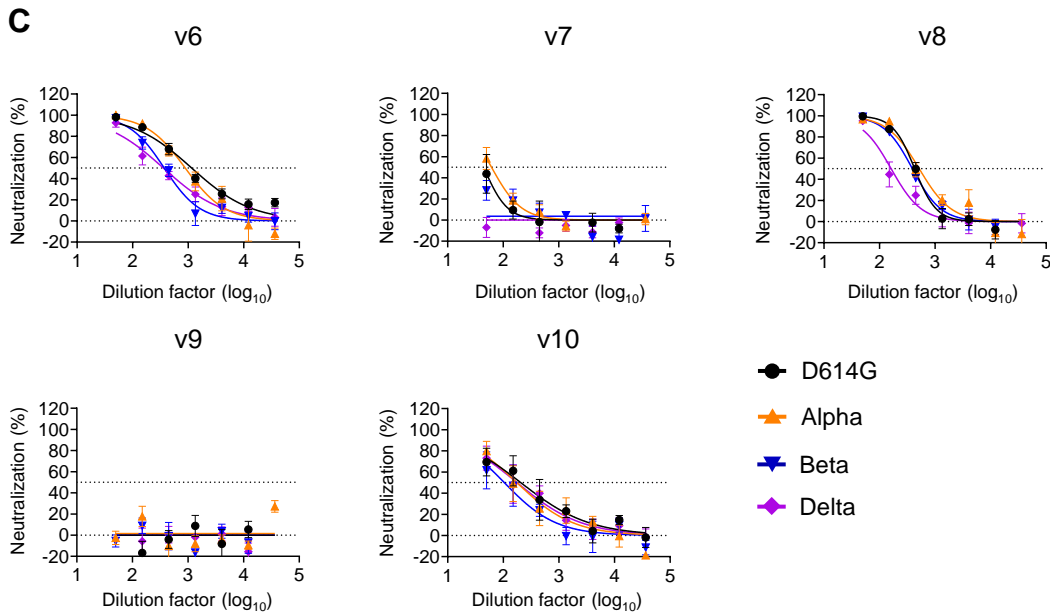
Mean IC_{50}

Variants	v6	v7	v8	v9	v10
D614G	5897	43469	8759	898	5517
Alpha	8512	37993	4298	433	4085
Beta	3926	14420	4434	585	4384
Delta	3339	12070	4305	367	3474

Fold-change IC_{50} relative to D614G

Variants	v6	v7	v8	v9	v10
Alpha	1.44	-1.14	-2.04	-2.07	-1.35
Beta	-1.50	-3.00	-1.98	-1.56	-1.26
Delta	-1.77	-3.60	-2.03	-2.45	-1.59

Color scale: 2 (green) to -4 (red)



D

Mean IC_{50}

Variants	v6	v7	v8	v9	v10
D614G	1252	92	683	50	455
Alpha	1475	74	407	50	357
Beta	475	55	401	50	200
Delta	431	50	159	50	361

Fold-change IC_{50} relative to D614G

Variants	v6	v7	v8	v9	v10
Alpha	1.18	-1.24	-1.68	1	-1.27
Beta	-2.64	-1.67	-1.70	1	-2.28
Delta	-2.90	-1.84	-4.30	1	-1.26

Color scale: 2 (green) to -5 (red)