

1 A single-dose live-attenuated YF17D-vectored SARS-CoV2 vaccine candidate

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34 **Abstract**

35 The explosively expanding COVID-19 pandemic urges the development of safe, efficacious
36 and fast-acting vaccines to quench the unrestrained spread of SARS-CoV-2. Several promising
37 vaccine platforms, developed in recent years, are leveraged for a rapid emergency response to
38 COVID-19¹. We employed the live-attenuated yellow fever 17D (YF17D) vaccine as a vector
39 to express the prefusion form of the SARS-CoV-2 Spike antigen. In mice, the vaccine
40 candidate, tentatively named YF-S0, induces high levels of SARS-CoV-2 neutralizing
41 antibodies and a favorable Th1 cell-mediated immune response. In a stringent hamster SARS-
42 CoV-2 challenge model², vaccine candidate YF-S0 prevents infection with SARS-CoV-2.
43 Moreover, a single dose confers protection from lung disease in most vaccinated animals even
44 within 10 days. These results warrant further development of YF-S0 as a potent SARS-CoV-2
45 vaccine candidate.

46

47 **Vaccine design and rationale**

48 Protective immunity against SARS-CoV-2 and other coronaviruses is believed to depend on
49 neutralizing antibodies (nAbs) targeting the viral Spike (S) protein ^{3,4}. In particular, nAbs
50 specific for the N-terminal S1 domain containing the Angiotensin Converting Enzyme 2
51 (ACE2) receptor binding domain (RBD) interfere with and have been shown to prevent viral
52 infection in several animal models^{5,6}.

53 The live-attenuated YF17D vaccine is known for its outstanding potency to rapidly induce
54 broad multi-functional innate, humoral and cell-mediated immunity (CMI) responses that may
55 result in life-long protection following a single vaccine dose in nearly all vaccinees ^{7,8}. These
56 favorable characteristics of the YF17D vaccine translate also to vectored vaccines based on the
57 YF17D backbone ⁹. YF17D is used as viral vector in two licensed human vaccines [Imojev[®]
58 against Japanese encephalitis virus (JEV) and Dengvaxia[®] against dengue virus (DENV)]. For
59 these two vaccines, genes encoding the YF17D surface antigens prM/E, have been swapped
60 with those of JEV or DENV, respectively. Potent Zika virus vaccines based on this ChimeriVax
61 approach are in preclinical development ¹⁰.

62 YF17D is a small (+)-ss RNA live-attenuated virus with a limited vector capacity, but it has
63 been shown to tolerate insertion of foreign antigens at two main sites in the viral polyprotein ¹¹.
64 Importantly, an insertion of foreign sequences is constrained by (i) the complex topology and
65 post-translational processing of the YF17D polyprotein; and, (ii) the need to express the antigen
66 of interest in an immunogenic, likely native, fold, to yield a potent recombinant vaccine.

67 Using an advanced reverse genetics system for the generation of recombinant flaviviruses^{12,13},
68 a panel of YF17D-based COVID-19 vaccine candidates (YF-S) was designed. These express
69 codon-optimized versions of the S protein [either in its native cleavable S1/2, or non-cleavable
70 prefusion S0 conformation or its S1 subdomain] of the prototypic SARS-CoV-2 Wuhan-Hu-1

71 strain (GenBank: MN908947.3), as in-frame fusion within the YF17D-E/NS1 intergenic region
72 (YF-S1/2, YF-S0 and YF-S1) (Figure 1A, Figure S1). As outlined below, variant YF-S0 was
73 finally selected as lead vaccine candidate based on its superior immunogenicity, efficacy and
74 favorable safety profile.

75 Infectious live-attenuated YF-S viruses were rescued by plasmid transfection into baby hamster
76 kidney (BHK-21) cells. Transfected cells presented with a virus-induced cytopathic effect;
77 infectious virus progeny was recovered from the supernatant and further characterized. Each
78 construct results in a unique plaque phenotype, smaller than that of the parental YF17D (Figure
79 1B), in line with some replicative trade-off posed by the inserted foreign sequences. S or S1 as
80 well as YF17D antigens were readily visualized by double staining of YF-S infected cells with
81 SARS-CoV-2 Spike and YF17D-specific antibodies (Figure 1C). The expression of S or S1 by
82 the panel of YF-S variants was confirmed by immunoblotting of lysates of freshly infected
83 cells. Treatment with PNGase F allowed to demonstrate a proper glycosylation pattern (Figure
84 1D).

85 In line with a smaller plaque phenotype, intracranial (i.c.) inoculation of YF17D or the YF-S
86 variants in suckling mice confirmed the attenuation of the different YF-S as compared to the
87 empty vector YF17D (Figure 2A and B and Figure S2). Mouse pups inoculated i.c. with 100
88 plaque forming units (PFU) of the parental YF17D stopped growing (Figure S2A) and
89 succumbed to infection within seven days (median day of euthanasia; MDE) (Figure 2A),
90 whereas pups inoculated with the YF-S variants continued to grow. From the group inoculated
91 with YF-S0 only half needed to be euthanized (MDE 17,5 days). For the YF-S1/2 and YF-S1
92 groups MDE was 12 and 10 days respectively; thus in particular YF-S0 has a markedly reduced
93 neurovirulence. Likewise, YF-S0 is also highly attenuated in type I and II interferon receptor
94 deficient AG129 mice, that are highly susceptible to (a neurotropic) YF17D infection^{13,14}.

95 Whereas 1 PFU of YF17D resulted in neuro-invasion requiring euthanasia of all mice (MDE
96 16 days) (Figure 2B), a 1000-fold higher inoculum of YF-S0 did not result in any disease
97 (Figure S2C) and only 1 in 12 animals that received a 10,000 higher inoculum needed to be
98 euthanized (Figure 2B). In summary, a set of transgenic replication-competent YF17D variants
99 (YF-S) was generated that express different forms of the SARS-CoV-2 S antigen and that are
100 highly attenuated in mice in terms of neurovirulence and neurotropism as compared to YF17D.

101

102 **Immunogenicity and protection against SARS-CoV-2 infection and COVID-19-like** 103 **disease in a stringent hamster model.**

104 To assess the potency of the various vaccine constructs, a stringent hamster challenge model
105 was developed². Animals were vaccinated at day 0 with 10^3 PFU (i.p. route) of the different
106 constructs or the negative controls and were boosted 7 days later with the same dose (Figure
107 3A). At day 21 post-vaccination, all hamsters vaccinated with YF-S1/2 and YF-S0 (n=12 from
108 two independent experiments) had seroconverted to high levels of S-specific IgG and virus
109 nAbs (Figure 3B,C; see Figure S3 for benchmarking of SARS-CoV-2 serum neutralization test,
110 SNT). For YF-S1/2 \log_{10} geometric mean titers (GMT) for IgG and nAbs were 3.2 (95% CI,
111 2.9-3.5) and 1.4 (95% CI, 1.1-1.9) respectively, while in the case of YF-S0 GMT values for
112 IgG and nAbs of 3.5 (95% CI, 3.3-3.8) and 2.2 (95% CI, 1.9-2.6) were measured, with rapid
113 seroconversion kinetics (50% seroconversion rate < 2 weeks; Figure 3D). By contrast, only 1
114 out of 12 hamsters that had received YF-S1 seroconverted and this with a low level of nAbs.
115 This indicates the need for a full-length S antigen to elicit an adequate humoral immune
116 response.

117 Next, vaccinated hamsters were challenged intranasally (either at day 23 or day 28 post
118 vaccination) with 2×10^5 PFU of SARS-CoV-2. At day 4 post-infection, high viral loads were

119 detected in lungs of sham-vaccinated controls and animals vaccinated with YF17D as matched
120 placebo (Figure 4A, B). Infection was characterized by a severe lung pathology with multifocal
121 necrotizing bronchiolitis, leukocyte infiltration and edema, resembling findings in patients with
122 severe COVID-19 bronchopneumonia (Figure 4A specimen pictures and 4B radar plot;
123 Supplementary- histogram). By contrast, hamsters vaccinated with YF-S0 were protected
124 against this aggressive challenge (Figure 3E-F). As compared to sham-vaccinated controls, YF-
125 S0 vaccinated animals had a median reduction of 5 log₁₀ (IQR, 4.5-5.4) in viral RNA loads (p
126 <0.0001; Figure 3D), and of 5.3 log₁₀ (IQR, 3.9-6.3) for infectious SARS-CoV-2 virus in the
127 lungs (p <0.0001; Figure 3E). Moreover, infectious virus was no longer detectable in 10 of 12
128 hamsters (two independent experiments), and viral RNA was reduced to non-quantifiable levels
129 in their lungs. Residual RNA measured in 2 out of 12 animals may equally well represent
130 residues of the high-titer inoculum as observed in non-human primate models¹⁵⁻¹⁸. Vaccination
131 with YF-S0 (two doses of 10³ PFU) also efficiently prevented systemic viral dissemination; in
132 most animals, no or only very low levels of viral RNA were detectable in spleen, liver, kidney
133 and heart four days after infection (Figure S4A). Similarly and in full support, a slightly
134 different dose and schedule used for vaccination (5 x 10³ PFU of YF-S0 at day 0 and 7
135 respectively) resulted in all vaccinated hamsters (n=7) in respectively a 6 log₁₀ (IQR, 4.6-6.6)
136 and 5.7 log₁₀ (IQR, 5.7-6.6) reduction of viral RNA and infectious virus titers as compared to
137 sham (Figure S5). Finally, vaccination with YF-S0 may induce saturating levels of nAbs
138 thereby conferring sterilizing immunity, as demonstrated by the fact that in about half of the
139 YF-S0 vaccinated hamsters no anamnestic antibody response was observed following challenge
140 (Figure 3G and S4B-D (paired nAb analysis)). By contrast, in hamsters vaccinated with the
141 second-best vaccine candidate YF-S1/2, nAb levels further increased following SARS-CoV-2
142 infection (in 11 out of 12 animals) whereby a plateau was only approached after challenge.

143 The lungs of YF-S0 vaccinated animals remained normal, or near to normal with no more signs
144 of bronchopneumonia which is markedly different to sham-vaccinated animals (n=12 from two
145 independent experiments; Figure 4). The specific disease scores and biomarkers quantified²
146 included (i) a reduction or lack of detectable lung pathology as observed by histological
147 inspection (Figure 4A,B, Figure S6A); and, (ii) a significant improvement of the individual lung
148 scores ($p = 0.002$) (Figure 4C, Figure S6B) and respiratory capacity (*i.e.** 32% less of lung
149 volume obstructed; $p = 0.0323$; Figure 4D) in YF-S0 vaccinated animals as derived by micro-
150 computed tomography (micro-CT) of the chest. In addition, immunization with YF-S0 resulted
151 in an almost complete, in most cases full, normalization of the expression of cytokines, *e.g.*, IL-
152 6, IL-10, or IFN- γ in the lung, linked to disease exacerbation in COVID-19 (Figure 4E,F and
153 Figure S7)¹⁹⁻²¹. Even the most sensitive markers of viral infection, such as the induction of
154 antiviral Type III interferons (IFN- λ)²², or the expression of IFN-stimulated genes (ISG) such
155 as MX2 and IP-10 in YF-S0 vaccinated animals showed no elevation as compared to levels in
156 the lungs of untreated healthy controls (Figure 4F and Figure S7).

157 Overall, YF-S0 that expresses the non-cleavable S variant outcompeted construct YF-S1/2
158 expressing the cleavable version of S. This argues for the stabilized prefusion form of S serving
159 as a relevant protective antigen for SARS-CoV-2. Moreover, in line with its failure to induce
160 nAbs (Figure 3B), construct YF-S1 expressing solely the hACE2 receptor-binding S1 domain
161 (Figure 1D) did not confer any protection against SARS-CoV-2 challenge in hamsters (Figure
162 3E, F and Figure 4).

163

164 **Immunogenicity, in particular a favorable Th1 polarization of cell-mediated immunity in**
165 **mice**

166 Since there are very few tools available to study CMI in hamsters, humoral and CMI responses
167 elicited by the different YF-S constructs were studied in parallel in mice. Since YF17D does
168 not readily replicate in wild-type mice^{23,24}, *Ifnar*^{-/-} mice that are deficient in Type I interferon
169 signaling and that are hence susceptible to vaccination with YF17D, were employed^{10,24,25}.

170 Mice were vaccinated with 400 PFU (of either of the YF-S variants, YF17D or sham) at day 0
171 and were boosted with the same dose 7 days later (Figure 5A). At day 21 all YF-S1/2 and YF-
172 S0 vaccinated mice (n>9 in three independent experiments) had seroconverted to high levels of
173 S-specific IgG and nAbs with log₁₀ GMT of 3.5 (95% CI, 3.1-3.9) for IgG and 2.2 (95% CI,
174 1.7-2.7) for nAbs in the case of YF-S1/2, or 4.0 (95% CI, 3.7-4.2) for IgG and 3.0 (95% CI,
175 2.8-3.1) for nAbs in the case of YF-S0 (Figure 5B,C). Importantly, seroconversion to S-specific
176 IgG was detectable as early as 7 days after the first immunization (Figure 5D). Isotyping of IgG
177 revealed an excess of IgG2b/c over IgG1 indicating a dominant pro-inflammatory and hence
178 antiviral (Th1) polarization of the immune response (Figure 5E) which is considered important
179 for vaccine-induced protection against SARS-CoV-2²⁶⁻²⁸. Alike in hamsters, YF-S1 failed to
180 induce SARS-CoV-2 nAbs (Figure 5B,C). However, high levels of YF nAbs were conjointly
181 induced by all constructs confirming a consistent immunization (Figure S8).

182 To assess SARS-CoV-2-specific CMI responses that play a pivotal role for the shaping and
183 longevity of vaccine-induced immunity as well as in the pathogenesis of COVID-19^{29,30},
184 splenocytes from vaccinated mice were incubated with a tiled peptide library spanning the
185 entire S protein as recall antigen. In general, vaccination with any of the YF-S variants resulted
186 in marked S-specific T-cell responses with a favorable Th1-polarization as detected by IFN- γ
187 ELISpot (Figure 6A), further supported by an upregulation of T-bet (*TBX21*), in particular in
188 cells isolated from YF-S0 vaccinated mice (p = 0.0198, n = 5). This CMI profile was balanced
189 by a concomitant elevation of GATA-3 levels (*GATA3*; driving Th2; p = 0.016), but no marked
190 overexpression of ROR γ t (*RORC*; Th17) or FoxP3 (*FOXP3*; Treg) (Figure 6B). Intriguingly,

191 in stark contrast to its failure to induce nAbs in mice (Figure 5A,C), or protection in hamsters
192 (Figure 2 and 3), YF-S1 vaccinated animals had a greater number of S-specific splenocytes (p
193 < 0.0001 , $n = 7$) than those vaccinated with YF-S1/2 or YF-S0 (Figure 6A). Thus, even a
194 vigorous CMI may not be sufficient for vaccine efficacy. A more in-depth profiling of the T-
195 cell compartment by means of intracellular cytokine staining (ICS) and flow cytometry
196 confirmed the presence of S-specific IFN- γ and TNF- α expressing CD8⁺ T-lymphocytes, and
197 of IFN- γ expressing CD4⁺ (Figure 6E) and γ/δ T lymphocytes (Figure 6F), in particular in YF-
198 S0 immunized animals. A specific and pronounced elevation of other markers such as IL-4
199 (Th2 polarization), IL-17A (Th17), or FoxP3 (regulatory T-cells) was not observed for YF-S1/2
200 or YF-S0. This phenotype is supported by t-SNE plot analysis of the respective T-cell
201 populations in YF-S1/2 and YF-S0 vaccinated mice (Figure 6G and S8 tSNE) showing an
202 increased percentage of IFN- γ expressing cells. It further revealed, firstly, a similar composition
203 of either CD4⁺ cell sets, comprising an equally balanced mixture of Th1 (IFN- γ ⁺ and/or TNF-
204 α ⁺) and Th2 (IL-4⁺) cells, and possibly a slight raise in Th17 cells in the case of the YF-S0
205 vaccinated animals. Likewise, secondly, for both constructs the CD8⁺ T-lymphocyte population
206 was dominated by IFN- γ or TNF- α expressing cells, in line with the matched transcriptional
207 profiles (Figure 6B). Of note, though similar in numbers, both vaccines YF-S0 and YF-S1/2
208 showed a distinguished (non-overlapping) profile regarding the respective CD8⁺ T lymphocyte
209 populations expressing IFN- γ . In fact, YF-S0 tended to induce S-specific CD8⁺ T-cells with a
210 stronger expression of IFN- γ (Figure 6G and S8). In summary, YF-S0 induces a vigorous and
211 balanced CMI response in mice with a favorable Th1 polarization, dominated by SARS-CoV-
212 2 specific CD8⁺ T-cells expressing high levels of IFN- γ when encountering the SARS-CoV-2
213 S antigen.

214

215 **Protection and short time to benefit after single-dose vaccination**

216 Finally, vaccination of hamsters using a single-dose of YF-S0 induced high levels of nAbs and
217 bAbs (Figure 7B and 7C) in a dose- and time-dependent manner. Furthermore, it appears a
218 single 10^4 PFU dose of YF-S0 yielded higher levels of nAbs (\log_{10} GMT 2.8; 95% CI: 2.5-3.2)
219 at 21 days post-vaccination compared to the antibody levels in a prime-boost vaccination with
220 two doses of 10^3 PFU (\log_{10} GMT 2.2; 95% CI: 1.9-2.6) ($p = 0.039$, two tailed Mann-Whitney
221 test) (Figure 3B). Also, this single-dose regimen resulted in efficient and full protection against
222 SARS-CoV-2 challenge, assessed by absence of infectious virus in the lungs in 8 out of 8
223 animals (Figure 7E). It should be noted that viral RNA at quantifiable levels was present in only
224 1 out of 8 animals (Figure 7D). In addition, protective immunity was mounted rapidly. Already
225 10 days after vaccination, 5 out of 8 animals receiving 10^4 PFU of YF-S0 were protected against
226 stringent infection challenge (Figure 7D and 7E).

227

228 Discussion

229 Vaccines against SARS-CoV-2 need to be safe and result rapidly, ideally after one single dose,
230 in long-lasting protective immunity. Different SARS-CoV-2 vaccine candidates are being
231 developed, and several are vector-based. We report encouraging results of YF17D-vectored
232 SARS-CoV-2 vaccine candidates. The post-fusion (S1/2), pre-fusion (S0) as well as the RBD
233 S1 domain (S1) of the SARS-CoV-2 Spike protein were inserted in the YF17D backbone to
234 yield the YF-S1/2, YF-S0 and YF-S1, respectively (Figure S1). The YF-S0 vaccine candidate,
235 in particular, resulted in a robust humoral immune response in both, mice and Syrian hamsters.
236 Since SARS-CoV-2 replicates massively in the lungs of infected Syrian hamsters and results in
237 major lung pathology^{2,31-33} we selected this model to assess the potency of these three vaccine
238 candidates. YF-S0 resulted in efficient protection against stringent SARS-CoV-2 challenge,
239 comparable, if not more vigorous, to other vaccine candidates in non-human primate
240 models^{16,17,34}. In about 40% of the YF-S0 vaccinated animals no increase in nAb levels (> 2x)
241 following SARS-CoV-2 challenge was observed, suggestive for sterilizing immunity (no
242 anamnestic response). In experiments in which animals were challenged three weeks after
243 single 10⁴ PFU dose vaccination, no infectious virus was detected in the lungs. Considering the
244 severity of the model, it is remarkable, that in several animals that were challenged with SARS-
245 CoV-2 already 10 days after vaccination no infectious virus could be recovered from the lungs.
246 Reduction of viral replication mitigated lung pathology in infected animals with a concomitant
247 normalization of biomarkers associated with infection and disease (Figure 4 and S6). Likewise,
248 in lungs of vaccinated and subsequently challenged hamsters no elevation of cytokines, such as
249 IL-6, was noted (Figure 4F).

250 Moreover, YF-S0 showed in two mice models a favorable safety profile as compared to the
251 parental YF17D vector (Figure 2A and B). This is of importance as YF17D vaccine is contra-

252 indicated in elderly and persons with underlying medical conditions. These preliminary, though
253 encouraging, data suggest that YF-S0 might also be safe in those persons most vulnerable to
254 COVID-19.

255 In addition, cell-mediated immunity (CMI) studied in mice revealed that YF-S0, besides
256 efficiently inducing high titers of nAbs, favors a Th1 response. Such a Th1 polarization is
257 considered relevant in light of a disease enhancement supposedly linked to a skewed Th2
258 immune²⁹ or antibody-dependent enhancement (ADE)³⁵. ADE may occur when virus-specific
259 antibodies promote virus infection via various Fc γ receptor-mediated mechanisms, as suggested
260 for an inactivated RSV post-fusion vaccine candidate³⁶. A Th2 polarization may cause an
261 induction and dysregulation of alternatively activated 'wound-healing'
262 monocytes/macrophages^{26-28,37} resulting in an overshooting inflammatory response (cytokine
263 storm) thus leading to acute lung injury (ALI). No indication of such a disease enhancement
264 was observed in our models.

265 In conclusion, YF-S0 confers vigorous protective immunity against SARS-CoV-2 infection.
266 Remarkably, this immunity can be achieved within 10 days following a single dose vaccination.
267 In light of the threat SARS-CoV-2 will remain endemic with spikes of re-infection, as a
268 recurring plague, vaccines with this profile may be ideally suited for population-wide
269 immunization programs.

270

271 **Methods**

272 **Cells and viruses**

273 BHK-21J (baby hamster kidney fibroblasts) cells³⁷ were maintained in Minimum Essential
274 Medium (Gibco), Vero E6 (African green monkey kidney, ATCC CRL-1586) and HEK-293T
275 (human embryonic kidney cells) cells were maintained in Dulbecco's Modified Eagle Medium
276 (Gibco). All media were supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-
277 glutamine (Gibco), 1% sodium bicarbonate (Gibco). BSR-T7/5 (T7 RNA polymerase
278 expressing BHK-21)³⁸ cells were kept in DMEM supplemented with 0.5 mg/ml geneticin
279 (Gibco).

280 For all challenge experiments in hamsters, SARS-CoV-2 strain BetaCov/Belgium/GHB-
281 03021/2020 (EPI ISL 407976|2020-02-03) was used from passage P4 grown on Vero E6 cells
282 as described². YF17D (Stamaril[®], Sanofi-Pasteur) was passaged twice in Vero E6 cells before
283 use.

284 **Vaccine design and construction**

285 Different vaccine constructs were generated using an infectious cDNA clone of YF17D (in an
286 inducible BAC expression vector pShuttle-YF17D, patent number WO2014174078 A1)^{10,12,39}.
287 A panel of several SARS-CoV-2 vaccine candidates was engineered by inserting a codon
288 optimized sequence of either the SARS-CoV-2 Spike protein (S) (GenBank: MN908947.3) or
289 variants thereof into the full-length genome of YF17D (GenBank: X03700) as translational in-
290 frame fusion within the YF-E/NS1 intergenic region^{11,40} (Figure S1). The variants generated
291 contained (i) either the S protein sequence from amino acid (aa) 14-1273, expressing S in its
292 post-fusion and/or prefusion conformation (YF-S1/2 and YF-S0, respectively), or (ii) its
293 subunit-S1 (aa 14-722; YF-S1). To ensure a proper YF topology and correct expression of

294 different S antigens in the YF backbone, transmembrane domains derived from WNV were
295 inserted.

296 The SARS2-CoV-2 vaccine candidates were cloned by combining the S cDNA (obtained after
297 PCR on overlapping synthetic cDNA fragments; IDT) by a NEB Builder Cloning kit (New
298 England Biolabs) into the pShuttle-YF17D backbone. NEB Builder reaction mixtures were
299 transformed into *E.coli* EPI300 cells (Lucigen) and successful integration of the S protein
300 cDNA was confirmed by Sanger sequencing. Recombinant plasmids were purified by column
301 chromatography (Nucleobond Maxi Kit, Machery-Nagel) after growth over night, followed by
302 an additional amplification of the BAC vector for six hours by addition of 2 mM L-arabinose
303 as described¹⁰.

304 Infectious vaccine viruses were generated from plasmid constructs by transfection into BHK-
305 21J cells using standard protocols (TransIT-LT1, Mirus Bio). The supernatant was harvested
306 four days post-transfection when most of the cells showed signs of CPE. Infectious virus titers
307 (PFU/ml) were determined by a plaque assay on BHK-21J cells as previously described^{10,14}.
308 The presence of inserted sequences in generated vaccine virus stocks was confirmed by RNA
309 extraction (Direct-zol RNA kit, Zymo Research) followed by RT-PCR (qScript XLT, Quanta)
310 and Sanger sequencing, and by immunoblotting of freshly infected cells (see *infra*).

311 **Immunofluorescent staining**

312 *In vitro* antigen expression of different vaccine candidates was verified by immunofluorescent
313 staining as described previously by Kum et al. 2018. Briefly, BHK-21J cells were infected with
314 100 PFU of the different YF-S vaccine candidates. Infected cells were stained three days post-
315 infection (3dpi). For detection of YF antigens polyclonal mouse anti-YF17D antiserum was
316 used. For detection of SARS-CoV-2 Spike antigen rabbit SARS-CoV Spike S1 antibody

317 (40150-RP01, Sino Biological) and rabbit SARS-CoV Spike primary antibody (40150-T62-
318 COV2, Sino Biological) was used. Secondary antibodies were goat anti-mouse Alexa Fluor-
319 594 and goat anti-rabbit Alexa Fluor-594 (Life Technologies). Cells were counterstained with
320 DAPI (Sigma). All confocal fluorescent images were acquired using the same settings on a
321 Leica TCS SP5 confocal microscope, employing a HCX PL APO 63x (NA 1.2) water
322 immersion objective.

323 **Immunoblot analysis (Simple Western)**

324 Infected BHK21-J cells were harvested and washed once with ice cold phosphate buffered
325 saline, and lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific)
326 containing 1x protease inhibitor and phosphatase inhibitor cocktail (Thermo Fisher Scientific).
327 After centrifugation at 15,000 rpm at 4 °C for 10 minutes, protein concentrations in the cleared
328 lysates were measured using BCA (Thermo Fisher Scientific). Immunoblot analysis was
329 performed by a Simple Western size-based protein assay (Protein Simple) following
330 manufactures instructions. Briefly, after loading of 400 ng of total protein onto each capillary,
331 specific S protein levels were identified using specific primary antibodies (NB100-56578,
332 Novus Biologicals and 40150-T62-CoV2, Sino Biological Inc.), and HRP conjugated
333 secondary antibody (Protein Simple). Chemiluminescence signals were analyzed using
334 Compass software (Protein Simple). To evaluate the removal of N-linked oligosaccharides from
335 the glycoprotein, protein extracts were treated with PNGase F according to manufactures
336 instructions (NEB).

337 **Animals**

338 Wild-type Syrian hamsters (*Mesocricetus auratus*) and BALB/c mice and pups were purchased
339 from Janvier Laboratories, Le Genest-Saint-Isle, France. *Ifnar1*^{-/-} 41 and AG129⁴² were bred in-

340 house. Six- to ten-weeks-old female mice and wild-type hamsters were used throughout the
341 study.

342 **Animal Experiments**

343 Animals were housed in couples (hamsters) or per five (mice) in individually ventilated isolator
344 cages (IsoCage N – Biocontainment System, Tecniplast) with access to food and water *ad*
345 *libitum*, and cage enrichment (cotton and cardboard play tunnels for mice, wood block for
346 hamsters). Housing conditions and experimental procedures were approved by the Ethical
347 Committee of KU Leuven (license P015-2020), following Institutional Guidelines approved by
348 the Federation of European Laboratory Animal Science Associations (FELASA). Animals were
349 euthanized by 100 μ l (mice) or 500 μ l (hamsters) of intraperitoneally administered Dolethal
350 (200 mg/ml sodium pentobarbital, Vétquinol SA).

351 **Immunization and infection of hamsters.**

352 Hamsters were intraperitoneally (i.p) vaccinated with the indicated amount of PFUs of the
353 different vaccine constructs using a prime and boost regimen (at day 0 and 7). As a control, two
354 groups were vaccinated at day 0 and day 7 with either 10^3 PFU of YF17D or with MEM medium
355 containing 2% FBS (sham). All animals were bled at day 21 to analyze serum for binding and
356 neutralizing antibodies against SARS-CoV-2. At the indicated time after vaccination and prior
357 to challenge, hamsters were anesthetized by intraperitoneal injection of a xylazine (16 mg/kg,
358 XYL-M[®], V.M.D.), ketamine (40 mg/kg, Nimatek[®], EuroVet) and atropine (0.2 mg/kg,
359 Sterop[®]) solution. Each animal was inoculated intranasally by gently adding 50 μ l droplets of
360 virus stock containing 2×10^5 TCID₅₀ of SARS-CoV-2 on both nostrils. Animals were
361 monitored daily for signs of disease (lethargy, heavy breathing or ruffled fur). Four days after
362 challenge, all animals were euthanized to collect end sera and lung tissue in RNA later, MEM

363 or formalin for gene-expression profiling, virus titration or histopathological analysis,
364 respectively.

365 **Immunization of mice.**

366 *Ifnar1*^{-/-} mice were i.p. vaccinated with different vaccine constructs by using a prime and boost
367 of each 4 x 10² PFU (at day 0 and 7). As a control, two groups were vaccinated (at day 0 and
368 7) with either YF17D or sham. All mice were bled weekly and serum was separated by
369 centrifugation for indirect immunofluorescence assay (IIFA) and serum neutralization test
370 (SNT). Three weeks post first-vaccination, mice were euthanized, spleens were harvested for
371 ELISpot, transcription factor analysis by qPCR and intracellular cytokine staining (ICS).

372 **SARS-CoV-2 RT-qPCR**

373 The presence of infectious SARS-CoV-2 particles in lung homogenates was quantified by
374 qPCR². Briefly, for quantification of viral RNA levels and gene expression after challenge,
375 RNA was extracted from homogenized organs using the NucleoSpin™ Kit Plus (Macherey-
376 Nagel), following the manufacturer's instructions. Reactions were performed using the iTaq™
377 Universal Probes One-Step RT-qPCR kit (BioRad), with primers and probes (Integrated DNA
378 Technologies) listed in Supplementary Table S1. The relative RNA fold change was calculated
379 with the 2^{-ΔΔC_q} method⁴³ using housekeeping gene β-actin for normalization.

380 **End-point virus titrations**

381 To quantify infectious SARS-CoV-2 particles, endpoint titrations were performed on confluent
382 Vero E6 cells in 96-well plates. Lung tissues were homogenized using bead disruption
383 (Precellys®) in 250 μL minimal essential medium and centrifuged (10,000 rpm, 5 min, 4 °C) to

384 pellet the cell debris. Viral titers were calculated by the Reed and Muench method⁴⁴ and
385 expressed as 50% tissue culture infectious dose (TCID₅₀) per mg tissue.

386 **Histology**

387 For histological examination, lung tissues were fixed overnight in 4% formaldehyde and
388 embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin and
389 analyzed blindly for lung damage by an expert pathologist.

390 **Micro-computed tomography (CT) and image analysis**

391 To monitor the development of lung pathology after SARS-CoV-2 challenge, hamsters were
392 imaged using an X-cube micro-computed tomography (CT) scanner (Molecubes) as described
393 before². Quantification of reconstructed micro-CT data were performed with DataViewer and
394 CTan software (Bruker Belgium). A semi-quantitative scoring of micro-CT data was performed
395 as primary outcome measure and imaging-derived biomarkers (non-aerated lung volume) as
396 secondary measures, as previously described^{2,45-48}.

397

398 **Neurovirulence in suckling mice and neurotropism in AG129 mice**

399 BALB/c mice pups and AG129 mice were respectively intracranially or i.p. inoculated with the
400 indicated PFU amount of YF17D and YF-S vaccine constructs and monitored daily for
401 morbidity and mortality for 21 days post inoculation.

402 **Detection of total binding IgG and IgG isotyping by indirect immunofluorescent assay** 403 **(IIFA)**

404 To detect SARS-CoV-2 specific antibodies in hamster and mouse serum, an in-house developed
405 indirect IFA (IIFA) was used. Using CRISPR/Cas9, a CMV-SARS-CoV-2-Spike-Flag-IRES-
406 mCherry-P2A-BlastiR cassette was stably integrated into the ROSA26 safe harbor locus of
407 HEK293T cells⁴⁹. To determine SARS-CoV-2 Spike binding antibody end titers, 1/2 serial
408 serum dilutions were made in 96-well plates on HEK293T-Spike stable cells and HEK293T wt
409 cells in parallel. Goat-anti-mouse IgG Alexa Fluor 488 (A11001, Life Technologies), goat-anti-
410 mouse IgG1, IgG2b and IgG2c Alexa Fluor 488 (respectively 115-545-205, 115-545-207 and
411 115-545-208 from Jackson ImmunoResearch) were used as secondary antibody. After
412 counterstaining with DAPI, fluorescence in the blue channel (excitation at 386 nm) and the
413 green channel (excitation at 485 nm) was measured with a Cell Insight CX5 High Content
414 Screening platform (Thermo Fischer Scientific). Specific SARS2-CoV-2 Spike staining is
415 characterized by cytoplasmic (ER) enrichment in the green channel. To quantify this specific
416 SARS-CoV-2 Spike staining the difference in cytoplasmic vs. nuclear signal for the HEK293T
417 wt conditions was subtracted from the difference in cytoplasmic vs. nuclear signal for the
418 HEK293T SARS-CoV-2 Spike conditions. All positive values were considered as specific
419 SARS-CoV-2 staining. The IIFA end titer of a sample is defined as the highest dilution that
420 scored positive this way. Because of the limited volume of serum, IIFA end titers for all
421 conditions were determined on minipools of two to three samples.

422 **Pseudotyped virus seroneutralization test (SNT)**

423 SARS-CoV-2 VSV pseudotypes were generated as described previously⁵⁰⁻⁵². Briefly, HEK-
424 293T cells were transfected with a pCAGGS-SARS-CoV-2 Δ 18-Flag expression plasmid
425 encoding SARS-CoV-2 Spike protein carrying a C-terminal 18 amino acids deletion^{53,54}. One
426 day post-transfection, cells were infected with VSV Δ G expressing a GFP (green fluorescent
427 protein) reporter gene (MOI 2) for 2h. The medium was changed with medium containing anti-

428 VSV-G antibody (I1, mouse hybridoma supernatant from CRL-2700; ATCC) to neutralize any
429 residual VSV-G virus input⁵⁵. 24h later supernatant containing SARS-CoV-2 VSV pseudotypes
430 was harvested.

431 To quantify SARS-CoV-2 nAbs, serial dilutions of serum samples were incubated for 1 hour at
432 37 °C with an equal volume of SARS-CoV-2 pseudotyped VSV particles and inoculated on
433 Vero E6 cells for 18 hours. Neutralizing titers (SNT₅₀) for YFV were determined with an in-
434 house developed fluorescence based assay using a mCherry tagged variant of YF17D virus^{10,39}.
435 To that end, serum dilutions were incubated in 96-well plates with the YF17D-mCherry virus
436 for 1h at 37 °C after which serum-virus complexes were transferred for 72 h to BHK-21J cells.
437 The percentage of GFP or mCherry expressing cells was quantified on a Cell Insight CX5/7
438 High Content Screening platform (Thermo Fischer Scientific) and neutralization IC₅₀ values
439 were determined by fitting the serum neutralization dilution curve that is normalized to a virus
440 (100%) and cell control (0%) in Graphpad Prism (GraphPad Software, Inc.).

441 **SARS-CoV-2 plaque reduction neutralization test (PRNT)**

442 Sera were serially diluted with an equal volume of 70 PFU of SARS-CoV-2 before incubation
443 at 37 °C for 1h. Serum-virus complexes were added to Vero E6 cell monolayers in 24-well
444 plates and incubated at 37 °C for 1h. Three days later, overlays were removed and stained with
445 0.5% crystal violet after fixation with 3.7% PFA. Neutralization titers (PRNT₅₀) of the test
446 serum samples were defined as the reciprocal of the highest test serum dilution resulting in a
447 plaque reduction of at least 50%.

448 **Antigens for T cell assays**

449 PepMix™ Yellow Fever (NS4B) (JPT-PM-YF-NS4B) and subpool-1 (158 overlapping 15-
450 mers) of PepMix™ SARS-CoV-2 spike (JPT-PM-WCPV-S-2) were used as recall antigens for
451 ELISpot and ICS. Diluted Vero E6 cell lysate (50 µg/mL) and a combination of PMA (50
452 ng/mL) (Sigma-Aldrich) and Ionomycin (250 ng/mL) (Sigma-Aldrich) served as negative and
453 positive control, respectively.

454 **Intracellular cytokine staining (ICS) and flow cytometry**

455 Fresh mouse splenocytes were incubated with 1.6 µg/mL Yellow Fever NS4B peptide; 1.6
456 µg/mL Spike peptide subpool-1; PMA (50 ng/mL)/Ionomycin (250 ng/mL) or 50 µg/mL Vero
457 E6 cell for 18h at 37 °C. After treatment with brefeldin A (Biolegend) for 4h, the splenocytes
458 were stained for viability with Zombie Aqua™ Fixable Viability Kit (Biolegend) and Fc-
459 receptors were blocked by the mouse FcR Blocking Reagent (Miltenyi Biotec)(0.5µL/well) for
460 15 min in the dark at RT. Cells were then stained with extracellular markers BUV395 anti-CD3
461 (17A2) (BD), BV785 anti-CD4 (GK1.5) (Biolegend), APC/Cyanine7 anti-CD8 (53-6.7)
462 (Biolegend) and PerCP/Cyanine5.5 anti-TCR γ/δ (GL3) (Biolegend) in Brilliant Stain Buffer
463 (BD) before incubation on ice for 25 min. Cells were washed once with PBS and
464 fixed/permeabilized for 30 min by using the FoxP3 transcription factor buffer kit (Thermo
465 Fisher Scientific) according to the manufacturer's protocol. Finally, cells were intracellularly
466 stained with following antibodies: PE anti-IL-4 (11B11), APC anti-IFN- γ (XMG1.2),
467 PE/Dazzle™ 594 anti-TNF- α (MP6-XT22), Alexa Fluor® 488 anti-FOXP3 (MF-14), Brilliant
468 Violet 421 anti-IL-17A (TC11-18H10.1) (all from Biolegend) and acquired on a BD
469 LSRFortessa™ X-20 (BD). All measurements were calculated by subtracting from non-
470 stimulated samples (incubated with non-infected Vero E6 cell lysates) from corresponding
471 stimulated samples. The gating strategy employed for ICS analysis is depicted in Fig. S9. The
472 strategy used for comparative expression profiling of vaccine-induced T-cell populations by t-

473 distributed Stochastic Neighbor Embedding (t-SNE) analysis is outlined in Fig. S8. All flow
474 cytometry data were analysed using FlowJo Version 10.6.2 (LLC)). t-SNE plot was generated
475 in Flowjo after concatenating spike-specific CD4 and CD8 T cell separately based on gated
476 splenocyte samples.

477 **ELISpot**

478 ELISpot assays for the detection of IFN- γ -secreting mouse splenocytes were performed with
479 mouse IFN- γ kit (ImmunoSpot[®] MIFNG-1M/5, CTL Europe GmbH). IFN- γ spots were
480 visualized by stepwise addition of a biotinylated detection antibody, a streptavidin-enzyme
481 conjugate and the substrate. Spots were counted using an ImmunoSpot[®] S6 Universal Reader
482 (CTL Europe GmbH) and normalized by subtracting spots numbers from control samples
483 (incubated with non-infected Vero E6 cell lysates) from the spot numbers of corresponding
484 stimulated samples. Negative values were corrected to zero.

485 **qPCR for transcription factor profile.**

486 Spike peptide-stimulated splenocytes split were used for RNA extraction by using the
487 sNucleoSpin[™] Kit Plus kit (Macherey-Nagel). cDNA was generated by using a high-capacity
488 cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed
489 using the TaqMan gene expression assay (Applied Biosystems) on an ABI 7500 fast platform.
490 Expression levels of TBX21, GATA3, RORC, FOXP3 (all from Integrated DNA Technologies)
491 were normalized to the expression of GAPDH (IDT). Relative gene expression was assessed
492 by using the $2^{-\Delta\Delta Cq}$ method.

493 **Statistical analysis**

494 GraphPad Prism (GraphPad Software, Inc.) was used for all statistical evaluations. The number
495 of animals and independent experiments that were performed is indicated in the figure legends.
496 Statistical significance was determined using the non-parametric Mann-Whitney U-test and
497 Kruskal-Wallis test if not otherwise stated. Values were considered significantly different at P
498 values of ≤ 0.05 .

499

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501

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519

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552

553 **Declaration of Interests**

554 L.S.F., J.N., and K.D. are named as inventors on patent describing the invention and use of coronavirus
555 vaccines. All other authors have nothing to disclose.

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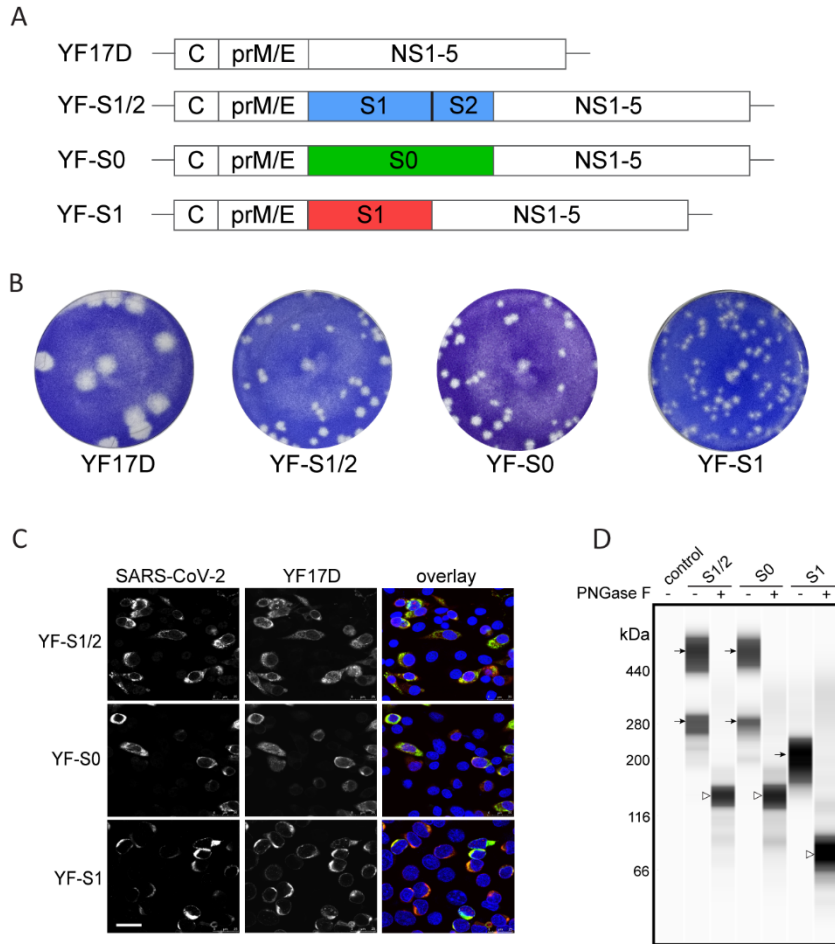
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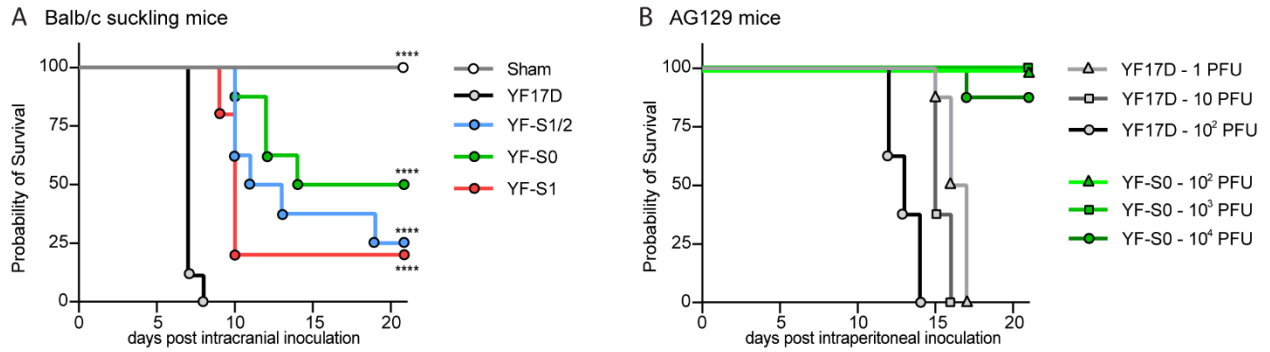
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710

711 **Fig. 1. Vaccine design and antigenicity.** (A) Schematic representation of YF17D-based SARS-CoV-2
 712 vaccine candidates (YF-S). YF-S1/2 expresses the native cleavable post-fusion form of the S protein
 713 (S1/2), YF-S0 the non-cleavable pre-fusion conformation (S0), and YF-S1 the N-terminal (receptor
 714 binding domain) containing S1 subunit of the S protein. For molecular details in vaccine design see
 715 Methods section. (B) Representative pictures of plaque phenotypes from different YF-S vaccine
 716 constructs on BHK-21 cells in comparison to YF17D. (C) Confocal immunofluorescent images of BHK-
 717 21 cells three days post-infection with different YF-S vaccine constructs staining for SARS-CoV-2
 718 Spike antigen (green) and YF17D (red) (nuclei stained with DAPI, blue; white scalebar: 25 μ m). (D)
 719 Immunoblot analysis of SARS-CoV-2 Spike (S1/2, S0 and S1) antigen and SARS Spike expression after
 720 transduction of BHK-21 cells with different YF-S vaccine candidates. Prior to analysis, cell lysates were
 721 treated with PNGase F to remove their glycosylation or left untreated (black arrows – glycosylated forms
 722 of S; white arrows – de-glycosylated forms).

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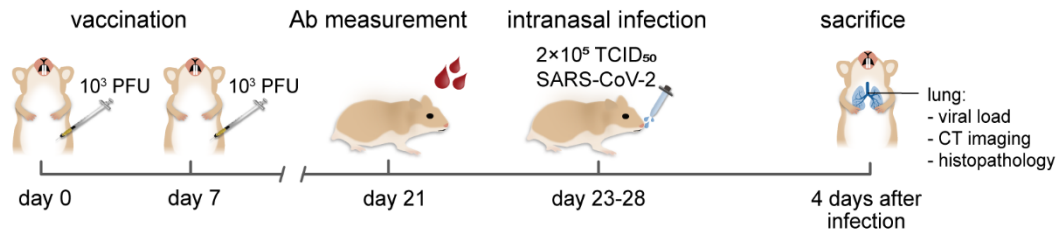


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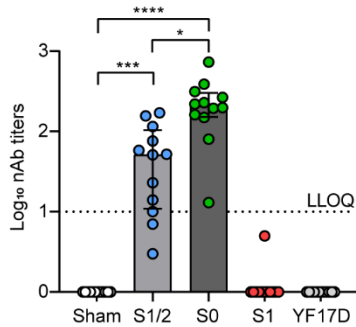
725 **Fig. 2. Attenuation of YF-S vaccine candidates. (A)** Survival curve of suckling Balb/c mice (up to 21
726 days) after intracranial (i.c.) inoculation with 100 PFU of vaccine candidates YF-S1/2 (n=8, blue), YF-
727 S0 (n=8, green), YF-S1 (n=8, red) in comparison to sham (n=10, grey) or YF17D (n=9, black). **(B)**
728 Survival curve of AG129 mice (up to 21 days) after intraperitoneal (i.p.) inoculation with a dose range
729 of YF-S0 (10^2 , 10^3 and 10^4 PFU; green) in comparison to YF17D (1, 10 and 10^2 PFU black and grey).
730 Statistical significance between groups was calculated by the Log-rank Mantel-Cox test (**** $P <$
731 0.0001).

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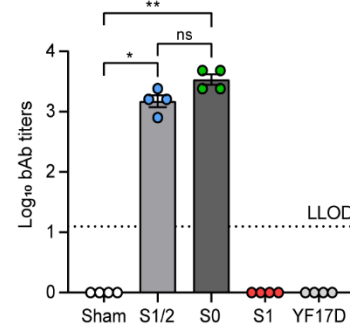
A Vaccination and challenge schedule



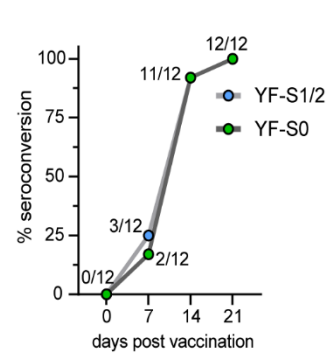
B nAb titers



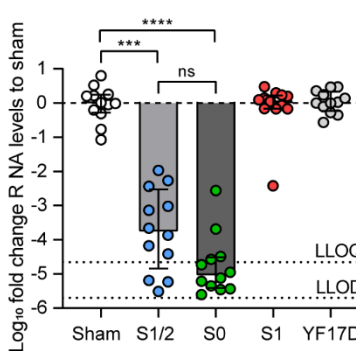
C bAb titers



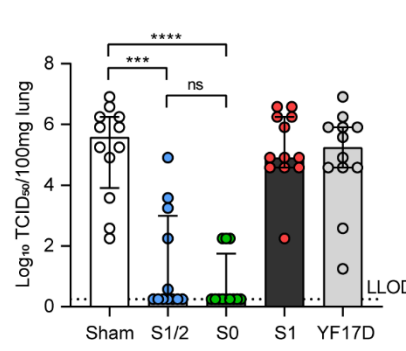
D Seroconversion rates



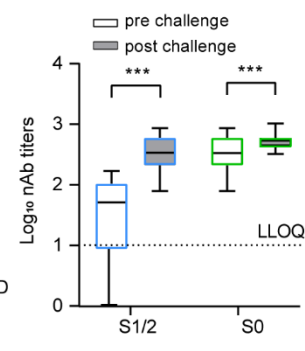
E Viral RNA in lungs



F Infectious virus in lungs



G nAb titers pre/post challenge



733

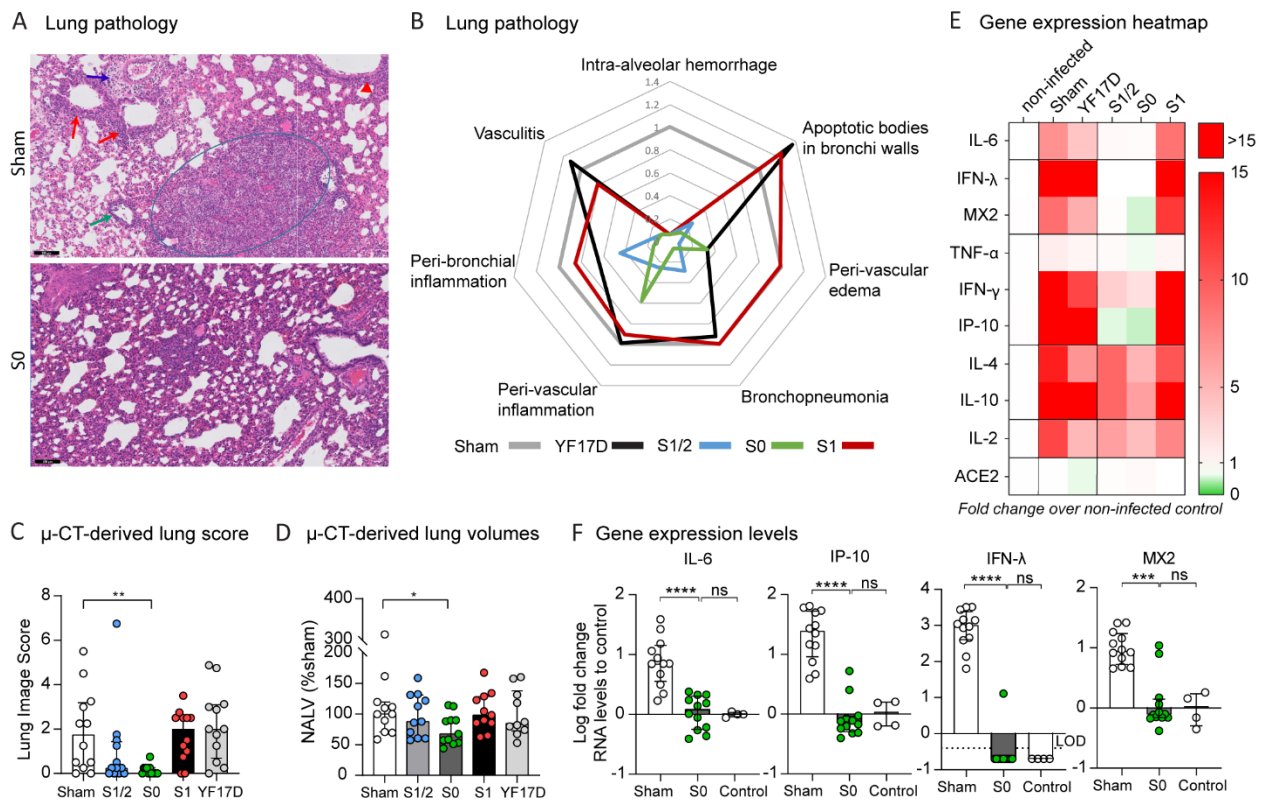
734 **Fig. 3. Immunogenicity and protective efficacy of YF-S vaccine candidates in hamsters. (A)**
 735 **Schematic representation of vaccination and challenge schedule.** Syrian hamsters were immunized
 736 twice i.p. at day 0 and 7 with 10³ PFU each of vaccine constructs YF-S1/2 (blue, n=12), YF-S0 (green,
 737 n=12), YF-S1 (red, n=12), sham (white, n=12) or YF17D (grey, n=12) (two independent experiments).
 738 Subsequently, animals were intranasally inoculated with 2 × 10⁵ TCID₅₀ of SARS-CoV-2 and followed
 739 up for four days. **(B-D) Humoral immune responses.** Neutralizing antibodies (nAb) **(B)** and total
 740 binding IgG (bAb) **(C)** in hamsters vaccinated with different vaccine candidates (sera collected at day
 741 21 post-vaccination in both experiments; minipools of sera of three animals each were analyzed for
 742 quantification of bAb). **(D)** Seroconversion rates at indicated days post-vaccination with YF-S1/2 and
 743 YF-S0 (number of animals with detectable bAbs at each time point are referenced). **(E, F) Protection**
 744 **from SARS-CoV-2 infection.** Viral loads in lungs of hamsters four days after intranasal infection were
 745 quantified by RT-qPCR **(E)** and virus titration **(F)**. Viral RNA levels were determined in the lungs,
 746 normalized against β-actin and fold-changes were calculated using the 2^(-ΔΔC_q) method compared to the
 747 median of sham-vaccinated hamsters. Infectious viral loads in the lungs are expressed as number of
 748 infectious virus particles per 100 mg of lung tissue. **(G) Anamnestic response.** Comparison of the levels
 749 of nAbs prior and four days after challenge. For a pairwise comparison of responses in individual
 750 animals see Supplementary Figure S4C and D. Dotted line indicating lower limit of quantification
 751 (LLOQ) or lower limit of detection (LLOD) as indicated. Data shown are medians ± IQR. Statistical

752 significance between groups was calculated by the non-parametric ANOVA, Kruskal-Wallis with
 753 uncorrected Dunn's test (B-F), or a non-parametric two-tailed Wilcoxon matched-pairs rank test (G) (ns
 754 = Non-Significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

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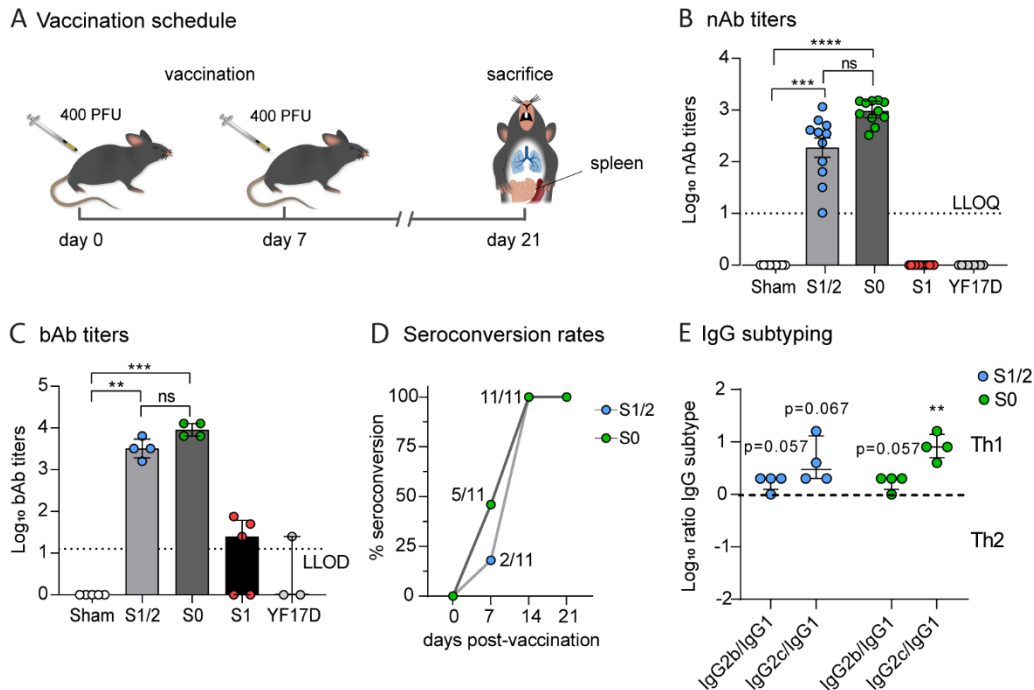


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759 **Fig. 4. Protection from lung disease in YF-S vaccinated hamsters. (A) Representative H&E images**
 760 **of the lungs of a diseased (sham-vaccinated and infected) and a YF-S0-vaccinated and challenged**
 761 **hamster. Peri-vascular edema (blue arrow); peri-bronchial inflammation (red arrows); peri-vascular**
 762 **inflammation (green arrow); bronchopneumonia (circle), apoptotic body in bronchial wall (red**
 763 **arrowhead). (B) A spider-web plot showing histopathological score for signs of lung damage (peri-**
 764 **vascular edema, bronchopneumonia, peri-vascular inflammation, peri-bronchial inflammation,**
 765 **vasculitis, intra-alveolar hemorrhage and apoptotic bodies in bronchus walls) normalized to sham (grey).**
 766 **Black scalebar: 100 μm (C-D) Micro-CT-derived analysis of lung disease. Five transverse cross**
 767 **sections at different positions in the lung were selected for each animal and scored to quantify lung**
 768 **consolidations (C) or used to quantify the non-aerated lung volume (NALV) (D), as functional**
 769 **biomarker reflecting lung consolidation. (E) Heat-map showing differential expression of selected**
 770 **antiviral, pro-inflammatory and cytokine genes in lungs of sham- or YF-S-vaccinated hamsters after**
 771 **SARS-CoV-2 challenge four days p.i. (n=12 per treatment group) relative to non-treated non-infected**
 772 **controls (n=4) (scale represents fold-change over controls). RNA levels were determined by RT-qPCR**
 773 **on lung extracts, normalized for β-actin mRNA levels and fold-changes over the median of uninfected**
 774 **controls were calculated using the $2^{-(\Delta\Delta Cq)}$ method. (F) Individual expression profiles of mRNA levels**
 775 **of IL-6, IP-10, IFN-λ and MX2, with data presented as median ± IQR relative to the median of non-**
 776 **treated non-infected controls. For IFN-λ, where all control animals had undetectable RNA levels, fold-**
 777 **changes were calculated over the lowest detectable value (LLOD – lower limit of detection; dotted line).**

778 Statistical significance between conditions was calculated by the non-parametric ANOVA, Kruskal-
 779 Wallis with uncorrected Dunn's test (ns = Not-Significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P <$
 780 0.001).

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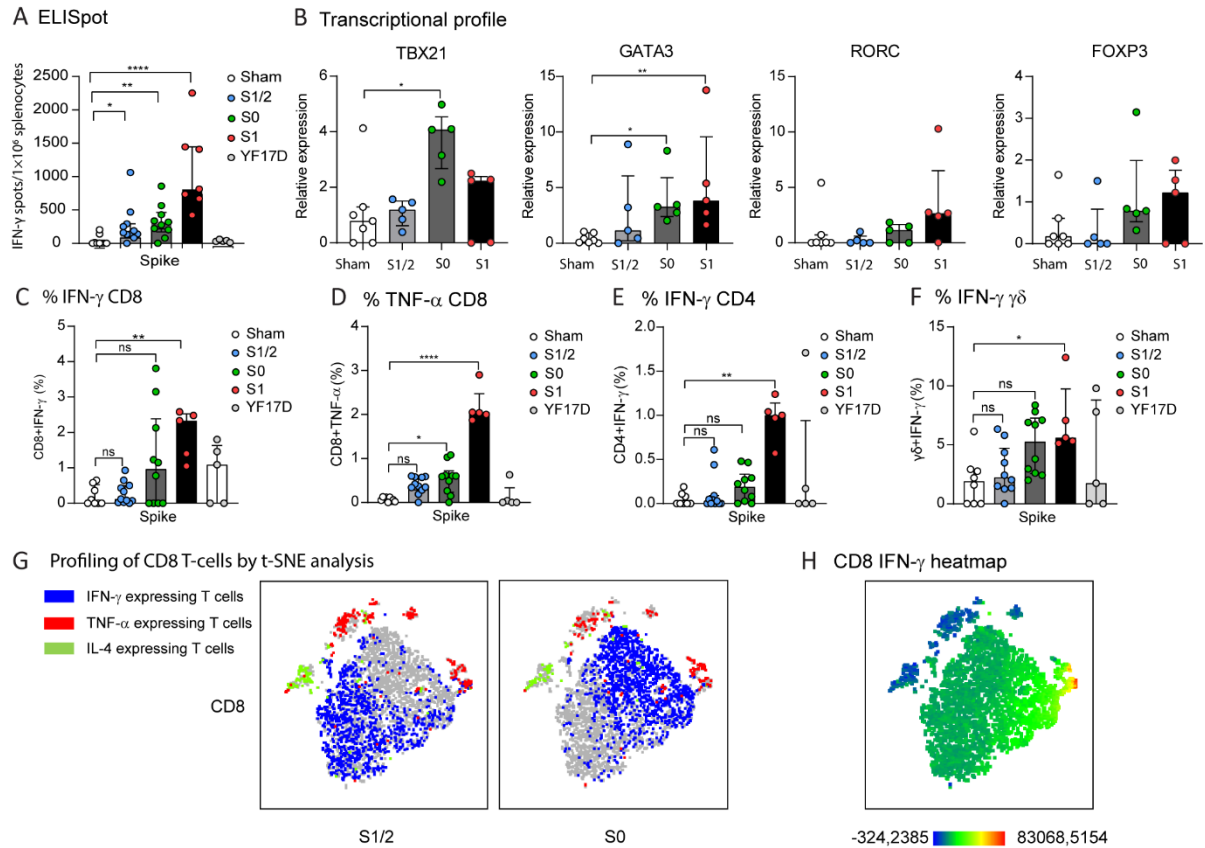


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783 **Fig. 5. Humoral immune response elicited by YF-S vaccine candidates in mice. (A) Schematic**
 784 **presentation of immunization and challenge schedule.** *Ifnar*^{-/-} mice were vaccinated twice i.p. with
 785 400 PFU each at day 0 and 7 in five groups: constructs YF-S1/2 (blue, n=11), YF-S0 (green, n=11), YF-
 786 S1 (red, n=13), sham (white, n=9) or YF17D (grey, n=9). **(B, C) SARS-CoV-2 specific antibody levels.**
 787 Titers of nAbs **(B)** and bAbs **(C)** at day 21 post-vaccination. **(D) Seroconversion rates.** Rates at
 788 indicated days post-vaccination with YF-S1/2 and YF-S0 (number of animals with detectable bAbs at
 789 each time point are referenced). For quantification of bAbs, minipools of sera of two to three animals
 790 each were analyzed. Dotted lines indicate lower limit of quantification (LLOQ) or lower limit of
 791 detection (LLOD). **(E) IgG for YF-S1/2 (blue) and YF-S0 (green).** Ratios of IgG2b or IgG2c over
 792 IgG1 (determined for minipools of two to three animals each) plotted and compared to a theoretical limit
 793 between Th1 and Th2 response (dotted line indicates IgG2b/c over IgG1 ratio of 1). Data shown are
 794 medians ± IQR from three independent vaccination experiments (n > 9 for each condition). Statistical
 795 significance between groups was calculated by a non-parametric ANOVA, Kruskal-Wallis with
 796 uncorrected Dunn's test (B-C) or parametric One-Sample T-test (D) (ns = Not-Significant, $P > 0.05$, * P
 797 < 0.05 , ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

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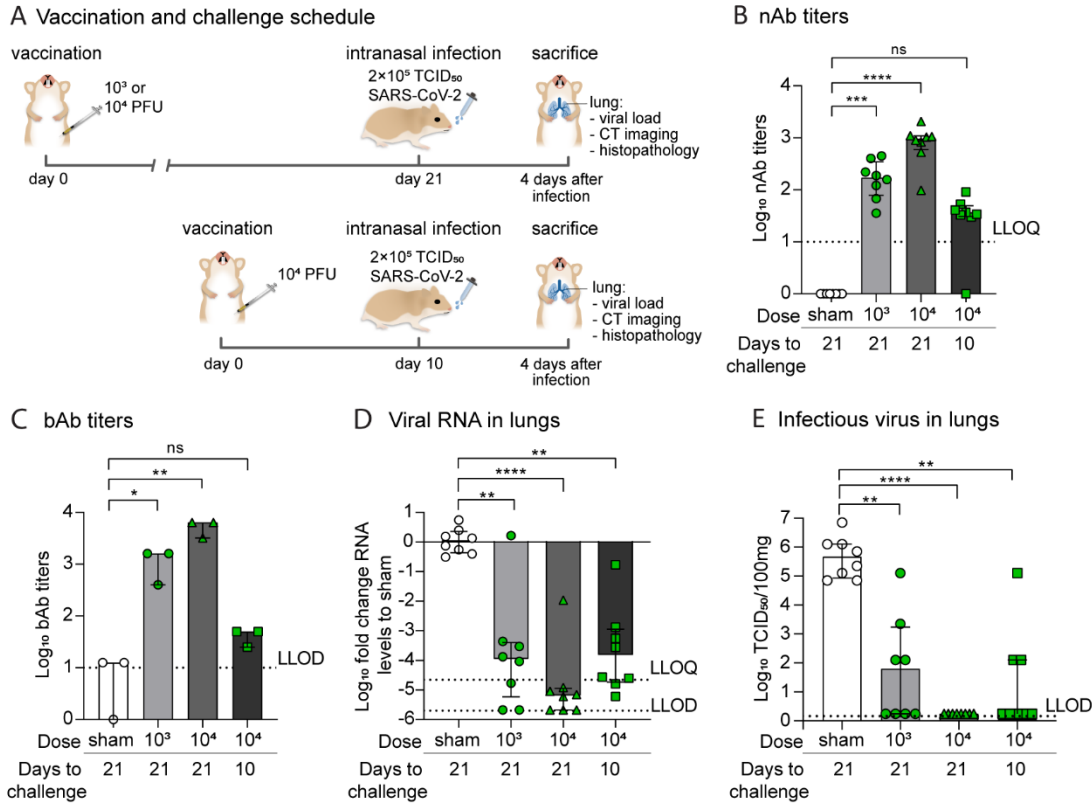


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801 **Fig. 6. Cell-mediated immune (CMI) responses of YF-S vaccine candidates in mice.** Spike-specific
 802 T-cell responses were analyzed by ELISpot and intracellular cytokine staining (ICS) of splenocytes
 803 isolated from *ifnar*^{-/-} mice 21 days post-prime (*i.e.*, two weeks post-boost) immunization with YF-S1/2
 804 (blue), YF-S0 (green), YF-S1 (red) in comparison to sham (white) or YF17D (grey). **(A) Quantitative**
 805 **assessment of SARS-CoV-2 specific CMI response by ELISpot.** Spot counts for IFN- γ -secreting cells
 806 per 10⁶ splenocytes after stimulation with SARS-CoV-2 Spike peptide pool. **(B) Transcriptional**
 807 **profile induced by YF-S vaccination.** mRNA expression levels of transcription factors (TBX21,
 808 GATA3, RORC, FOXP3) determined by RT-qPCR analysis of Spike peptide-stimulated splenocytes
 809 ($n=5-7$ per condition). Data were normalized for GAPDH mRNA levels and fold-changes over median
 810 of uninfected controls were calculated using the $2^{(-\Delta\Delta C_q)}$ method. **(C-F)** Percentage of IFN- γ **(C)** and
 811 TNF- α **(D)** expressing CD8, and IFN- γ expressing CD4 **(E)** and γ/δ **(F)** T-cells after stimulation with
 812 SARS-CoV-2 Spike peptide pool. All values normalized by subtracting spots/percentage of positive
 813 cells in corresponding unstimulated control samples. Data shown are medians \pm IQR. Statistical
 814 significance between groups was calculated by the non-parametric ANOVA, Kruskal-Wallis with
 815 uncorrected Dunn's test (ns = Not-Significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **(G, H)**
 816 **Profiling of CD8 T-cells from YF-S1/2 and YF-S0 vaccinated mice by t-SNE analysis.** t-distributed
 817 Stochastic Neighbor Embedding (t-SNE) analysis of spike-specific CD8 T-cells positive for at least one
 818 intracellular marker (IFN- γ , TNF- α , IL-4) from splenocytes of *ifnar*^{-/-} mice immunized with YF-S1/2 or
 819 YF-S0 ($n=6$ per group) after overnight stimulation with SARS-CoV-2 Spike peptide pool. Blue dots
 820 indicate IFN- γ expressing T-cells, red dots TNF- α expressing T-cells, and green dots IL-4 expressing
 821 CD8 T-cells. **(H) Heatmap** of IFN- γ expression density of spike-specific CD8 T-cells from YF-S1/2
 822 and YF-S0 vaccinated mice. Scale bar represents IFN- γ expressing density (blue – low expression, red
 823 – high expression) (see Figure S8 for full analysis).

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827 **Fig. 7. Single shot vaccination in hamsters using the YF-S0 lead vaccine candidate. (A) Schematic**
 828 **presentation of experiment.** Three groups of hamsters were vaccinated only once i.p. with sham (white;
 829 $n=8$) or YF-S0 at two different doses; 1×10^3 PFU (low, green circles; $n=8$) and 10^4 PFU (high, green
 830 triangles; $n=8$) of YF-S0 at 21 days prior to challenge. A fourth group was vaccinated with the high 10^4
 831 PFU dose of YF-S0 at 10 days prior to challenge (green squares; $n=8$). **(B-C) Humoral immune**
 832 **responses following single dose vaccination.** Titers of nAb **(B)** and bAb **(C)** in sera collected from
 833 vaccinated hamsters immediately prior to challenge (minipools of sera of two to three animals analyzed
 834 for quantification of bAb). **(D, E) Protection from SARS-CoV-2 infection.** Protection from challenge
 835 with SARS-CoV-2 after vaccination with YF-S0 in comparison to sham vaccinated animals, as
 836 described for two-dose vaccination schedule (Figure 3 and Figure S5); log₁₀-fold change relative to sham
 837 vaccinated in viral RNA levels **(D)** and infectious virus loads **(E)** in the lung of vaccinated hamsters at
 838 day four p.i. as determined by RT-qPCR and virus titration, respectively. Dotted line indicating lower
 839 limit of quantification (LLOQ) or lower limit of detection (LLOD) as indicated. Data shown are medians
 840 \pm IQR. Statistical significance between groups was calculated by the non-parametric ANOVA, Kruskal-
 841 Wallis with uncorrected Dunn's test (ns = Non-Significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, **** $P <$
 842 0.0001).

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