

Lineage-mosaic and mutation-patched spike proteins for broad-spectrum COVID-19 vaccine

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Abstract

The widespread SARS-CoV-2 in humans results in the continuous emergence of new variants. Recently emerged Omicron variant with multiple spike mutations sharply increases the risk of breakthrough infection or reinfection, highlighting the urgent need for new vaccines with broad-spectrum antigenic coverage. Using inter-lineage chimera and mutation patch strategies, we engineered a recombinant monomeric spike variant (STFK1628x), which showed high immunogenicity and mutually complementary antigenicity to its prototypic form (STFK). In hamsters, a bivalent vaccine comprised of STFK and STFK1628x elicited high titers of broad-spectrum antibodies to neutralize all 14 circulating SARS-CoV-2 variants, including Omicron; and fully protected vaccinees from intranasal SARS-CoV-2 challenges of either the ancestral strain or immune-evasive Beta variant. Strikingly, the vaccination of hamsters with the bivalent vaccine completely blocked the within-cage virus transmission to unvaccinated sentinels, for either the ancestral SARS-CoV-2 or Beta variant. Thus, our study provides new insights and antigen candidates for developing next-generation COVID-19 vaccines.

Introduction

The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) poses an unbearable public health burden. The SARS-CoV-2 spike mainly contains three immunogenic domains as targets of neutralizing antibody (nAb), the N-terminal domain (NTD), the receptor-binding domain (RBD), and the subunit 2 (S2), thereby serving as the essential antigen of COVID-19 vaccines. Though several COVID-19 vaccines are available, the constant emergence of SARS-CoV-2 variants is challenging against the protective efficacy of vaccination. Viral genome mutations may alter the biological phenotypes of SARS-CoV-2 in many aspects, such as viral infectivity, pathogenicity, and antigenicity. Critically, the

1 amino-acid substitutions in the antigenic sites of the spike protein may enable
2 viruses to escape from naturally acquired and vaccine-induced immunity (1).
3 Among the variants currently identified as variants of concern (VOCs) or
4 variants of interest (VOIs), many were able to cause immune escape. The Beta
5 (B.1.351) variant, first identified in South Africa, was found to cause a 6.5-8.6-
6 fold decrease in nAb titers raised by existing mRNA vaccines (2). Besides, the
7 Gamma (P.1), Delta (B.1.617.2), and Mu (B.1.621) variants also caused a 3.8-
8 4.8-, 2.9- and 9.1-fold nAb decrease, respectively, according to previous reports
9 (3, 4). The recently emerged Omicron (B.1.1.529) variant, being firstly identified
10 in November 2021 and rapidly designated as a new VOC, has shown elevated
11 risks in causing risk of breakthrough infection or reinfection due to its multiple
12 spike mutations altering viral antigenicity (5-9).

13 Most of the currently licensed COVID-19 vaccines were designed based on the
14 SARS-CoV-2 prototype spike; their vaccine effectiveness (VE) appeared to be
15 compromised in countering variants with immune evasion. The phase 3 clinical
16 trial of AZD1222 indicated that this vaccine was ineffective against mild-to-
17 moderate COVID-19 disease due to the Beta variant (10). For Omicron, a
18 recent study showed that the efficacy of two doses of BNT162b2 against
19 symptomatic illnesses caused by this variant was only about 30%, whereas
20 AZD1222 did not show a significant protective effect (11). Several vaccine
21 manufacturers have announced their plans of antigen updates in developing
22 vaccines with broad-spectrum protection (12, 13). An ideal goal is to develop a
23 new antigen providing a broad-spectrum coverage for all SARS-CoV-2 variants
24 that resist nAbs raised by the prototypic spike. However, it remains hugely
25 challenging to achieve this goal.

26 In this study, using inter-lineage chimera and mutation patch strategies, we
27 generated a serial of monomeric spike ectodomain proteins harboring multi-site
28 mutations from different VOC/VOI variants. Our evaluations demonstrated a
29 chimeric spike protein of STFK1628x, containing NTD from B.1.620 lineage,

1 RBD-S2 from the Gamma variant, and additional RBD mutation patches from
2 the Delta variant, showed mutually complementary antigenicity to the ancestral
3 spike-derived STFK protein. The bivalent vaccine of STFK plus STFK1628x
4 exhibited high immunogenicity to elicit high titers of broad-spectrum neutralizing
5 antibodies to protect against *in vivo* challenges with the ancestral SARS-CoV-
6 2 and Beta variant in hamsters. More importantly, we further evidenced the new
7 bivalent vaccine could block the within-cage virus transmission from vaccinated
8 hamsters to unvaccinated sentinels. Overall, our findings shed light on the
9 understandings of antigenic and immunogenic characteristics of SARS-CoV-2
10 spike variants, also providing antigen candidates for developing next-
11 generation COVID-19 vaccines.

12 **Results**

13 **Monomeric spike ectodomain STFK protein is highly immunogenic in** 14 **rodents and nonhuman primates**

15 Several studies had demonstrated the immunogenicity of recombinant SARS-
16 CoV-2 spike ectodomain protein in trimeric forms (14-17). However, introducing
17 the exogenous trimerization domain may elicit an unexpected immune
18 response(14). Moreover, the recombinant trimer protein may dissociate during
19 the cell cultures and downstream purification process, which decreases the
20 yield of homogeneous trimeric proteins for vaccine production. Therefore, we
21 tried to design and produce monomeric spike proteins for the COVID-19
22 vaccine to address these issues. Although numerous high-resolution structures
23 of SARS-CoV-2 spike trimers were reported, the detailed structure of the S2 C-
24 terminus, particularly for those after amino acid (aa) 1146, was not resolved.
25 We tested eight constructs of furin site mutated spike ectodomain with
26 progressively truncated C-terminus in Chinese hamster ovary (CHO) cells.
27 Interestingly, the C-terminal truncation to various positions between aa1152 and
28 aa1192 resulted in higher expression levels and purification yields than the
29 construct encompassing the entire ectodomain (S1208) (**Fig. 1A**). In addition,

1 the C-terminal truncated spike proteins presented comparable, even better
2 ACE2 binding activity to the trimeric StriFK protein (17) (**fig. S1A**). To minimize
3 the potential epitope loss associated with C-terminal truncation, we finally
4 chose the construct of S1192 encompassing aa 1-1192 (hereafter designated
5 STFK) as an immunogen candidate for further study. As expected, the STFK
6 was presented in monomeric form, as evidenced by the SEC-HPLC and native-
7 PAGE analyses (**Fig. 1B**). In contrast to the trimeric StriFK, the STFK elicited
8 significantly higher nAb titers against the pseudotyped virus (PsV) in mice at
9 weeks 1 ($P = 0.002$) and 2 ($P = 0.028$) after the 1st prime vaccine dose,
10 suggesting the advantage of the STFK for induction of rapid humoral response.
11 After the 2nd dose immunization, both STFK and StriFK-based vaccines
12 generated comparable nAb titers (**Fig. 1C**).

13 To determine the structural basis for the excellent immunogenicity of the STFK,
14 we resolved the cryo-electron microscopy (cryo-EM) structure of the STFK in
15 complex with three previously reported nAbs 36H6, 83H7, and 85F7 (18, 19).
16 Following the previous classifications of the nAbs targeting epitopes (Class I-V)
17 (20, 21), the 36H6, 83H7, and 85F7 were categorized into Class II, V, and III,
18 according to their binding modes, respectively (**fig. S2** and **S3**). The 3.81Å
19 resolution structure of the immune-complex confirmed the monomeric form of
20 the STFK, which could interact with three antigen-binding fragments (Fabs) of
21 nAbs simultaneously (**Fig. 1D**, **fig. S2A**, and **Table S1**). The STFK is
22 structurally similar to the monomeric form dissociated from a spike trimer (22).
23 Due to the conformational flexibility in the monomeric form, the S2 subunit was
24 not visualized in the reconstruction. However, in contrast to the trimeric spike,
25 the STFK presents a more exposed RBD and NTD, thereby making the nAb
26 epitopes more accessible and may contribute to its advantage for eliciting rapid
27 nAb response.

28 Next, we evaluated the dose-dependent immunogenicity of the STFK-based
29 vaccine with the FH002C adjuvant in the BALB/c mice, rhesus monkeys, and

1 golden hamsters. In our previous study, the FH002C, a risedronate-modified
2 new adjuvant, showed potent immunostimulatory effects for hormonal and
3 cellular immune responses (17). In BALB/c mice, STFK vaccinations generated
4 a dose-dependent response for the anti-spike IgG, anti-RBD IgG (**fig. S1B**),
5 and neutralizing antibodies at 0.01 to 10 µg dose levels (**Fig. 1E**). Two injections
6 of STFK at a dose level as low as 0.1 µg induced a potent nAb response
7 showing geometric mean titers (GMT) of 3.9 log₁₀ against the PsV and 362
8 against the authentic virus (200 TCID₅₀) that were 3.5- and 2.3-fold higher than
9 that of the NIBSC 20/136 anti-SARS-CoV-2 standard (1,000 IU/mL) in the
10 corresponding assays (**Fig. 1E**). In rhesus monkeys, STFK vaccinations at 1 or
11 15 µg dose levels also elicited strong humoral immune responses (**Fig. 1F** and
12 **fig. S1C**), as shown that immunized animals presented high nAb titers against
13 either PsV (GMT=4.1 and 4.5 log₁₀ for 1 and 15 µg groups, respectively) or
14 authentic virus (GMT=588 and 1,351 for 1 and 15 µg groups, respectively). In
15 addition, hamsters that received STFK vaccines of 0.1-10 µg per dose showed
16 comparable and >4.0 log₁₀ of nAb titers at week-2 after the boost dose (**Fig. 1G**
17 and **fig. S1D**). Overall, the nAb titers (referred to as PsV nAb GMTs) elicited by
18 1 µg of FH002C-adjuvanted STFK vaccine were about 4.2 to 9.3-fold higher
19 than that of the NIBSC 20/136 standard in mice, monkeys, and hamsters. Apart
20 from humoral immunity, vaccinated mice also presented potent spike-specific T
21 cell responses ($P < 0.001$) (**fig. S1E**). These data demonstrated the promising
22 immunogenicity of the monomeric STFK recombinant protein in rodents and
23 nonhuman primates.

24 **Engineered STFK variant protein provides broad antigenic coverage** 25 **which compensates with prototypic spike**

26 We investigated the impacts of 14 VOC/VOI variants on nAbs raised by the
27 prototypic STFK in animals. Notably, all sera from ten monkeys and eight
28 hamsters at week-2 after 2-dose vaccinations showed detectable nAbs against
29 all PsVs bearing VOC/VOI spike variants, including the newly emerged

1 Omicron (**Fig. 1H**). However, by contrast to that against D614G lineage, STFK-
2 elicited nAb titers in monkeys were markedly decreased (>5-fold) for Beta
3 (6.5×), Mu (B.1.621) (14×), B.1.620 (17×), Omicron (34×); were mild to
4 moderately reduced (2- to 5-fold) for A.VOI.V2 (4.4×), Gamma (2.9×), Eta
5 (B.1.525) (3.2×), Iota_484K (B.1.526) (2.8×), Iota_477N (B.1.526) (2.4×), and
6 Kappa (B.1.617.1) (2.0×) (**Fig. 1H and 1I**). For Alpha, Delta, Epsilon (B.1.429),
7 and Lambda (C.37) variants, the nAb titers only slightly changed a (<2-fold).
8 Moreover, sera from immunized hamsters presented highly similar ($R^2=0.889$,
9 $P < 0.001$) cross-neutralizing profiles to that of monkeys (**Fig. 1I**). These results
10 are consistent with findings in humans that the E484K-harboring variants and
11 the Omicron may markedly evade nAbs raised by the prototypic spike.

12 Following the approach as graphically depicted in **Fig. 2A**, we aimed to develop
13 a new STFK antigen providing complementary antigenic coverage to the
14 prototypic protein to address the concerns for the evasive variants (**Fig. 2A**).
15 As the Mu and Omicron variants had not emerged when our experiment started,
16 we firstly tested mutated STFK antigens based on the spikes of Beta
17 (STFK1351), Gamma (STFK1128), and B.1.620 (STFK1620) variants (**fig. S4**).
18 Compared to those immunized with STFK, hamsters vaccinated with
19 STFK1351, STFK1128, and STFK1620 showed 1.0-3.0×, 1.3-6.2×, and 1.7-
20 5.5× increased nAb titers (GMTs) in neutralizing four immune-escape variants
21 (Gamma, A.VOI.V2, Beta, and B.1.620) (**fig. S5**). The STFK1128 exhibited
22 better immunogenicity than the other two, as it raised ~4.0 log₁₀ of nAb GMT in
23 neutralizing its parental virus (Gamma) (**fig. S5**). In contrast, Beta variant-
24 derived STFK1351 was poorly immunogenic.

25 We then introduced circulating RBD mutations absent in the Gamma variant
26 but present in other VOC/VOI viruses into the STFK1128 backbone for the
27 following engineering. We used the mutations of L452R (noted in Delta, Kappa,
28 Epsilon), S477N (presented in Iota_477N and B.1.620), T478K (Delta-derived),
29 and E484Q (Kappa-derived, to replace the E484K in STFK1128) to generate

1 six new antigens (**fig. S6**). Hamster immunization tests revealed that the
2 STFK1128e (L452R/S477N/E484Q), STFK1128f (L452R/T478K/E484K), and
3 STFK1128g (L452R/T478K/E484Q) displayed improvements on the cross-
4 neutralization spectrum in comparison to the STFK1128 (**fig. S6C**). As the Delta
5 became the dominant variant worldwide since June 2021, we selected the
6 STFK1128f exhibiting higher titers of nAbs to neutralize both Beta and Delta
7 viruses as a candidate for further optimization.

8 Besides RBD mutations, NTD deletions presented in several VOC/VOI variants
9 may also contribute to their immune-escape potentials. To cover the mutated
10 NTD epitopes, we designed two inter-lineage chimeric constructs of
11 STFK1328x and STFK1628x; the former included the NTD of STFK1351 (Beta),
12 and the latter had the NTD of STFK1620 (B.1.620). Both constructs shared the
13 RBD-S2 domain of STFK1128f, except a K417N in STFK1328x. Remarkably,
14 the STFK1628x could elicit a broad and potent neutralizing antibody response
15 in hamsters (**fig. S7A**), which showed 4.1-12.8× increased nAb titers (GMT)
16 than prototypic STFK in neutralizing the A.VOI.V2 (4.1×), Gamma (7.6×), Beta
17 (12.8×) and B.1.620 (8.5×). In contrast to its parental STFK1128f, the
18 STFK1628x also exhibited higher nAb titers against most VOC/VOI variants
19 (**Fig. 2B** and **fig. S7B**). These data supported the STFK1628x as a promising
20 antigen candidate for the updated COVID-19 vaccine. As our previous study
21 suggested that the aa 439-448 was another hot-spot region in addition to aa484
22 (19), we further made two modified STFK1628x versions, designated
23 STFK1628y and STFK1628z, that included N440K and G446V, respectively
24 (**Fig. 2B** and **fig. S7B**). The STFK1628y and STFK1628z displayed distinct
25 antigenic profiles in hamsters (**fig. S7**). Notably, in contrast to the prototypic
26 antigen, the STFK1628x and STFK1628y elicited significantly increased nAb in
27 neutralizing two newly emerged variants, Mu (5.5×, $P = 0.002$ for STFK1628x;
28 4.0×, $P = 0.010$ for STFK1628y) and Omicron (7.4×, $P = 0.002$ for STFK1628x;
29 30.7×, $P = 0.010$ for STFK1628y) (**fig. S7C**). Following these data, we

1 formulated a bivalent vaccine using the STFK1628x and the prototypic STFK
2 at a mass ratio of 1:1. To most of the VOC/VOI variants, hamsters immunized
3 with the bivalent vaccine showed significantly ($P < 0.05$) increased nAb levels
4 to that elicited by the prototypic antigen in neutralizing the D614G virus (~ 4.0
5 \log_{10}) (**Fig. 2B** and **2C**). Strikingly, the bivalent vaccine yielded a nAb GMT of
6 2,130 (ID_{50} range: 9,61 to 4,763) to the highly immune-evasive Omicron, which
7 was about 36-fold higher than the NIBSC 20/136 immunoglobulin standard
8 ($ID_{50}=60$ to Omicron). Taken together, STFK plus STFK1628x provided a full-
9 spectrum neutralization coverage to all VOC/VOI variants.

10 We also obtained a 3.88 Å cryo-EM structure of the STFK1628x in complexed
11 with three nAbs 83H7, 85F7, and 2B4 (**Fig. 2D**, **fig. S2B**, and **Table S1**). As the
12 T478K abolishes the activity of 36H6 nAb, we replaced the 36H6 with a class
13 IV mAb of 2B4 with cross-SARS-CoV-1/2 neutralization potency (**fig. S3D**) (18).
14 As expected, the STKF1628x presented a similar structure to STFK but showed
15 distinguished densities on the mutation sites, such as 417, 452, 478, 484, and
16 501, corresponding to its alternative antigenic profile (**Fig. 2E**).

17 **The bivalent vaccine protects hamsters against intranasal SARS-CoV-2** 18 **challenges**

19 To assess the ability of the STFK-based vaccine to mediate protection against
20 SARS-CoV-2, we intranasally challenged hamsters that received STFK,
21 STFK1628x, or bivalent vaccines (**Fig. 3A**). For either challenge with the
22 ancestral strain or Beta variant, vaccinated hamsters showed an average of
23 2.2-4.6% weight increase to their baseline levels by the end of a 7-day follow-
24 up (**Fig. 3B** and **3C**). By contrast, unvaccinated animals showed a maximum
25 weight loss of 14.8% and 13.8% by 7 days post-infection (dpi) in the ancestral
26 strain and Beta variant challenges, respectively. Moreover, 1 of 8 and 5 of 8
27 animals died from ancestral SARS-CoV-2 and beta variant infections,
28 respectively, but none in the vaccinated groups (**Fig. 3D** and **3E**).

29 At 7 dpi, the median viral RNA levels of control hamsters challenged by the

1 prototypic virus were 7.26 (range 5.24-7.78) log₁₀ in the lung, 6.88 (range 6.12-
2 7.32) log₁₀ in the nasal turbinate, and 6.29 (range 5.04-6.67) log₁₀ copies/mL in
3 the trachea (**Fig. 3F** and **fig. S8A**). By contrast, hamsters that received
4 vaccinations of either STFK, STFK1628x, or the bivalent version showed
5 significant ($P < 0.01$ for each comparison) viral RNA reductions by >5.0 log₁₀,
6 2.0-3.0 log₁₀, 3.0-4.0 log₁₀ copies/mL in tissues of the lung, nasal turbinate, and
7 trachea, respectively (**Fig. 3F** and **fig. S8A**). To protect the prototypic virus
8 challenge, the three vaccine candidates appeared with comparable efficacy ($P >$
9 0.05) in decreasing viral loads of the respiratory tract tissues (**Fig. 3F** and **fig.**
10 **S6A**). In the Beta variant challenges, control hamsters also showed high levels
11 of viral RNA similar to that infected with the prototypic virus in their respiratory
12 tract tissues. For vaccinated animals, the medians of pulmonary viral loads
13 were 1.05, 0.35, and 3.36 log₁₀ copies/mL in the STFK1628x, bivalent and
14 STFK groups, corresponding to reductions of 6.27 ($P < 0.0001$), 6.96 ($P <$
15 0.0001), and 3.96 ($P = 0.01$) log₁₀ to controls, respectively (**Fig. 3G**). In tissues
16 of nasal turbinate and trachea, no statistically significant difference in viral RNA
17 suppression was observed among the 3 vaccination groups (**fig. S8B**), but
18 hamsters immunized with the bivalent vaccine presented relatively lower viral
19 loads than the others.

20 In addition to mediating virological suppressions, the three vaccines also
21 protected hamsters from lung disease caused by SARS-CoV-2 infections.
22 Pathological examinations revealed most unvaccinated hamsters presented
23 severe pulmonary diseases at 7 dpi regardless of the challenged virus type (**Fig.**
24 **3H** and **3I**, **fig. S8C** and **S8D**). In contrast, gross lung observations and
25 pulmonary pathology scorings demonstrated all vaccinated animals were free
26 from moderate-to-severe pneumonia, with the only exception noted in one from
27 the STFK group challenged with Beta variant (**Fig. 3I**). These data indicated
28 that the STFK, STFK1628x, and the bivalent vaccines effectively protected
29 hamsters against the SARS-CoV-2 challenge. Moreover, the STFK1628x and

1 the bivalent vaccine showed better protective efficacy against the Beta variant
2 challenge in hamsters.

3 **The bivalent vaccine blocks SARS-CoV-2 transmissions in hamsters**

4 In addition to protecting vaccine users from SARS-CoV-2 infection and
5 pathology, an ideal COVID-19 vaccine should also reduce viral shedding and
6 transmission by vaccinated individuals exposed to the virus. For assessments,
7 index hamsters were immunized with either STFK1628x or the bivalent vaccine
8 and were challenged intranasally with ancestral SARS-CoV-2 or Beta variant.
9 One day later, naïve hamsters as sentinels were cohoused with index animals
10 for 24 hours (**Fig. 4A**). After a subsequent 7-day follow-up, sentinels of the
11 unvaccinated index hamsters showed an average weight loss of 4.0% and 1.7%
12 in the ancestral virus and beta variant challenges, respectively. In contrast,
13 sentinels of vaccinated indexes receiving either the STFK1628x or the bivalent
14 version exhibited gradually increased weights (**Fig. 4B and 4C**). By the end of
15 the experiment, all sentinels of the unvaccinated indexes had detectable viral
16 RNA with approximate levels of 6.0-7.0 log₁₀ copies/mL in the respiratory tract
17 tissues (**Fig. 4D and 4E**). By contrast, 4 (50%) and 1 (12.5%) sentinels
18 cohoused with STFK1628-vaccinated hamsters, when challenged with the Beta
19 variant and the ancestral SARS-CoV-2 respectively, showed detectable viral
20 RNA in either tissue from the lung, nasal turbinate, or trachea. Remarkably, for
21 either challenge of the two viruses, no sentinel hamster of the indexes
22 immunized with the bivalent vaccine showed detectable viral RNA in any tissues
23 from the lung, nasal turbinate, and trachea (**Fig. 4D and 4E**). These data
24 supported complete protection from SARS-CoV-2 transmission conferred by
25 the bivalent vaccine in hamsters.

26

27 **Discussion**

28 With the widespread SARS-CoV-2 infections in large populations of humans
29 and other susceptible animals, the emergence of antigenic drift variants seems

1 inevitable. The newly identified Omicron variant with >30 spike mutations
2 brought great challenges to established immunity by vaccination or resolved
3 infections. Recent studies revealed that the Omicron had led to widespread
4 escape from nAbs acquired from infections by the ancestral SARS-CoV-2 or
5 other VOC variants, including Alpha, Beta, Gamma, and Delta (23, 24),
6 exhibiting a distinctly altered antigenicity. This variant is also highly resistant
7 against nAbs elicited by double immunizations of the authorized COVID-19
8 vaccine (25). Though some studies have found the 3rd dose of vaccination may
9 increase the nAb titer against Omicron, it was still lower than that against other
10 variants (e.g., Delta) by several folds (26, 27). Next-generation COVID-19
11 vaccines with pan-variants protection are urgently required to deal with the
12 growing threat of Omicron-like immune-escape variants.

13 Adding another spike antigen of an immune-escape variant is the most common
14 approach for developing an updated COVID-19 vaccine. Before Omicron
15 emergence, the Beta and Gamma were two noticeable VOCs with increased
16 immune evasion concerns; therefore, most previous studies used spikes from
17 one of the two variants as the added antigen. The mRNA-1273.351, a Beta
18 variant-based mRNA vaccine developed by Moderna, could elicit a potent nAb
19 response against the Beta variant in mice but was inferior to that derived from
20 the prototype-based mRNA-1273 in neutralizing the Gamma and Epsilon
21 variants (27). Neutralizing antibodies elicited by vaccines formulated with either
22 Beta or Gamma-derived spikes in animals appeared to be less effective against
23 the Delta variant (28, 29). Consistent findings were also noted in the presented
24 study (fig. S5). Moreover, in our data, neither spike antigens from variants of
25 Beta (STFK1351), Gamma (STFK1128), nor B.1.620 (STFK1620) induced
26 markedly improved nAb against Omicron in hamsters (fig. S7). Therefore, it is
27 unlikely to generate broad-spectrum antigenic coverage by using a pre-existing
28 naturally occurring variant.

29 Here, we provided a progressive approach to enlarge the cross-variants

1 antigenic covering of a recombinant spike protein. This approach followed inter-
2 lineage chimera and mutation patch strategies. As the NTD, RBD, and S2
3 contain neutralizing antibody-targeting epitopes, inter-lineage chimeric spike
4 antigens might confer cross-variants antibody response (30). The engineered
5 STFK1628x, providing mutually-complementary antigenic coverage to the
6 prototypic spike, was a chimeric construct of the B.1.620-derived NTD and the
7 Gamma variant derived RBD-S2. The B.1.620 NTD harbored amino-acid
8 deletions of $\Delta 69-70$, $\Delta 145$, and $\Delta 242-244$, correspond to changed epitopes on
9 the N2, N3, and N5 loops (31). Notably, Alpha, Eta, and Omicron variants
10 shared the $\Delta 69-70$ and $\Delta 145$, whereas the Beta variant contained $\Delta 242-244$.
11 The RBD-S2 of the two engineered antigens were derivatives of the Gamma
12 spike with additional antigenic mutation patches of L452R and T478K. Our and
13 others' studies both suggested the Gamma-derived spike was highly
14 immunogenic to elicit potent nAb response against E484K-harboring variants,
15 such as Beta and Gamma (32). As two Delta-derived characteristic mutations,
16 the L452R and T478K introduction showed added value to improve the
17 neutralization response against the Delta variant (fig. S6). In contrast to STFK,
18 the monovalent STFK1628x and the STFK/STFK1628x-combined bivalent
19 vaccines conferred improved protection against viral infection and transmission
20 of SARS-CoV-2 Beta variant in hamsters (Fig. 3 and 4). Given the potent cross-
21 variants neutralization response elicited by the STFK/STFK1628x-combined
22 bivalent vaccine in hamsters, we can expect the broad-spectrum protection of
23 the new vaccine.

24 Remarkably, the STFK1628x and its STFK1628y derivative elicited significantly
25 higher nAb titers against the Omicron variant than the prototypic STFK (fig.
26 S7D), possibly attributed to the shared mutations of $\Delta 69-70$, K417N/T, T478K,
27 E484K/A, and N501Y. In comparison to STFK1628x, the STFK1628y has an
28 additional N440K mutation but presented a 4-fold higher nAb titer to neutralize
29 Omicron (fig. S7D), suggesting the N440K plays an essential role in changing

1 the antigenicity of this variant. Notably, as both antigens were generated before
2 Omicron emergence, these results were encouraging and suggested the
3 possibilities of prospective antigen design and vaccine preparations against an
4 unknown future variant. Timely and comprehensive assessments for the
5 antigenic influence of newly emerged spike mutations are essential for guiding
6 vaccine antigen design.

7 Although trimerized spike antigens were commonly used in current COVID-19
8 vaccines, our study found that the monomeric spike protein is also highly
9 immunogenic in rodents and nonhuman primates. The CHO-derived C-terminal
10 truncated STFK proteins maintained comparable ACE2 binding activity as the
11 trimeric StriFK, conferred potent immune responses, and complete protection
12 against SARS-CoV-2 in hamsters. The monomeric form with more exposed
13 protein surfaces may enable more accessibilities of cryptic epitopes and
14 thereby facilitating the rapid and diverse antibody response. Removing the
15 additional motif required for protein trimerization eliminates unexpected
16 immune responses targeting the trimeric domain. In addition, the high yield of
17 STFK in the CHO-cell expression system (800-1,000 mg/L from stable cell lines)
18 is beneficial to reduce manufacturing costs and deal with the global shortage of
19 COVID-19 vaccines.

20 In summary, our study provides a new way to design new antigens for next-
21 generation COVID-19 vaccines aiming to confer broad-spectrum protection.

1 **Materials and Methods**

2 **Constructs, protein expressions, and purifications**

3 Trimeric S-ectodomain proteins of SARS-CoV-2 (StriFK) were expressed and
 4 purified as previously described (17). Expression cassettes encoding
 5 monomeric S-ectodomain proteins (containing mutated furin-site, RRAR to
 6 GSAS) involved in the study were generated by site-directed mutagenesis via
 7 PCR cloning based on the parental codon-optimized StriFK (17). The
 8 expression cassettes of S1152 to S1208 with C-terminal 8×His-tag were cloned
 9 into the EIRBsMie vector (19). The tag-free STFK and STFK variants were
 10 constructed into a pGS01b vector, modified from the pCGS3 vector containing
 11 glutamine synthetase (GS) selection marker (Sigma Aldrich). All STFK
 12 constructs had an N1192M modification to reduce potential protein aggregation,
 13 possibly attributed the K933-N1192 interaction (33). As previously described,
 14 transient protein expressions were performed using the ExpiCHO expression
 15 system (Thermo Fisher Scientific). Stable cell lines expressing the STFK,
 16 STFK1628x, and STKF1628y were generated via transfections of the PGS01b-
 17 vectored constructs into CHOZN GS^{-/-} cells (Sigma Aldrich) following GS
 18 selections and single-cell clonings. Polyhistidine-tagged proteins (S1152 to
 19 S1208) purified from culture supernatants were collected on day 7 after
 20 transfection using Ni Sepharose 6FF (Cytiva). The tag-free STFK proteins were
 21 purified by using Q-FF Sepharose ion-exchange chromatography (Cytiva).
 22 Recombinant human ACE2 (human Fc tag, rACE2) protein also was produced
 23 in ExpiCHO-S cells and purified by protein-A affinity chromatography column
 24 (Cytiva) as previously described (17).

25

26 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Native-PAGE**

27 SDS-PAGE analyses were performed using 4-12% SurePAGE (Genscript), the
 28 precast mini polyacrylamide gels. For native-PAGE, the protein samples were
 29 mixed with the Native Sample Buffer (BIO-RAD) in equal volume and then

subjected to electrophoresis using the 7.5% Mini-Protein TGX Precast Protein Gels (Bio-RAD) in a non-denaturing buffer. Gel images were captured using FUSION FX7 Spectra multispectral imaging system (Vilber).

Size exclusion chromatography (SEC-HPLC)

The SEC-HPLC analysis shown in Fig. 1B was performed using a TSK-GEL G3000PWXL column on an HPLC system (Waters Alliance) and conducted as described previously(17).

ACE2 binding assays

A capture antibody 45C3 (developed in our laboratory) recognizes spike S2 domain was coated in 96-well microplates at 200ng per well. Plates were incubated overnight at 4 °C and then blocked with ELISA-blocking buffer (Wantai BioPharm). The STFK proteins were twofold serially diluted from 10 µg mL⁻¹ to 9.8 ng mL⁻¹ in duplicate and then added to wells (100 µL). After incubation for 1 hour at 25°C followed by washing with PBST buffer, rACE2 protein solution (100 µL per well, 1 µg mL⁻¹) was added to the wells. Subsequently, the microplates were incubated for 1 hour at 25°C. After washing five times, HRP-conjugated anti-human IgG (Thermo Fisher Scientific) solutions were added and incubated for 1 hour at 25°C. Following washing five times, tetramethylbenzidine chromogen (TMB) solution (Wantai BioPharm) was added into microplates 100 µL per well. After a further 10 minutes of incubation at 25°C, 2 M H₂SO₄ was added to stop the chromogen reaction, and the optical density (OD₄₅₀₋₆₃₀) value was measured. The half-maximal effective concentration (EC₅₀) was calculated by the 4-parameter logistic (4PL) regression using GraphPad Prism 8 software.

Vaccine preparations

Recombinant spike protein subunit vaccines used in this study were composed

1 of spike proteins and a nitrogen bisphosphonate-modified zinc-aluminum hybrid
2 adjuvant (FH002C), which was described detailly in our previous study(17).
3 Briefly, the proteins were mixed with an equal volume of 2× concentration
4 FH002C adjuvant to achieve the final desired concentration of antigen in the
5 final formulation. All vaccine formulations were mixed well and stored at 2-8 °C
6 until use.

7

8 **Experimental animals**

9 BALB/c and C57BL/6 mice were purchased from Shanghai SLAC Laboratory
10 Animal Co., Ltd. Lakeview Golden (LVG) Syrian hamsters were purchased from
11 Charles River Laboratories (Beijing). The animals were fed in Specific-
12 pathogen-free circumstances. The mouse and hamster studies were carried out
13 in strict accordance with the recommendations of the Guide for the Care and
14 Use of Laboratory Animals under the approval of the Institutional Animal Care
15 and Use Committee of Xiamen University. The rhesus monkey experiment was
16 conducted at the Key Laboratory of Technical Evaluation of Fertility Regulation
17 for Nonhuman Primate Inc in Fujian province.

18

19 **Mouse immunizations**

20 Six to eight-week-old BALB/c mice (n=6 per group) were immunized with STFK
21 vaccines at 0.01, 0.1, 1, or 10 µg per dose in 150 µL through intramuscular
22 injection following a two-dose schedule at weeks 0 and 3. Sera were collected
23 at week 4 via retro-orbital bleeding to measure antibody titers. For T cell
24 response assessment, six to eight-week-old C57BL/6 mice were immunized
25 with STFK vaccine at 10 µg per dose in 150 µL through intramuscular injection.
26 Immunized mice were sacrificed on day 7 after immunization to collect
27 splenocytes for further assay.

28

29 **Rhesus monkey immunizations**

1 Ten rhesus monkeys were allocated randomly into two groups (three females
2 and two males per group). Groups of monkeys were injected with 1 µg or 15 ug
3 of STFK vaccine per dose (150 µL) via the intramuscular route for 2 doses. All
4 monkeys were vaccinated at weeks 0 and 4. Two weeks after boosting, serum
5 samples were collected for antibody analyses, including measurement of anti-
6 spike IgG, anti-RBD IgG, pseudovirus neutralizing antibody, and authentic
7 neutralizing antibody titers.

8

9 **Hamster immunizations**

10 Six to eight-week-old hamsters were used to evaluate the immunogenicity and
11 protective effect of the vaccine candidates. Each group contained four males
12 and four females. Groups of hamsters were immunized intramuscularly twice
13 with the vaccine candidates at 10 µg per dose in 200 µl, three weeks apart. All
14 serum samples were collected at week-2 after the 2nd dose via retro-orbital
15 bleeding to measure the antibody titers.

16

17 **Pseudovirus (PsV) neutralization assays**

18 The nAb titers against the ancestral spike-pseudotyped virus presented in Fig.
19 1C and 1E-1G were determined by using a vesicular stomatitis virus (VSV)
20 system as previously described (34). The International Standard for anti-SARS-
21 CoV-2 immunoglobulin (NIBSC code: 20/136) was obtained from National
22 Institute for Biological Standards and Control, UK (35).

23 The lentiviral-based pseudovirus (LV) neutralization assay was used to
24 determine vaccine-elicited nAbs against circulating SARS-CoV-2 variants (Fig.
25 1H, 2, S5, S6, and S7). The lentiviral pseudoviruses bearing spikes from SARS-
26 CoV-2 variants were generated as described previously (19, 36), including
27 D614G (site-directed mutagenesis), Alpha (B.1.1.7, GISAID accession number:
28 EPI_ISL_601443), Beta (B.1.351, EPI_ISL_700428), Gamma (P.1,
29 EPI_ISL_792680), Delta (B.1.617.2, EPI_ISL_1662451), Omicron (B.1.1.529,
30 BA.1, EPI_ISL_6704867), Iota_484K (B.1.526_484K, EPI_ISL_1009654),

1 Iota_477N (B.1.526_477N, EPI_ISL_995145), Epsilon (B.1.429,
2 EPI_ISL_873881), Eta (B.1.525, EPI_ISL_762449), Kappa (B.1.617.1,
3 EPI_ISL_1595904), A.VOI.V2 (EPI_ISL_1347941), Lambda (C.37,
4 EPI_ISL_2921532), Mu (B.1.621, EPI_ISL_3933281) and B.1.620
5 (EPI_ISL_1620228). The PsV nAb measurements were performed as
6 previously described using Opera Phenix or Operetta CLS High-Content
7 Analysis System (PerkinElmer) (19).

8

9 **Authentic SARS-CoV-2 neutralization assay**

10 The nAb titers of sera from immunized animals against authentic SARS-CoV-2
11 were detected using a cytopathic effect (CPE)-based microneutralization assay
12 as previously described (37). The ancestral virus (BetaCoV/Jiangsu/JS02/2020,
13 EPI_ISL_411952) was used. Briefly, serum samples were twofold serially
14 diluted from 1:4 to 1:8192 in duplicate with DMEM medium. All prepared
15 samples were mixed with the virus of 200 TCID₅₀ and incubated for 2 hours at
16 37 °C. The mixtures (150 µL per well) were added to a monolayer of Vero cells
17 in a 96-well plate and incubated at 37 °C supplying with 5% CO₂. Three-day
18 later, the cytopathic effect was assessed with microscopic examinations. The
19 neutralizing titer of serum was expressed as the reciprocal of the maximal
20 sample dilution that protects at least 50% of cells from CPE.

21

22 **Enzyme-linked immunospot (ELISpot) assay**

23 According to the manufacturer's instructions, the assays were performed with
24 mouse IFN-γ ELISpot plates kits (Dakewe Biotech, 2210005). In brief, single-
25 cell suspensions were obtained from mouse spleen (10⁶ cells per well) through
26 grinding in 70 µm cell strainers and were seeded in anti-mouse IFN-γ antibody
27 precoated ELISpot plates. Then, cells were incubated with pooled peptides of
28 SARS-CoV-2 spike (15-mer peptides with 11aa overlap covering the entire
29 spike protein; GenScript) and cultured at 37°C with 5% CO₂ for 20 hours. Spots

were counted and analyzed by using CTL-ImmunoSpot S5 (Cellular Technology Limited). The numbers of IFN- γ -secreting cells were calculated by subtracting phosphate-buffered saline (PBS)-stimulated wells from spike peptide pool-stimulated wells.

Anti-RBD, anti-spike IgG measurements

Microplates pre-coated with recombinant antigens of RBD or spike ectodomain were provided by Beijing Wantai Biological Pharmacy. The measurements were performed following previously described procedures (17), with the only difference that the cutoff (CO) value was set as 0.1 (OD₄₅₀₋₆₃₀). The IgG titer of each sample was determined as the cutoff index (OD₄₅₀₋₆₃₀/CO) at the dilution limit multiplied by the maximum dilution folds. Representative data from technical replicates were performed at least twice for plotting.

Cryo-EM sample preparation and data collection

Fabs of 36H6, 83H7, 2B4, and 85F7 were prepared by papain digestion of the mAbs and further purified with MabSelect SuRe (Cytiva). Aliquots (3 μ l) of 3.5 mg/mL mixtures of purified STFK or STFK1628x proteins in complex with excess Fab fragments of nAbs were incubated in 0.01% (v/v) Digitonin (Sigma) and then loaded onto glow-discharged (60 s at 20 mA) holey carbon Quantifoil grids (R1.2/1.3, 200 mesh, Quantifoil Micro Tools) using a Vitrobot Mark IV (ThermoFisher Scientific) at 100% humidity and 4°C. Data were acquired using the SerialEM software on an FEI Tecnai F30 transmission electron microscope (ThermoFisher Scientific) operated at 300 kV and equipped with a Gatan K3 direct detector. Images were recorded in the 36-frame movie mode at a nominal 39,000 \times magnification at super-resolution mode with a pixel size of 0.339 Å. The total electron dose was set to 60 e⁻ Å⁻², and the exposure time was 4.5 s.

Image processing and 3D reconstruction

1 Drift and beam-induced motion correction were performed with MotionCor2 (38)
2 to produce a micrograph from each movie. Contrast transfer function (CTF)
3 fitting and phase-shift estimation were conducted with Gctf (39). Micrographs
4 with astigmatism, obvious drift, or contamination were discarded before
5 reconstruction. The following reconstruction procedures were performed by
6 using Cryosparc V3 (40). In brief, particles were automatically picked by using
7 the "Blob picker" or "Template picker". Several rounds of reference-free 2D
8 classifications were performed, and the selected good particles were then
9 subjected to ab-initio reconstruction, heterogeneous refinement and final non-
10 uniform refinement. The resolution of all density maps was determined by the
11 gold-standard Fourier shell correlation curve, with a cutoff of 0.143. Local map
12 resolution was estimated with ResMap (41).

13

14 **Atomic model building, refinement, and 3D visualization**

15 The initial models of nAbs were generated from homology modeling by Accelrys
16 Discovery Studio software (available from: <https://www.3dsbiovia.com>). The
17 structure from the prototypic trimeric spike (PDB no. 6VSB) (42) was used as
18 the initial modes of our proteins. We initially fitted the templates into the
19 corresponding final cryo-EM maps using Chimera (43), and further corrected
20 and adjusted them manually by real-space refinement in Coot (44). The
21 resulting models were then refined with phenix.real_space_refine in PHENIX
22 (45). These operations were executed iteratively until the problematic regions,
23 Ramachandran outliers, and poor rotamers were either eliminated or moved to
24 favored regions. The final atomic models were validated with Molprobit (46,
25 47). All figures were generated with Chimera or ChimeraX (48).

26

27 **SARS-CoV-2 virus challenges in hamster**

28 Two weeks after boosting, hamsters were challenged with the ancestral SARS-
29 CoV-2 of hCoV-19/China/AP8/2020 (EPI_ISL_1655937) or Beta variant of

1 hCoV-19/China/AP100/2021 (EPI_ISL_2779639). For the intranasal challenge,
2 hamsters were challenged with 1×10^4 PFU of SARS-CoV-2 virus (diluted in 100
3 μ L of PBS) through the intranasal route under anesthesia. For virus
4 transmission-blocking study, vaccinated- or unvaccinated-hamsters were
5 intranasally inoculated with 1×10^4 PFU of ancestral SARS-CoV-2 or Beta
6 variant as indexes. One day post-infection, index hamsters were cohoused with
7 naïve sentinels for one day (Fig. 4A). The daily diet was limited to 7 g per 100
8 g of body weight to prevent animals from overeating. All hamsters were
9 monitored for body weight until being humanely euthanized on day 7 after
10 challenge or exposure. The respiratory tissues of hamsters were collected for
11 viral RNA quantification or histopathological assessments. All challenge
12 experiments were conducted in the Animal Biosafety Level 3 (ABSL-3) facility.

13

14 **SARS-CoV-2 RNA quantification**

15 Viral RNA levels in tissues from hamsters were determined by SARS-CoV-2
16 RT-PCR Kit (Wantai BioPharm). For each animal, two pieces (separately) of
17 lung tissue (0.1~0.2 g each), one piece of the trachea tissue (0.1~0.2 g), and a
18 half of nasal turbinate (0.8~1.2 g) were respectively homogenized with
19 TissueLyser II (Qiagen) in 1 ml PBS. Viral RNA in tissue lysates was extracted
20 using the QIAamp Viral RNA Mini Kit (Qiagen) and subjected to qRT-PCR
21 assays. Representative data from technical replicates were obtained from at
22 least two independent experiments for plotting.

23

24 **Histopathology**

25 The lung tissues from challenged hamsters were fixed with neutral buffered
26 formalin for 48 hours and processed routinely into paraffin blocks. Then tissues
27 were sectioned to 3 μ m by microtome (Leica). Next, the fixed lung sections
28 were stained with hematoxylin and eosin (Maxim Biotechnology). Whole-slide
29 images of the lung sections were captured with the EVOS M7000 Images

1 System (Thermo Fisher Scientific). Microscopic evaluation of pathological lung
2 lesions was performed blindly by pathologists following a semiquantitative
3 scoring system with the inclusion of three indicators (49): (i) alveolar septum
4 thickening and consolidation; (ii) hemorrhage, exudation, pulmonary edema,
5 and mucous; and (iii) recruitment and infiltration of inflammatory immune cells.
6 For each hamster, three or four lobes of the lung were assessed independently,
7 and the average score was calculated to indicate the overall pathological
8 severity.

9

10 **Statistical analysis**

11 The Mann-Whitney *U* test was used for the comparison between two
12 independent samples. The uncorrected Kruskal-Wallis test, Dunnett's Multiple
13 Comparison test, or uncorrected Fisher's LSD test was applied to analyze
14 differences among more than two groups. A two-sided log-rank test was applied
15 to compare the difference in survival. Statistical differences were considered to
16 be significant for two-tailed *P* values of < 0.05 . All statistical analyses were
17 conducted in GraphPad Prism 8 software.

18

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2 **Author contributions:** H.Z., Q.Y., Y.G., N.S.X. conceptualized and designed the
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5 experiments. H.C., Z.L., H.Z., X.W., Y.W. performed the rhesus monkey
6 experiments. Z.W., Y.H., D.C., M.L., H.L.G., J.G., H.X. performed the ELISA and
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11 Y.G., N.S.X. revised the manuscript. All authors read and approved the final
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13

14 **Competing interests:** Q.Y., Y.W., S.W., Y.Z., M.W., K.W., Z.W., J.X., T.Z., J.Z.
15 and N.S.X. are coinventors on a patent in the application for the spike
16 constructs and their applications described in this study. The other authors
17 declare no competing interests

18 **Data and materials availability:** Structure coordinates are deposited in the
19 Protein Data Bank under accession codes 7WP6 (STKF:36H6:83H7:85F7) and
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21 have been deposited in the Protein Data Bank under accession numbers EMD-
22 32676 (STKF:36H6:83H7:85F7), EMD-32678 (STFK1628x:83H7:85F7:2B4).
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27

28

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20

21

22

Figure 1

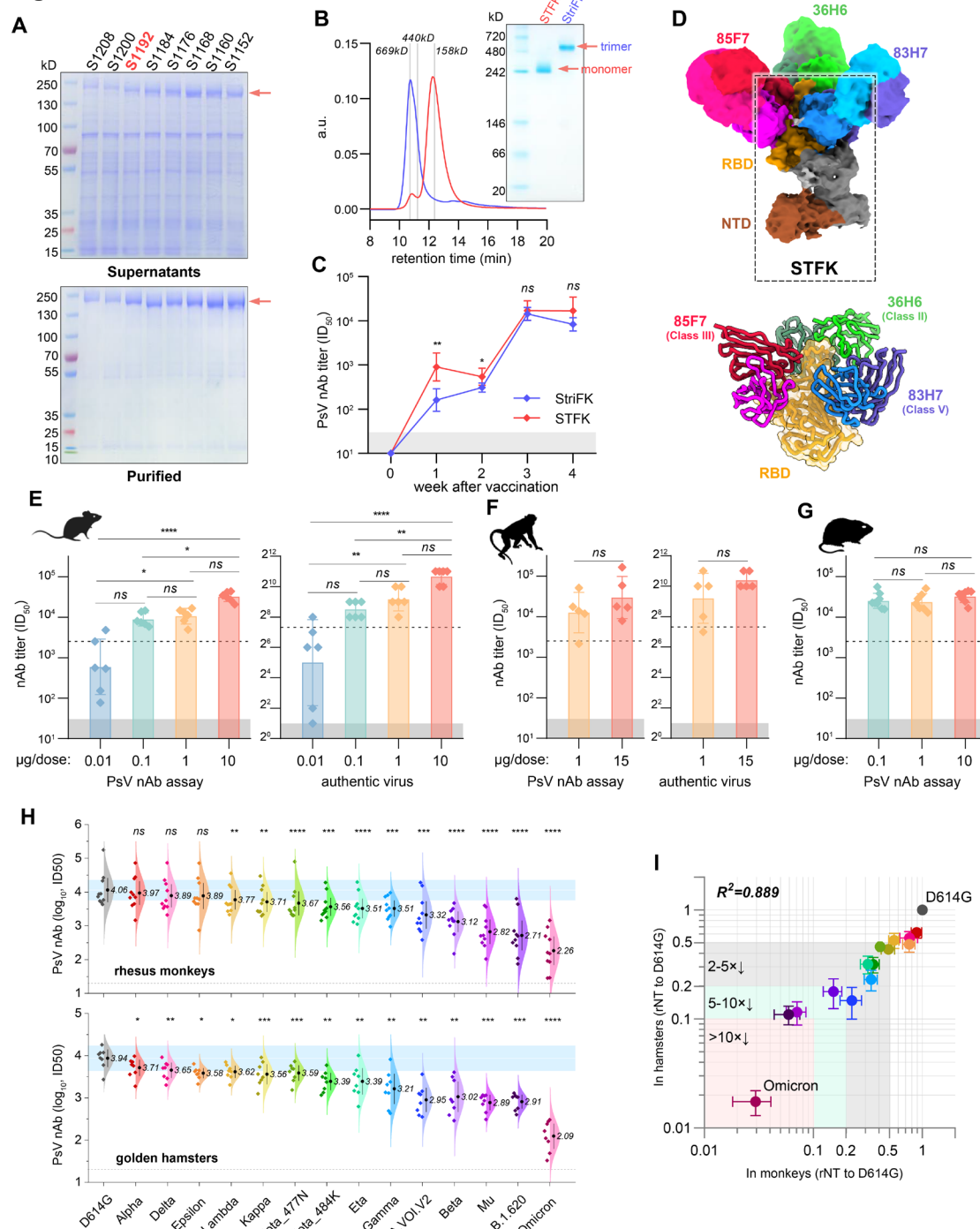


Fig. 1. The monomeric STFK is highly immunogenic in rodents and nonhuman primates. (A) Reduced SDS-PAGE analyses for supernatants (top panel) and purified proteins (bottom panel) produced from constructs encoding progressive truncations from the C terminus of the furin site with mutated spike ectodomain in CHO cells. S1208, aa 1-1208; S1200, aa 1-1200; S1192, aa 1-1192; S1184, aa 1-1184; S1176, aa 1-1176; S1168, aa 1-1168; S1160, aa 1-

1 1160; S1152, aa 1-1152. **(B)** Analyses of the monomeric STFK (aa 1-1192) and
2 trimeric StrFK by SEC-HPLC (left panel) and Native-PAGE (right panel). **(C)**
3 Comparison of the PsV nAb titers elicited by the STFK and StrFK in mice.
4 BALB/c mice were immunized twice with 1 µg antigen at weeks 0 and 2. **(D)**
5 3.81 Å cryo-EM density map and corresponding atomic model of the STFK in
6 complex of nAbs 36H6, 83H7, and 85F7. The black dotted box highlights the
7 monomeric STFK protein. **(E-G)** Serum nAb titers against pseudotyped (left
8 panel) and authentic SARS-CoV-2 viruses (right panel) of **(E)** BALB/c mice (n=6)
9 or **(F)** Rhesus monkeys (n=5) received 2 shots of STFK vaccinations at different
10 antigen doses. **(G)** Serum nAb titers against VSV-based PsV of hamsters
11 vaccinated at 0.1 (n=8), 1 (n=7), or 10 µg (n=8) of STFK per dose. The
12 immunization schedule was week 0/3 for **(E, G)** and week 0/4 for **(F)**. Sera were
13 analyzed at weeks 4 **(E)**, 6 **(F)**, and 5 **(G)**. The dotted lines show the PsV nAb
14 titers of WHO International Standard for anti-SARS-CoV-2 immunoglobulin
15 using the same assays (NIBSC 20/136). **(H)** The nAb titers of sera from STFK-
16 vaccinated rhesus monkeys (pooled of 1 and 15 µg groups, top panel) and
17 hamsters (10 µg group, bottom panel) against lentiviral-pseudotyped SARS-
18 CoV-2 spike variants compared to that against the ancestral D614G strain. The
19 numbers showed the nAb GMT (log₁₀) values. **(I)** Comparison of the cross-
20 neutralizing activities of vaccinated hamsters (X-axis) and rhesus monkeys (Y-
21 axis) against various lentiviral-pseudotyped SARS-CoV-2 variants. The relative
22 nAb titer (rNT) was calculated as its ID₅₀ ratio against a variant to the D614G
23 control for each sample. Data in (D-H) were plotted as the geometric mean with
24 SD. Dark shadows in (D-G) indicate the limit of detection (LOD). The dotted line
25 in (H) indicates the LOD. Blue shadows in (H) represent the range of 50%-200%
26 (within 2-fold changes) of the nAb GMT against D614G (as white line indicated).
27 Uncorrected Kruskal-Wallis test (D, E, and G), Mann-Whitney *U* test (F), or
28 Dunnett's Multiple Comparison test (H) were used for intergroup statistical
29 comparisons. Asterisks indicate statistical significance (*****P* < 0.0001; ****P* <

- 1 0.001; ** $P < 0.01$; * $P < 0.05$; ns, not significant). Silhouettes indicating the
- 2 species in (E-G) were from PhyloPic.org and available under the Public Domain
- 3 Dedication 1.0 license.

Figure 2

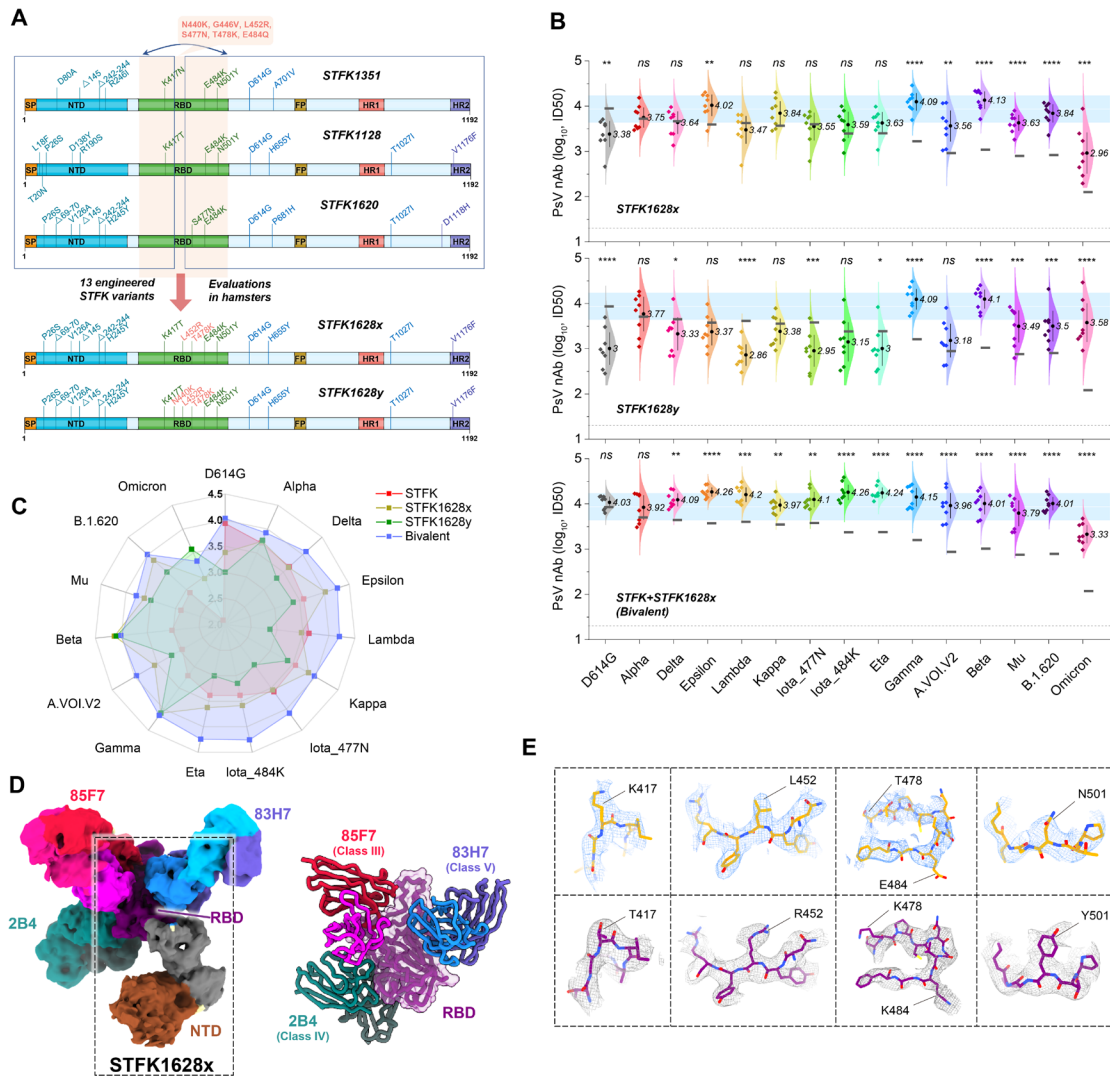


Fig. 2. Neutralizing antibody responses elicited by engineered STFK variants in hamsters. (A) Schematic of the progressive approach to enlarge the cross-variants antigenic covering of a recombinant spike protein via inter-lineage chimera and mutation patch strategies. **(B)** The nAb titers of sera from hamsters (n=8) receiving vaccination of STFK1628x, STFK1628y, or a bivalent version of STFK+STFK1628x to neutralize lentiviral-pseudotyped SARS-CoV-2 variants. Dark horizontal lines indicate the nAb GMTs induced by the prototypic STFK vaccine against the corresponding variants. Blue shadows represent the range of 50%-200% (within 2-fold changes) of the nAb GMT against D614G (as white line indicated) induced by prototypic STFK. Dotted lines indicate the LOD (ID₅₀=20) of the assay. Data were plotted as the

1 geometric mean with SD. Uncorrected Fisher's LSD tests were used for
2 statistical comparisons (STFK v.s. modified STFK vaccine). Asterisks indicate
3 statistical significance (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns,
4 not significant). **(C)** A spider plot showed the nAb GMTs (\log_{10}) against different
5 SARS-CoV-2 variants of hamsters immunized with STFK, STFK1628x,
6 STFK1628y, or the bivalent vaccine. Data were summarized from panel (B). **(D)**
7 3.88 Å cryo-EM density map and corresponding atomic model of the
8 STFK1628x in complex of nAbs 83H7, 85F7, and 2B4. The black dotted box
9 highlights the monomeric STFK1628x protein. **(E)** Comparisons of
10 representative density maps of residues involved in RBD mutations on the
11 STKF (top panel) and STFK1628x proteins (bottom panel).

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Figure 3

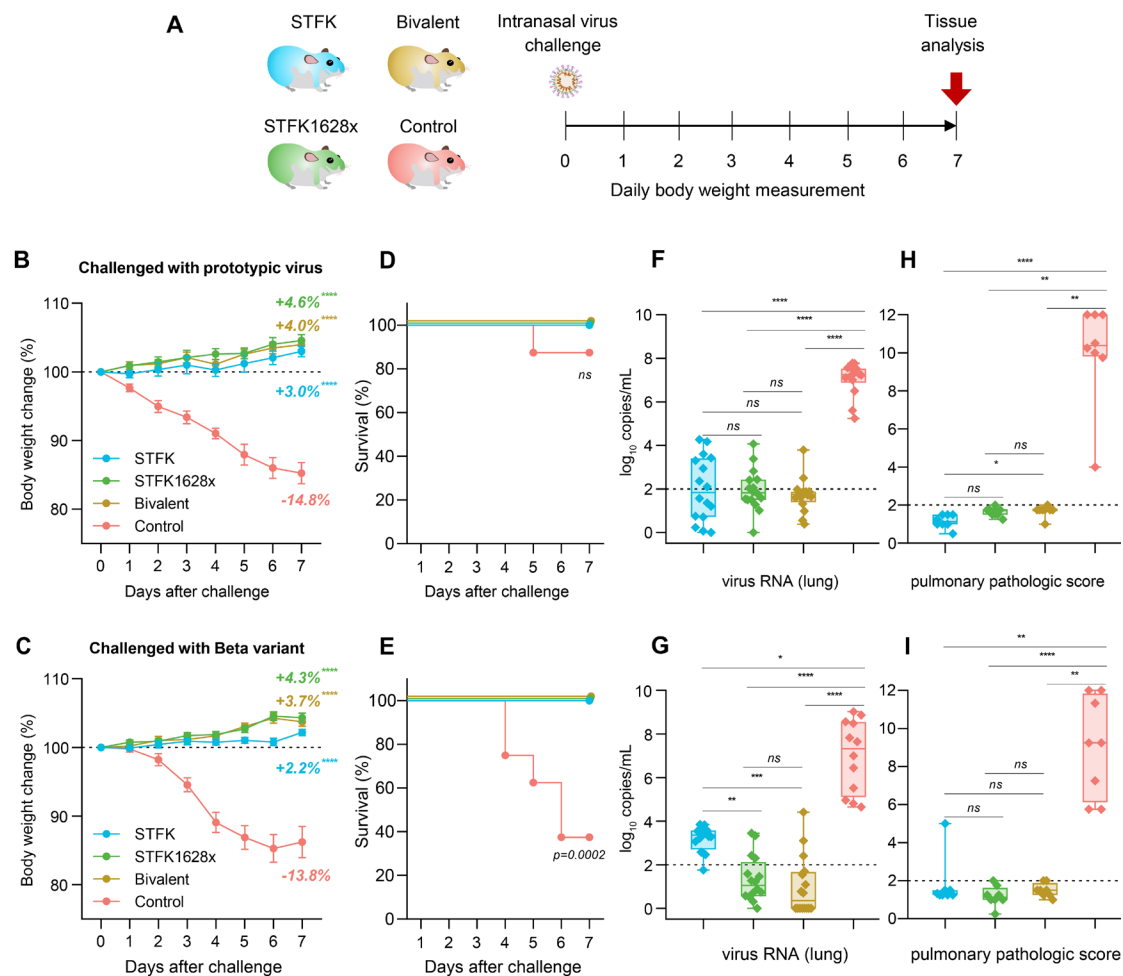


Fig. 3. The STFK, STFK1628x, and bivalent vaccines offer protection against ancestral SARS-CoV-2 and Beta variant intranasally challenged in hamsters. (A) Schematic representation of the intranasal virus challenge evaluation for vaccine effectiveness. A total of 64 hamsters were used for two independent tests. Each group (indicated in different colors) included eight hamsters (4 males and 4 females) that received 2-dose vaccinations (at weeks 0/3) before virus challenges. Hamsters were intranasally challenged with 1×10^4 PFU of ancestral SARS-CoV-2 or Beta variant (B.1.351) at week 5. After a 7-day weight monitoring follow-up, animals were euthanized for tissue analyses. Weight changes (**B, C**), survival curves (**D, E**), lung viral RNA levels (in two independent lung tissues, LOD=2 \log_{10} copies/mL) (**F, G**), and pulmonary pathological scores (**H, I**) of hamsters challenged by ancestral SARS-CoV-2 (**B, D, F, H**) or beta variant (**C, E, G, I**) were shown. Data in (**B, C**) were plotted as

1 means±SEM. Data in (**F-I**) were shown as box and whisker plots; the median,
 2 first quartile, third quartile, minimum, and maximum values were plotted.
 3 Dunnett's Multiple Comparison test (**B, C**), two-sided log-rank test (**D, E**), or
 4 uncorrected Kruskal-Wallis test (**F-I**) were used for intergroup statistical
 5 comparisons. Asterisks indicate statistical significance (**** $P < 0.0001$; *** $P <$
 6 0.001 ; ** $P < 0.01$; * $P < 0.05$; ns, not significant).
 7

Figure 4

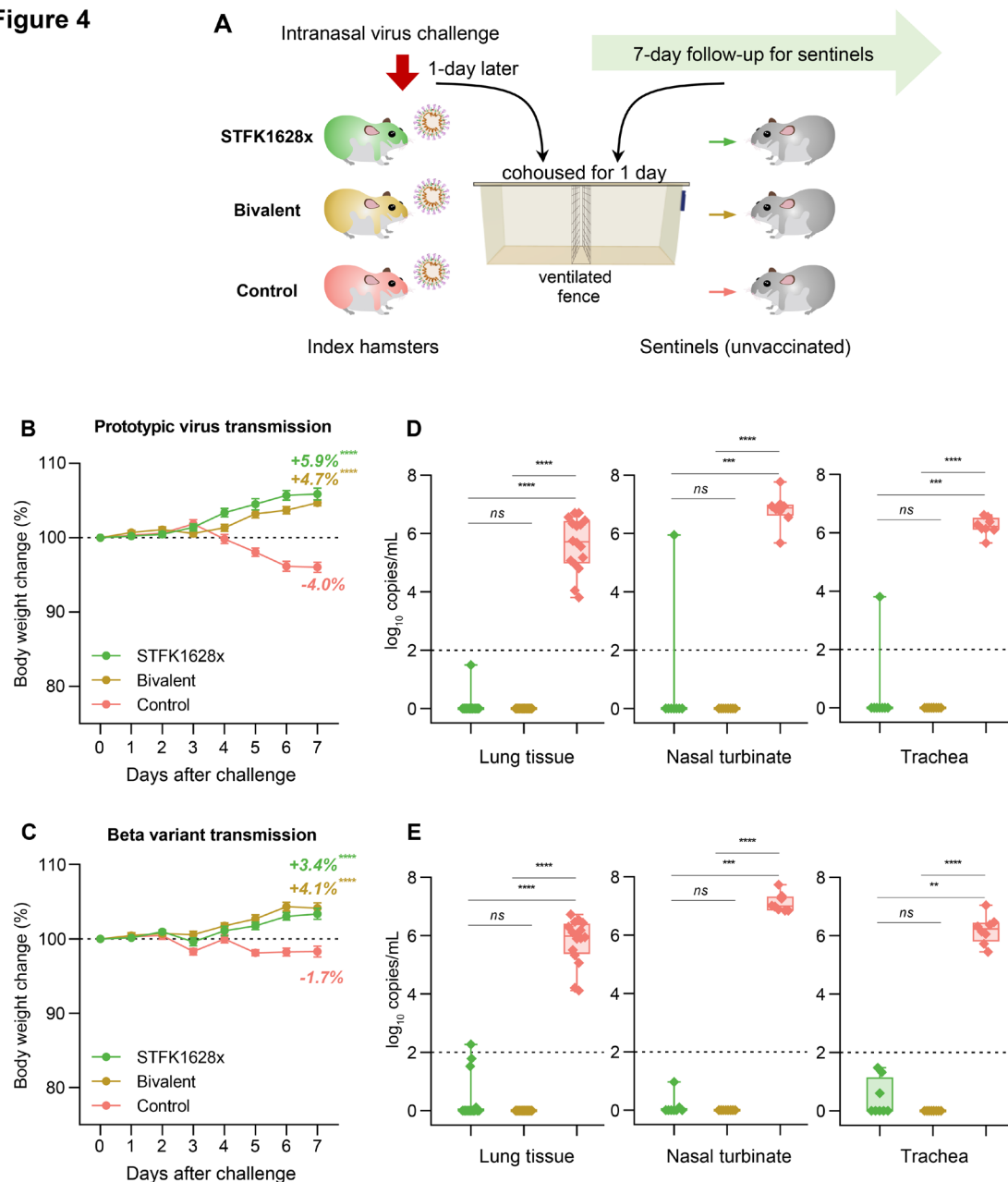


Fig. 4. The monovalent STFK1628x and the STFK/STFK1628x-combined bivalent vaccines prevent SARS-CoV-2 transmission among hamsters. (A)

Schematic diagram of the experimental design. Vaccinated- (STFK1628x or bivalent vaccine) and unvaccinated-index hamsters (n=4) were intranasally challenged with 1×10^4 PFU of ancestral SARS-CoV-2 or Beta variant. One day later, each 2 index hamsters were cohoused with 4 naïve sentinels for one day (separated by a double-layer ventilated fence in the same cage). Sentinel hamsters (n=8) were followed for 7-day and then euthanized for tissue analyses.

Weight changes (**B, C**) and viral RNA levels in the respiratory tract tissues (**D,**

1 **E**) of sentinels after cohoused with index hamsters challenged by ancestral
2 SARS-CoV-2 (**B, D**) or beta variant (**C, E**) were shown. Viral RNA levels in
3 tissues of the lung (in two independent tissues), nasal turbinate, and trachea
4 were measured by qRT-PCR (LOD=2 log₁₀ copies/mL). Data in (B and D) were
5 plotted as means ± SEM. Data in (C and E) were shown as box and whisker
6 plots; median, first quartile, third quartile, minimum value, and maximum value
7 were plotted. Dunnett's Multiple Comparison test (**B, C**), or uncorrected
8 Kruskal-Wallis test (**D, E**) were used for intergroup statistical comparisons, and
9 asterisks indicate statistical significance (*****P* < 0.0001; ****P* < 0.001; ***P* <
10 0.01; **P* < 0.05; ns, not significant).

11

Figure S1

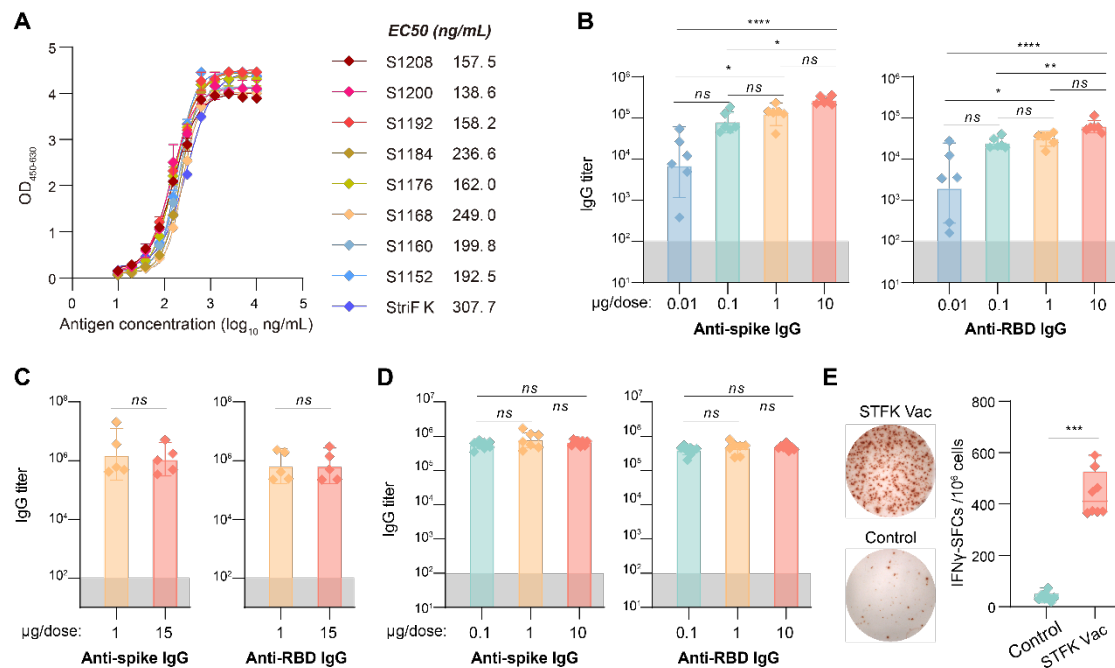


fig. S1. Evaluations for the recombinant STFK for *in vitro* binding with human rACE2 and *in vivo* immunogenicity. (A) ELISA-binding activities of recombinant spike proteins with human rACE2. (B-D) Anti-Spike and anti-RBD IgG titers in STFK-immunized (B) BALB/c mice, (C) rhesus monkeys, and (D) hamsters. (E) Spike-specific T cell response elicited by STFK-vaccination in C57BL/6 mice measured by ELISpot assays. Representative images (right panel) and the counts of IFN-γ spot-forming cells (left panel) were shown. Data in (B-D) were plotted as the geometric mean with SD. Data in (E) were shown as box and whisker plots; median, first quartile, third quartile, minimum value, and maximum value were plotted. Dark shadows in (B-D) indicate the LOD. Uncorrected Kruskal-Wallis test (B, D) or Mann-Whitney *U* test (C, E) were used for intergroup statistical comparisons. Asterisks indicate statistical significance (*****P* < 0.0001; ****P* < 0.001; ***P* < 0.01; **P* < 0.05; ns, not significant).

Figure S2

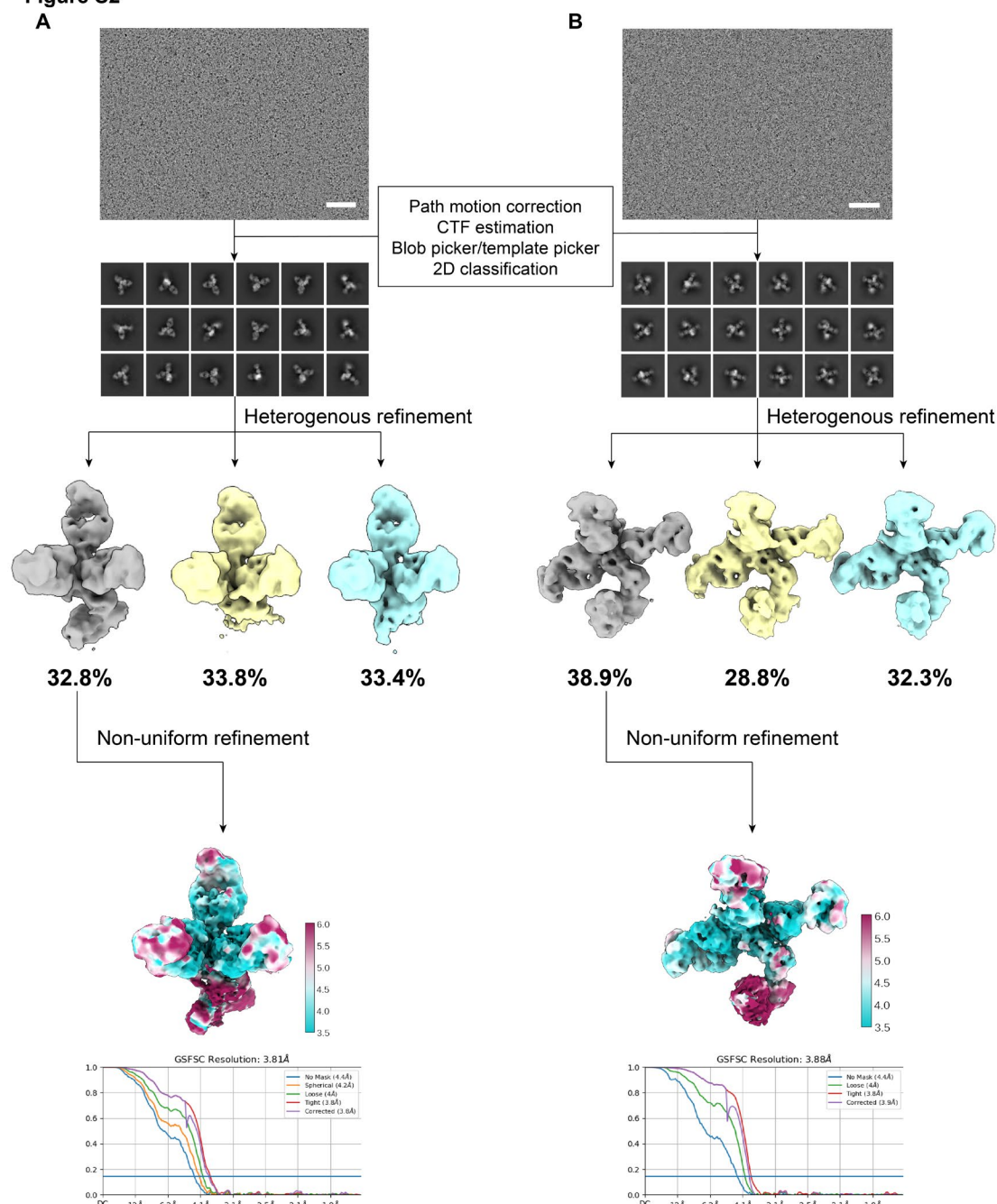


fig. S2. Flowcharts of cryo-EM images processing and 3D reconstructions of STFK:36H6:83H7:85F7 (A) and STFK1628x:83H7:85F7:2B4 (B). Fourier shell correlation (FSC) curves and local resolution analysis of 3D and reconstructions are shown, scale bar=50 nm.

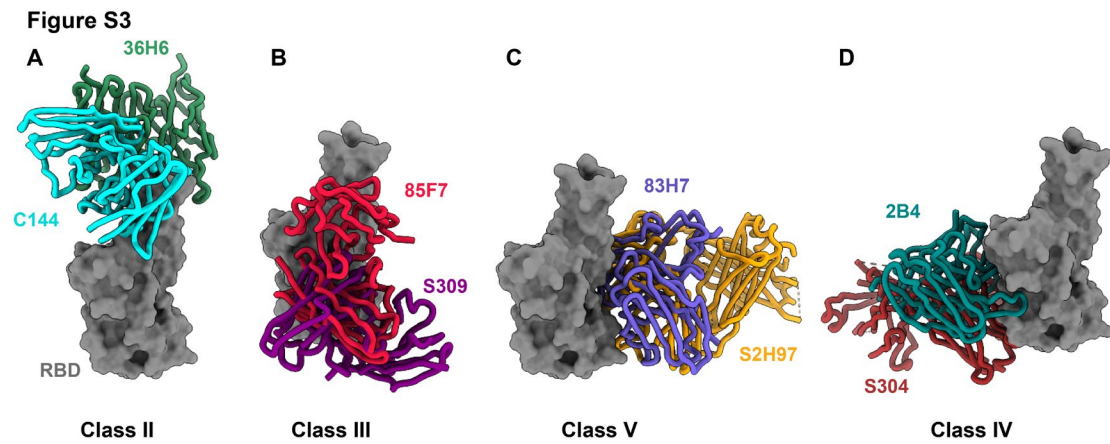


fig. S3. Comparison and classification of nAbs by their binding epitopes and modes. 36H6 (A), 85F7 (B), 83H7 (C), and 2B4 (D) were grouped into Class II, III, IV, and V nAbs, and their binding modes are similar to reported nAbs C144 (Class II, pdb no. 7K90), S309 (Class III, pdb no. 7R6W), S2H97 (Class V, pdb no. 7M7W) and S304 (Class IV, pdb no. 7R6X), respectively.

Figure S4

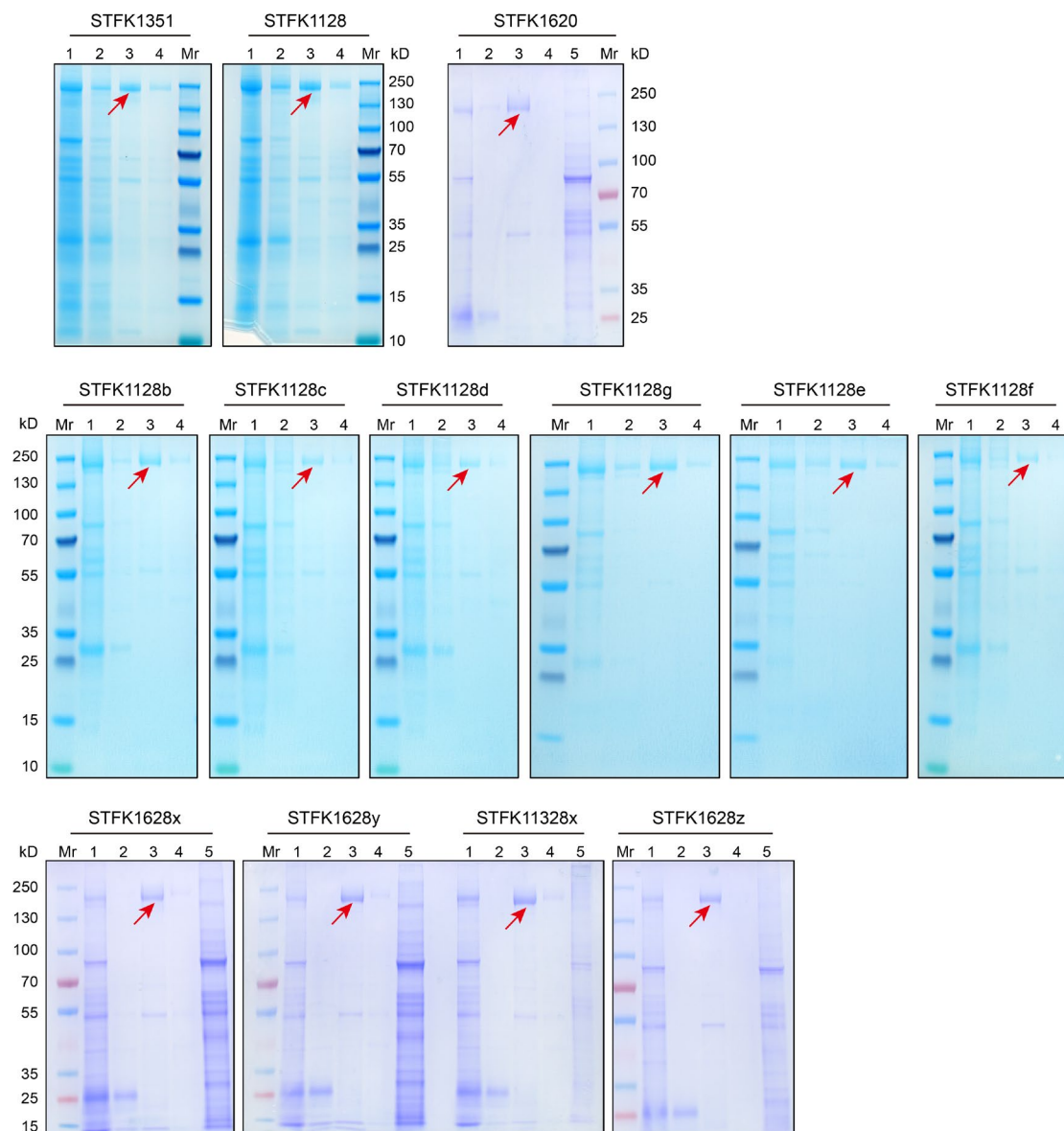


fig. S4. SDS-PAGE analyses for engineered STFK variants. Mr, protein ladder; lane 1, supernatants of transfected cells; lane 2, flow-through fraction from the Q-FF column; lane 3-4, the eluate fractions with buffer containing 100 mM NaCl; lane 5, eluate fraction with buffer containing 2 M NaCl. The red arrow indicates the target protein band.

Figure S5

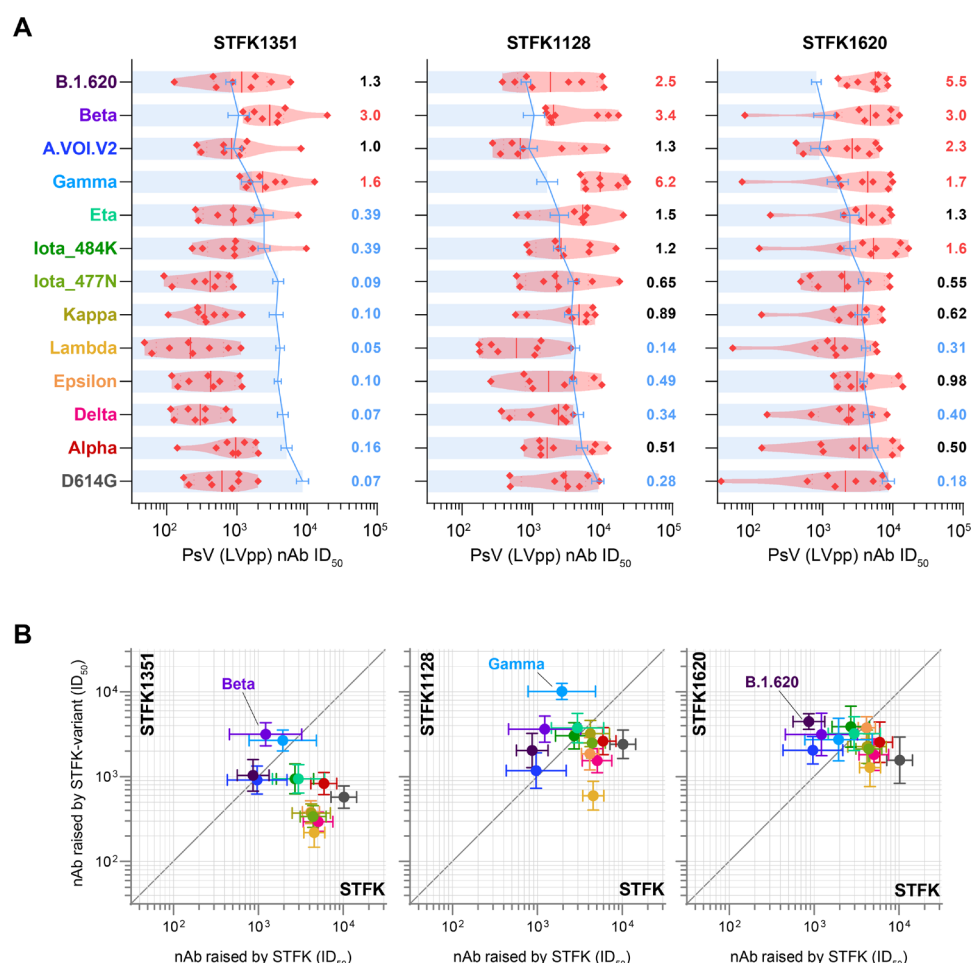
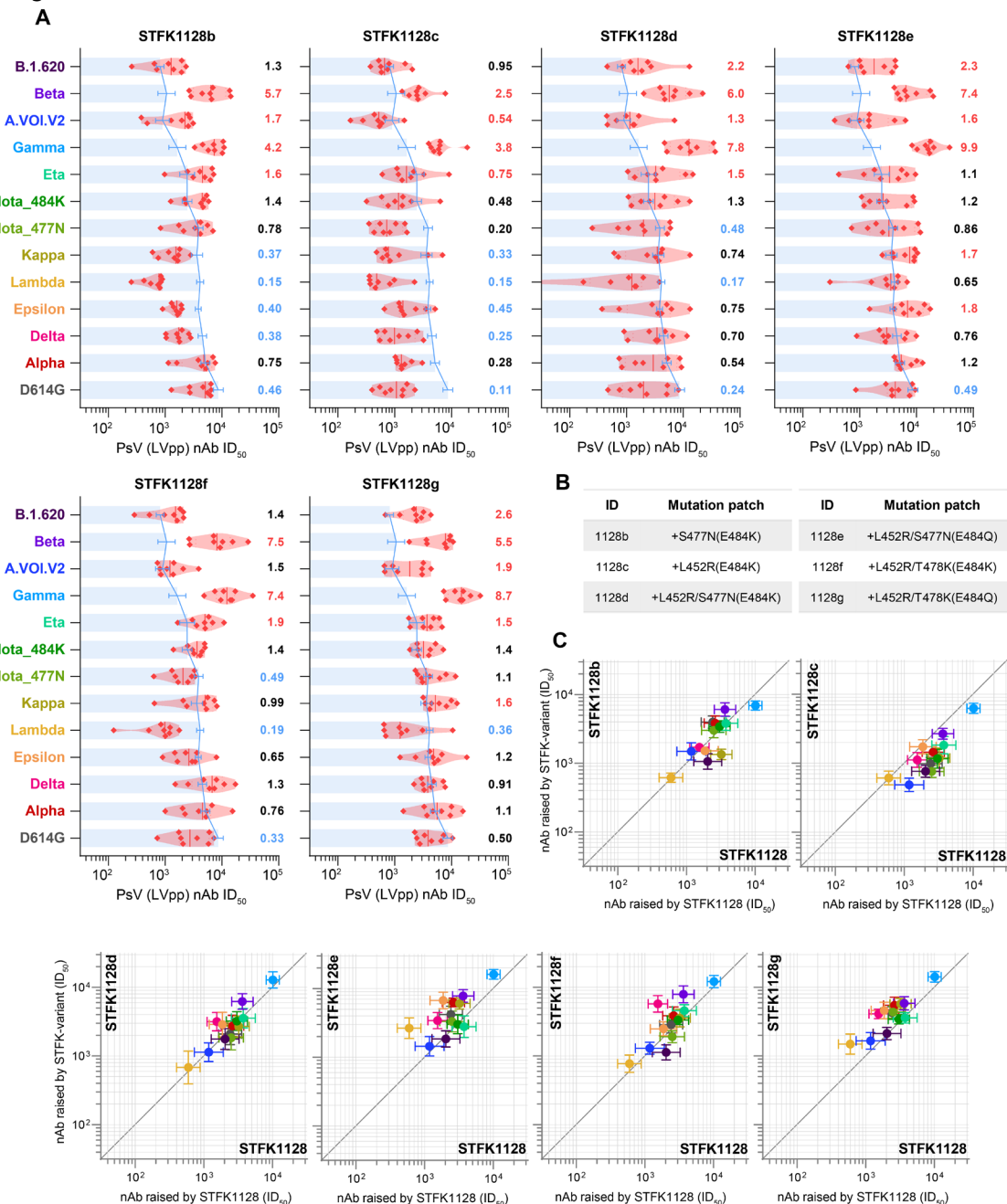


fig. S5. Neutralizing antibody responses elicited by STFK1351, STFK1128, and STFK1620 in hamsters. (A) The nAb titers of sera from hamsters (n=8) receiving vaccination of STFK1351, STFK1128, and STFK1620 to neutralize lentiviral-pseudotyped SARS-CoV-2 variants. The blue lines (bars) indicate the nAb GMTs (\pm SD) induced by the prototypic STFK vaccine against the corresponding variants. The numbers on the right represent the GMT fold-changes of nAb titers elicited by STFK variants to the prototypic STFK. The fold-changes were colored according to the values: <0.5 was in blue, 0.5-1.5 was in black, and >1.5 was in red. **(B)** The scatter plots compare the cross-neutralizing activities of nAbs raised by STFK variants (Y-axis) and prototypic STFK (X-axis). Data were plotted as the geometric mean with SEM. The diagonal line was Y=X.

Figure S6



1
2 **fig. S6. Neutralizing antibody responses elicited by STFK1128 derivatives**
3 **in hamsters. (A)** The nAb titers of sera from hamsters (n=8) receiving
4 vaccination of six STFK1128 derivatives **(B)** to neutralize lentiviral-pseudotyped
5 SARS-CoV-2 variants. The blue lines (bars) indicate the nAb GMTs (\pm SD)
6 induced by the prototypic STFK vaccine against the corresponding variants.
7 The numbers on the right represent the GMT fold-changes of nAb titers elicited
8 by STFK variants to the prototypic STFK. The fold-changes were colored

1 according to the values: <0.5 was in blue, $0.5-1.5$ was in black, and >1.5 was
2 in red. **(B)** Additional mutation patches in STFK1128 derivatives compared to its
3 parental construct. **(C)** The scatter plots show the comparison of nAbs against
4 13 variants raised by STFK1128 derivatives (Y-axis) and their parental STFK1128
5 (X-axis). Data were plotted as the geometric mean with SEM. The diagonal line
6 was $Y=X$.
7

Figure S7

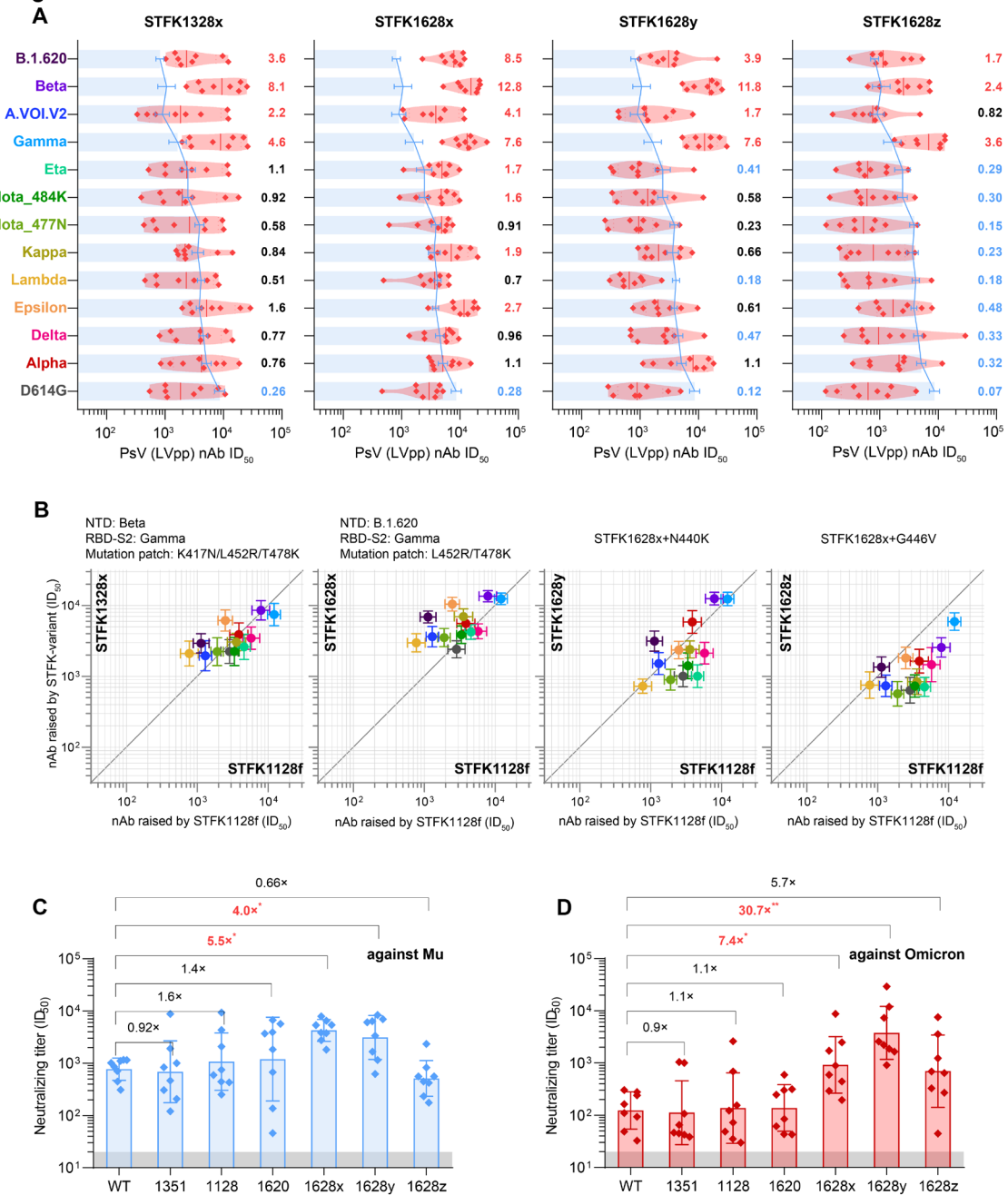


fig. S7. Neutralizing antibody responses elicited by inter-lineage chimeric STFK variants in hamsters. (A) The nAb titers of sera from hamsters (n=8) receiving vaccination of four STFK1128f-derived chimeric STFK variants to neutralize lentiviral-pseudotyped SARS-CoV-2 variants. The blue lines (bars) indicate the nAb GMTs (\pm SD) induced by the prototypic STFK vaccine against the corresponding variants. The numbers on the right represent the GMT fold-changes of nAb titers elicited by the chimeric STFK variants to the prototypic

1 STFK. The fold-changes were colored according to the values: <0.5 was in blue,
2 0.5-1.5 was in black, and >1.5 was in red. **(B)** The scatter plots compare the
3 cross-neutralizing activities of nAbs raised by chimeric STFK variants (Y-axis)
4 and their parental STFK1128f (X-axis). Data were plotted as the geometric
5 mean with SEM. The diagonal line was Y=X. A detailed information summary
6 of each chimeric variant was shown on the top of the panels. **(C, D)** The nAb
7 titers against the newly emerged variants of Mu **(C)** and Omicron **(D)** were
8 elicited by the engineered chimeric STFK variants in comparison to STFK
9 antigens based on naturally occurring variants. The numbers on the top of
10 panels represent the relative nAb GMT changes induced by the STFK variants
11 to the prototypic STFK. Dark shadows indicate the LOD. Uncorrected Kruskal-
12 Wallis tests were used for statistical comparison. Asterisks indicate statistical
13 significance (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not
14 significant).

15

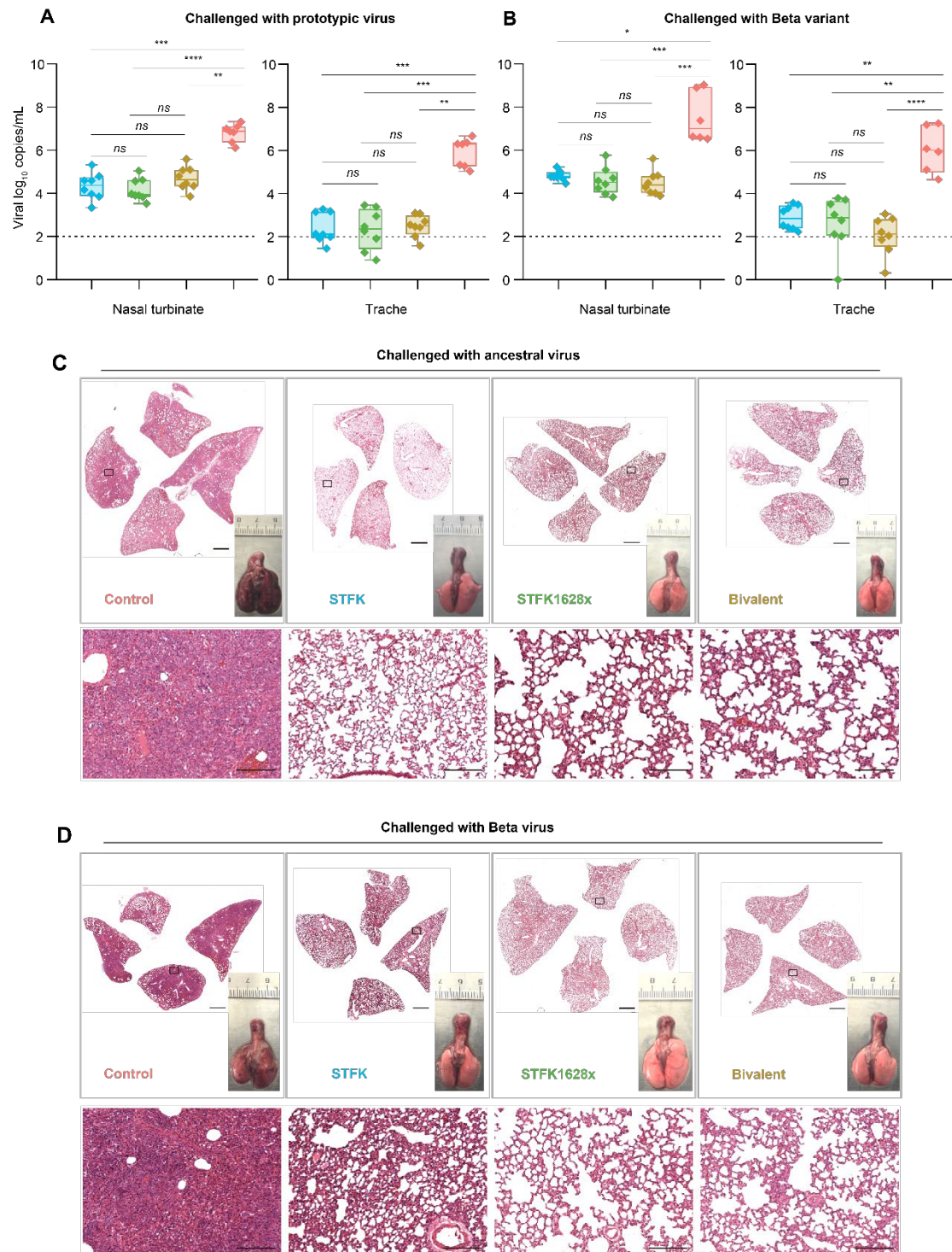


fig. S8. Tissue analyses for hamsters intranasally challenged with SARS-CoV-2. Animals were identical to that shown in Fig. 3. **(A-B)** Viral RNA levels in tissues of nasal turbinate (left panel) and trachea (right panel) collected from hamsters challenged with ancestral SARS-CoV-2 **(A)** or Beta variant **(B)**. **(C-D)** Representative H&E-stained lung sections were collected from ancestral

1 SARS-CoV-2 (**C**) or Beta variant (**D**) challenged hamsters. Views of the whole
2 lung lobes (four independent sections) and the gross observations of lung
3 tissues were presented in the top panel (scale bars, 2 mm), areas in the black
4 box were enlarged in the bottom panel (scale bars, 200 μ m). Uncorrected
5 Kruskal-Wallis tests were used for intergroup statistical comparison. Asterisks
6 indicate statistical significance (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P <$
7 0.05; ns, not significant).

8

1 **Table S1. Cryo-EM data collection, refinement and validation statistics of three-**
2 **antibody immune-complexes**

	STFK:36H6:83H7:85F7	STFK1628x:83H7:85F7:2B4
Data collection and processing		
Microscope	FEI TF30	FEI TF30
Camera	K3	K3
Magnification	39,000	39,000
Voltage (kV)	300	300
Electron exposure (e-/Å ²)	60	60
Defocus range (µm)	1.2-3.5	1.0-3.0
Pixel size (Å)	0.778	0.778
Micrographs (total)	3,479	4,191
Micrographs (used)	2,576	3,773
Total particle	1,146,590	1,684,307
Final particle images (no.)	162,177	115,589
Symmetry imposed	C1	C1
Map resolution (Å)	3.81	3.88
FSC threshold	0.143	0.143
Map sharpening B factor (Å ²)	-142.4	-119.8
Validation		
MolProbity score	1.96	1.99
Clashscore	7.71	8.64
Poor rotamers (%)	0.69	0.00
RMS (bonds)	0.0113	0.0087
RMS (angles)	1.36	1.32
Ramachadran plot		
Favored (%)	90.44	90.91
Allowed (%)	9.44	8.97
Disallowed (%)	0.12	0.12

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