1 Nanobodies recognizing conserved hidden clefts of all SARS-CoV-2 spike variants

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We are in the midst of the historic coronavirus infectious disease 2019 (COVID-19) pandemic caused by severe respiratory syndrome coronavirus 2 (SARS-CoV-2). Although countless efforts to control the pandemic have been attempted—most successfully, vaccination¹⁻³—imbalances in accessibility to vaccines, medicines, and diagnostics among countries, regions, and populations have been problematic. Camelid variable regions of heavy chain-only antibodies (VHHs or nanobodies)⁴ have unique modalities: they are smaller, more stable, easier to customize, and, importantly, less expensive to produce than conventional antibodies^{5,6}. We present

the sequences of nine alpaca nanobodies that detect the spike proteins of four SARS-CoV-2 variants of concern (VOCs)—namely, the alpha, beta, gamma, and delta variants. We show that they can quantify or detect spike variants via ELISA and lateral flow, kinetic, flow cytometric, microscopy, and Western blotting assays⁷. The panel of nanobodies broadly neutralized viral infection by pseudotyped SARS-CoV-2 VOCs. Structural analyses showed that a P86 clone targeted epitopes that were conserved yet unclassified on the receptor-binding domain (RBD) and located inside the N-terminal domain (NTD). Human antibodies have hardly accessed both regions; consequently, the clone buries hidden crevasses of SARS-CoV-2 spike proteins undetected by conventional antibodies and maintains activity against spike proteins carrying escape mutations.

We have been in the historic pandemic for about two years (2019-2021): numbers of confirmed deaths and infections still increase on a weekly basis. Researchers have developed many antibodies and nanobodies as weapons to fight against COVID-19⁸⁻¹¹; however, emerging SARS-CoV-2 variants of concern (VOCs) that carry mutations in the spike often escape the immune system and therapeutic antibodies¹²⁻¹⁴. These antibodies also serve as armor and shields, allowing us to survey infected individuals

and monitor circumstances via antigen test kits, enzyme-linked immunosorbent assays (ELISAs), dot blotting, and so forth. However, accessibility to diagnostic and monitoring kits is imbalanced worldwide because antibody-producing cells (e.g., hybridoma cells and engineered mammalian cells) are, in many cases, dominated by licenced companies that incur high costs of research, development, production, and distribution. Thus, we tried to create easily accessible and broadly neutralizing antibodies for SARS-CoV-2 VOCs.

Although many antibodies and nanobodies against the spike, especially the receptor-binding domain (RBD), have been established, those that are broadly neutralizing and detect all current variants have yet to be development. Few anti-SARS-CoV-2 spike *nanobodies* have been applied for immune assays, such as ELISAs and lateral flow and especially membrane dot blotting assays¹⁵. Here, we provide a panel of broadly neutralizing nanobodies that detect four SARS-CoV-2 VOCs. Our structural analyses show that two clones—P17 and P86—capped the receptor-binding domain (RBD) regardless of their up or down conformations. The most potent clone, P86, bridged a gap (Fig. 1a,b) between the down conformation of the RBD and the N-terminal domain (NTD) of a neighbouring protomer. The epitopes on the RBD recognized by the two clones partially overlapped; in particular, the epitope recognized

by P17 shifted away from the L452R mutation observed in the SARS-CoV-2 delta variant spike.

Purifying a SARS-CoV-2 spike protein retaining the furin cleavage site

The SARS-CoV-2 spike is one of the main targets for human antibodies^{16,17}. Its trimer complex is expressed on the surface of the virion and plays a role in fusion with host cells expressing the binding partner angiotensin-converting enzyme II (ACE2)^{18,19}. The most featured alteration of the SARS-CoV-2 spike compared to other coronavirus spikes is the generation of a furin cleavage site (-R-R-A-R⁶⁸⁵-: furin protease hydrolyses the C-terminal peptide bond of R⁶⁸⁵)²⁰ located directly between the S1 (residues 1-685) and S2 (residues 686-1,213) domains^{17,21}. Because it was difficult to obtain the extracellular domain of SARS-CoV-2 spike with the furin site from the supernatant of human embryonic kidney (HEK) cells, we attempted to purify it from cell lysate. We obtained a large complex of SARS-CoV-2 spike with detergents used in crystallizing membrane proteins (Extended Data Fig. 1a-c, and see Methods)²². As reported previously, although peaks were broad, buffers containing high salt and detergent concentrations maintained the SARS-CoV-2 spike trimer assembly (Extended Data Fig. 1b)²³. We noticed that a high salt concentration was necessary to stabilize the SARS-CoV-2 spike complexes

(Extended Data Fig. 1b)²⁴.

Immunization of alpacas and selection of nanobodies

To obtain spike-specific nanobodies, we immunized two alpacas with the purified whole extracellular domains of the SARS-CoV-2 spike complex from cell lysates²⁵ (Extended Data Fig. 1c, and see Methods). Serum titres of alpacas for SARS-CoV-2 spike increased and reached a plateau, indicating that both alpacas were effectively vaccinated (Extended Data Fig. 1d). We performed one round of biopanning with different proteins: the whole extracellular domain, the S2 domain alone of the SARS-CoV-2 spike, or the whole extracellular domain of the seasonal cold coronavirus HCoV-OC43 spike^{26,27} (see Methods). After deep-sequencing nanobody-coding genes of each panned sublibrary, we identified nine clones that were significantly enriched in sublibraries panned with the whole extracellular domain of SARS-CoV-2 compared with those panned with the others²⁸ (Fig. 1c,d, and see Methods). We expressed these clones in bacteria and purified them as C-terminally His-tagged dimers (Extended Data Fig. 2a,b); we then characterized their avidity, applicability, and neutralizing activity, as described below.

Kinetics and detectability of nanobody binding

First, we measured the binding kinetics of these clones for the whole extracellular domain of SARS-CoV-2 spike, which was the same construct that was used for immunization, via biolayer interferometry (Fig. 1e). We noticed that the salt concentrations in the kinetic assay buffers affected the avidities of the clones. Of note, high salt concentrations were necessary to stabilize the spike complex (Extended Data Fig. 1b). Four clones (P17, P86, C116, and P334) showed high avidity for the SARS-CoV-2 spike complex in hypertonic buffer; the others (C17, C49, P158, C246, and P543) showed high avidity in hypotonic buffer (Extended Data Fig. 2c,d).

Second, we checked these nanobodies via microscopy observation using HEK cells expressing the SARS-CoV-2 spike. Seven clones stained near the cell surface; two clones (C49 and C246) stained only an intracellular region (Extended Data Fig. 3a,b).

Third, flow cytometric analysis supported this observation: the seven clones detected the spike protein on the cell surface, but the C49 and C246 clones did not (Extended Data Fig. 3c). These results suggested that each clone recognizes the SARS-CoV-2 spike protein in a status-dependent manner.

Sensitivities of the clones for the RBD mutations of VOCs

The World Health Organization (WHO) has declared four strains to be VOCs, which have

mutations in the RBD: Alpha/UK/B.1.1.7 (N501Y); Beta/South Africa/B.1.351 (K417N, E484K, and N501Y); Gamma/Brazil/P.1 (K417T, E484K, and N501Y); and Delta/India/B.1.617.2 (L452R and T478K)^{29,30}. The SARS-CoV-2 virus strains having mutations in the residues E484 or L452 frequently escape neutralizing antibodies³¹⁻³³. We checked which clones could detect which spike variants via microscopy observation. Two clones (C17 and P17) exhibited severely reduced sensitivity for the spike variant carrying the E484K mutation; previously reported SARS72³⁴, mNb6³⁵, and Ty1³⁶ nanobodies also did not detect these variants. Although the Ty1 nanobody did not sense the spike variant having the L452R mutation, the clones provided in this study remained able to detect the L452R substitution (Extended Data Fig. 4).

Nanobodies for test kits

The finding that some clones recognized fluctuating, immature, or possibly unfolded SARS-CoV-2 spike proteins led us to consider whether the clones could detect the denatured SARS-CoV-2 spike variants via immunological assays. We sampled cell lysates expressing full-length C-terminally C9-tagged SARS-CoV-2 spike variants in Laemmli's sodium dodecyl sulfate (SDS) buffer under reducing conditions³⁷: The P158, P334, and P543 clones specifically detected SARS-CoV-2 spike proteins with the same

sensitivity as the mouse monoclonal C9-tagged antibody (Fig. 2a). The banding patterns suggested that each clone recognized a part of the S1 domain. These clones (P158, P334, and P543) detected SARS-CoV-2 spike variants—including alpha and beta variants—via Western blotting.

Nanobody-based ELISA and lateral flow assay

We then tried to develop a sandwich ELISA kit to detect the SARS-CoV-2 spike using these nanobodies. We tested eight clones as detector nanobodies with six blocking conditions on P158-coated plates; we found that P86 and P543 worked well (Extended Data Fig. 5). The detection limit of the SARS-CoV-2 spike was approximately $1 \mu\text{g ml}^{-1}$ when using P158 as the capture and P543 as the detector nanobodies³⁸ (Fig. 2b). Both P543 and P86 were able to specifically detect the SARS-CoV-2 spike original and beta variant in cellular homogenates: HEK cells expressing the full-length spikes were lysed and sequentially diluted (Fig. 2c). When the detection antibody was changed to Fc-tagged P543 (P543-Fc), the detection limit reached below 20 ng ml^{-1} (Fig. 2d). Moreover, antigen test kits based on a lateral flow membrane assay in which P158 lined a nitrocellulose membrane and P86 was conjugated to labelled beads were also able to detect 300 ng of the spike delta variant (Fig. 2e). We checked the capability of each clone via microscopy

observations using cells expressing SARS-CoV-2 spike variants carrying associated mutations. The clones that were useful for blotting (P158, P334, and P543) and P86 were still able to detect the SARS-CoV-2 spike carrying mutations in the S1 domain, including deletion mutations (del) of H69, V70, Y144, Y145, L242, A243, and L244 and point mutations of L18F, T20N, P26S, D138Y, R190S, D215G, R246I, N439Y, Y453F, T478K, and A570D³⁹⁻⁴² (Extended Data Fig. 6).

Neutralizing activity of the nanobodies against SARS-CoV-2 spike variants

We assayed whether these clones could neutralize virus infection using SARS-CoV-2 spike pseudotyped HIV-1-based viruses. We used K562 cells stably expressing human ACE2 and serine protease TMPRSS2. We also produced pseudotyped viruses encoding the luciferase gene and expressing the SARS-CoV-2 spike variants. Five (C17, P17, P86, C116, and C246) out of nine clones inhibited the pseudotyped virus expressing the spike (original WH-1: D614G) in a dose-dependent manner (Fig. 3a). Then, we also tested the potency of the five clones for the other SARS-CoV-2 variants—alpha, beta, and delta. The P17, P86, and C116 clones more potently neutralized the alpha variant than Ty1; the P86 and C246 clones significantly suppressed the beta variant; and P17 and C246 suppressed the delta variant (Fig. 3a). The IC₅₀ results were summarized and compared:

C246, which stained intracellular spike (Extended Data Fig. 4c), suppressed all variants tested; P86 neutralized beta but not delta variants; and P17 neutralized delta but not beta variants (Fig. 3b). Because it was unclear to us why P17 could be affected by the E484K mutation (Beta) and P86 by the L452R mutation (Delta), we finally determined the differences in the epitopes of the two clones.

The binding epitopes of the P86 and P17 clones

To determine the epitopes of the P86 and P17 clones on the SARS-CoV-2 spike, we performed a single-particle analysis of the spike-P86 and spike-P17 complexes using cryo-electron microscopy (cryo-EM). We observed 2-RBD-up (2-up+P86) and 3-RBD-up (3-up+P86) from the spike-P86 complex and 1-RBD-up (1-up+P17) and 2-RBD-up (2-up+P17) from the spike-P17 complex (Fig. 4a,b, Extended Data Fig. 7, and Extended Data Table 1). The densities of P86 and P17 were observed just the outside surfaces of both up- and down-RBDs—this arrangement was more apparent for the down-RBD (Extended Data Fig. 7). We observed that the densities of the clones bound to the down-RBD extended to the NTD of the neighbouring protomer (PDB entry: 7KRR)—more apparently in P86—the densities of P17 were too weak to determine the orientation (Fig. 4c,d).

To build an atomic model of 2-up+P86, we determined the crystal structure of P86 at 1.60 Å resolution (Extended Data Table 2). The asymmetric unit contained two structurally identical monomers (a root mean square deviation for C_α atoms of 0.20 Å); the three CDR regions were visualized well (Extended Data Fig. 8a,b). We fit the P86 crystal structure to the density near the down-RBD in a locally refined map. The map showed two featured regions: a loop-like density between the down-RBD and the NTD of the neighbouring protomer and an elongated density located on the top (Extended Data Fig. 8c). We fit the CDR3 and the C-terminal (C-ter.) region into the former and the latter, respectively, and the whole body was adequately accommodated. After manual model modifications, the P86 monomer fit well into the final sharpened map (Fig. 4c [left panel] and Extended Data Fig. 8c-e). The edge of the CDR3 region was inserted into the cleft between the down-RBD and the NTD of the neighbouring protomer, forming many hydrophilic and hydrophobic interactions (Fig. 4c [right panel]). Compared to the density of P86, the density of P17 was shifted towards the top of the RBD (Fig. 4e). Therefore, the interface between the down-RBD and P17 was near the receptor-binding motif (RBM: ACE2-binding region); on the other hand, the interface between the down-RBD and P86 shifted to the NTD of the neighbouring protomer (Fig. 4e).

To date, human antibodies target four epitopes, all on the RBD: Class-1 (RBM

class 1), Class-2 (RBM class 2), Class-3 (RBD core 1), and Class-4 (RBD core 2)⁴³⁻⁴⁵
(Extended Data Fig. 8f). The epitope of P86 was located outside of Class-2 and Class-3
and was on the opposite side of Class-1 and Class-4 (Fig. 4f and Extended Data Fig. 8f,g).
We compared two antibodies—DH1041(PDB entry: 7LAA) and Ty1 (PDB entry:
6ZXN)—that target the near-surface of the down-RBD where P86 is bound^{36,46}. We
superposed them on the single down-RBD within the 2-up+P86 model, which clearly
showed P86—especially the CDR1 and CDR3 loops—uniquely bound the down-RBD
and the NTD of the neighbouring protomer (Fig. 4c,g). We noticed that the P86-bound
down-RBD interfered with the ACE2 to access the neighbouring up-RBD (Extended Data
Fig. 8d). It seems that P86 is small enough to access the *hidden* cleft that is not recognized
by human antibodies.

Discussion

We provide the sequences of the nanobodies recognizing recently emerging SARS-CoV-
2 spike VOCs. Only sequence information or expression vectors are sufficient to produce
these clones in basic biological laboratories. We showed that the nanobodies detected the
spike variants via ELISA, immunochromatography, and blotting assays. Therefore, these
clones can be applied for surveillance of the virus in wastewater, monitoring infected

individuals (e.g., passengers, farm animals, or pets), and self-testing. Here, we showed that C246, which recognized immature or unfolded spikes, reduced the activity of the three SARS-CoV-2 VOCs. We have not yet determined the epitope of C246: we wondered whether C246 might inhibit the maturation of the virus rather than compete for association with the target⁴⁷.

The epitopes of P17 and P86 were shifted relative to each other (Fig. 4e), which caused different tolerances to the mutations—P17 neutralized the delta (L452R) variant, whereas P86 neutralized the beta (E484K) variant. The L452R variant may substantially reduce the avidity of P86 to the RBD: R452 is located near the centre of the P86 binding interface. By contrast, because P17 binds to the surface near the top of the RBD and R452 exists on the edge of the interface, P17 tolerates the long R452 side chain. E484 is part of the flexible *hook* rope of the RBD. Structural and molecular dynamics studies have suggested that the E484K mutation disorders the hook region⁴⁸. We assume that the Class-2 antibodies can only bind a solid state of the hook containing E484, whereas P86 is not severely influenced by states of the hook region containing K484 because P86 also contacts the conserved cleft between the RBD and the NTD of the neighbouring protomer. The epitope of P86 seems too narrow to be accessed by conventional antibodies. Indeed, the region of the RBD where P86 binds has not yet been classified as an epitope of human

253 antibodies⁴⁹ (Extended Data Fig. 8f,g). Therefore, we expect these clones to be similar to
 254 *lime in a gimlet* of antibody-based therapies.

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256 1 Amanat, F. *et al.* SARS-CoV-2 mRNA vaccination induces functionally diverse
 257 antibodies to NTD, RBD, and S2. *Cell* **184**, 3936-3948 e3910,
 258 doi:10.1016/j.cell.2021.06.005 (2021).

259 2 Wang, Z. *et al.* Naturally enhanced neutralizing breadth against SARS-CoV-2
 260 one year after infection. *Nature* **595**, 426-431, doi:10.1038/s41586-021-03696-9
 261 (2021).

262 3 Collier, D. A. *et al.* Sensitivity of SARS-CoV-2 B.1.1.7 to mRNA vaccine-
 263 elicited antibodies. *Nature* **593**, 136-141, doi:10.1038/s41586-021-03412-7
 264 (2021).

265 4 Hamers-Casterman, C. *et al.* Naturally occurring antibodies devoid of light
 266 chains. *Nature* **363**, 446-448, doi:10.1038/363446a0 (1993).

267 5 Pardon, E. *et al.* A general protocol for the generation of Nanobodies for
 268 structural biology. *Nat Protoc* **9**, 674-693, doi:10.1038/nprot.2014.039 (2014).

269 6 Saelens, X. & Schepens, B. Single-domain antibodies make a difference.
 270 *Science* **371**, 681-682, doi:10.1126/science.abg2294 (2021).

- 271 7 Czajka, T. F., Vance, D. J. & Mantis, N. J. Slaying SARS-CoV-2 One (Single-
272 domain) Antibody at a Time. *Trends Microbiol* **29**, 195-203,
273 doi:10.1016/j.tim.2020.12.006 (2021).
- 274 8 Huo, J. *et al.* Neutralizing nanobodies bind SARS-CoV-2 spike RBD and block
275 interaction with ACE2. *Nat Struct Mol Biol* **27**, 846-854, doi:10.1038/s41594-
276 020-0469-6 (2020).
- 277 9 Bessalah, S. *et al.* Perspective on therapeutic and diagnostic potential of camel
278 nanobodies for coronavirus disease-19 (COVID-19). *3 Biotech* **11**, 89,
279 doi:10.1007/s13205-021-02647-5 (2021).
- 280 10 Koenig, P. A. *et al.* Structure-guided multivalent nanobodies block SARS-CoV-2
281 infection and suppress mutational escape. *Science* **371**,
282 doi:10.1126/science.abe6230 (2021).
- 283 11 Raybould, M. I. J., Kovaltsuk, A., Marks, C. & Deane, C. M. CoV-AbDab: the
284 coronavirus antibody database. *Bioinformatics* **37**, 734-735,
285 doi:10.1093/bioinformatics/btaa739 (2021).
- 286 12 Chen, R. E. *et al.* In vivo monoclonal antibody efficacy against SARS-CoV-2
287 variant strains. *Nature* **596**, 103-108, doi:10.1038/s41586-021-03720-y (2021).
- 288 13 Schmidt, F. *et al.* High genetic barrier to SARS-CoV-2 polyclonal neutralizing

289 antibody escape. *Nature*, doi:10.1038/s41586-021-04005-0 (2021).

290 14 Wang, P. *et al.* Antibody resistance of SARS-CoV-2 variants B.1.351 and
291 B.1.1.7. *Nature* **593**, 130-135, doi:10.1038/s41586-021-03398-2 (2021).

292 15 Jovcevska, I. & Muyldermans, S. The Therapeutic Potential of Nanobodies.
293 *BioDrugs* **34**, 11-26, doi:10.1007/s40259-019-00392-z (2020).

294 16 Walls, A. C. *et al.* Unexpected Receptor Functional Mimicry Elucidates
295 Activation of Coronavirus Fusion. *Cell* **176**, 1026-1039 e1015,
296 doi:10.1016/j.cell.2018.12.028 (2019).

297 17 Walls, A. C. *et al.* Structure, Function, and Antigenicity of the SARS-CoV-2
298 Spike Glycoprotein. *Cell* **183**, 1735, doi:10.1016/j.cell.2020.11.032 (2020).

299 18 Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2
300 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280
301 e278, doi:10.1016/j.cell.2020.02.052 (2020).

302 19 Yurkovetskiy, L. *et al.* Structural and Functional Analysis of the D614G SARS-
303 CoV-2 Spike Protein Variant. *Cell* **183**, 739-751 e738,
304 doi:10.1016/j.cell.2020.09.032 (2020).

305 20 Tomiyama, K. *et al.* Relevant use of Klotho in FGF19 subfamily signaling
306 system in vivo. *Proc Natl Acad Sci U S A* **107**, 1666-1671,

- 307 doi:10.1073/pnas.0913986107 (2010).
- 308 21 Zhou, H. *et al.* Identification of novel bat coronaviruses sheds light on the
- 309 evolutionary origins of SARS-CoV-2 and related viruses. *Cell* **184**, 4380-4391
- 310 e4314, doi:10.1016/j.cell.2021.06.008 (2021).
- 311 22 Rasmussen, S. G. *et al.* Structure of a nanobody-stabilized active state of the
- 312 beta(2) adrenoceptor. *Nature* **469**, 175-180, doi:10.1038/nature09648 (2011).
- 313 23 Clausen, T. M. *et al.* SARS-CoV-2 Infection Depends on Cellular Heparan
- 314 Sulfate and ACE2. *Cell* **183**, 1043-1057 e1015, doi:10.1016/j.cell.2020.09.033
- 315 (2020).
- 316 24 Kawamoto, A. *et al.* Native flagellar MS ring is formed by 34 subunits with 23-
- 317 fold and 11-fold subsymmetries. *Nat Commun* **12**, 4223, doi:10.1038/s41467-
- 318 021-24507-9 (2021).
- 319 25 Dai, L. & Gao, G. F. Viral targets for vaccines against COVID-19. *Nat Rev*
- 320 *Immunol* **21**, 73-82, doi:10.1038/s41577-020-00480-0 (2021).
- 321 26 Shirakawa, K. *et al.* Phosphorylation of APOBEC3G by protein kinase A
- 322 regulates its interaction with HIV-1 Vif. *Nat Struct Mol Biol* **15**, 1184-1191,
- 323 doi:10.1038/nsmb.1497 (2008).
- 324 27 Tortorici, M. A. *et al.* Structural basis for human coronavirus attachment to sialic

325 acid receptors. *Nat Struct Mol Biol* **26**, 481-489, doi:10.1038/s41594-019-0233-
326 y (2019).

327 28 Xu, J. *et al.* Nanobodies from camelid mice and llamas neutralize SARS-CoV-2
328 variants. *Nature* **595**, 278-282, doi:10.1038/s41586-021-03676-z (2021).

329 29 Tegally, H. *et al.* Detection of a SARS-CoV-2 variant of concern in South Africa.
330 *Nature* **592**, 438-443, doi:10.1038/s41586-021-03402-9 (2021).

331 30 Mlcochova, P. *et al.* SARS-CoV-2 B.1.617.2 Delta variant replication and
332 immune evasion. *Nature*, doi:10.1038/s41586-021-03944-y (2021).

333 31 Deng, X. *et al.* Transmission, infectivity, and neutralization of a spike L452R
334 SARS-CoV-2 variant. *Cell* **184**, 3426-3437 e3428,
335 doi:10.1016/j.cell.2021.04.025 (2021).

336 32 Cele, S. *et al.* Escape of SARS-CoV-2 501Y.V2 from neutralization by
337 convalescent plasma. *Nature* **593**, 142-146, doi:10.1038/s41586-021-03471-w
338 (2021).

339 33 Dejnirattisai, W. *et al.* Antibody evasion by the P.1 strain of SARS-CoV-2. *Cell*
340 **184**, 2939-2954 e2939, doi:10.1016/j.cell.2021.03.055 (2021).

341 34 Wrapp, D. *et al.* Structural Basis for Potent Neutralization of Betacoronaviruses
342 by Single-Domain Camelid Antibodies. *Cell* **181**, 1436-1441,

doi:10.1016/j.cell.2020.05.047 (2020).

35 Schoof, M. *et al.* An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive Spike. *Science* **370**, 1473-1479, doi:10.1126/science.abe3255 (2020).

36 Hanke, L. *et al.* An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat Commun* **11**, 4420, doi:10.1038/s41467-020-18174-5 (2020).

37 Imura, A. *et al.* alpha-Klotho as a regulator of calcium homeostasis. *Science* **316**, 1615-1618, doi:10.1126/science.1135901 (2007).

38 Zhu, M., Gong, X., Hu, Y., Ou, W. & Wan, Y. Streptavidin-biotin-based directional double Nanobody sandwich ELISA for clinical rapid and sensitive detection of influenza H5N1. *J Transl Med* **12**, 352, doi:10.1186/s12967-014-0352-5 (2014).

39 Plante, J. A. *et al.* The variant gambit: COVID-19's next move. *Cell Host Microbe* **29**, 508-515, doi:10.1016/j.chom.2021.02.020 (2021).

40 McCallum, M. *et al.* N-terminal domain antigenic mapping reveals a site of vulnerability for SARS-CoV-2. *Cell* **184**, 2332-2347 e2316, doi:10.1016/j.cell.2021.03.028 (2021).

- 361 41 Planas, D. *et al.* Sensitivity of infectious SARS-CoV-2 B.1.1.7 and B.1.351
362 variants to neutralizing antibodies. *Nat Med* **27**, 917-924, doi:10.1038/s41591-
363 021-01318-5 (2021).
- 364 42 Thomson, E. C. *et al.* Circulating SARS-CoV-2 spike N439K variants maintain
365 fitness while evading antibody-mediated immunity. *Cell* **184**, 1171-1187 e1120,
366 doi:10.1016/j.cell.2021.01.037 (2021).
- 367 43 Barnes, C. O. *et al.* SARS-CoV-2 neutralizing antibody structures inform
368 therapeutic strategies. *Nature* **588**, 682-687, doi:10.1038/s41586-020-2852-1
369 (2020).
- 370 44 Piccoli, L. *et al.* Mapping Neutralizing and Immunodominant Sites on the
371 SARS-CoV-2 Spike Receptor-Binding Domain by Structure-Guided High-
372 Resolution Serology. *Cell* **183**, 1024-1042 e1021, doi:10.1016/j.cell.2020.09.037
373 (2020).
- 374 45 Corti, D., Purcell, L. A., Snell, G. & Veisler, D. Tackling COVID-19 with
375 neutralizing monoclonal antibodies. *Cell* **184**, 3086-3108,
376 doi:10.1016/j.cell.2021.05.005 (2021).
- 377 46 Li, D. *et al.* In vitro and in vivo functions of SARS-CoV-2 infection-enhancing
378 and neutralizing antibodies. *Cell* **184**, 4203-4219 e4232,

- 379 doi:10.1016/j.cell.2021.06.021 (2021).
- 380 47 Guttler, T. *et al.* Neutralization of SARS-CoV-2 by highly potent,
- 381 hyperthermostable, and mutation-tolerant nanobodies. *EMBO J*, e107985,
- 382 doi:10.15252/emboj.2021107985 (2021).
- 383 48 Gobeil, S. M. *et al.* Effect of natural mutations of SARS-CoV-2 on spike
- 384 structure, conformation, and antigenicity. *Science* **373**,
- 385 doi:10.1126/science.abi6226 (2021).
- 386 49 Cao, Y. *et al.* Potent Neutralizing Antibodies against SARS-CoV-2 Identified by
- 387 High-Throughput Single-Cell Sequencing of Convalescent Patients' B Cells.
- 388 *Cell* **182**, 73-84 e16, doi:10.1016/j.cell.2020.05.025 (2020).
- 389 50 Zhang, J. *et al.* Structural impact on SARS-CoV-2 spike protein by D614G
- 390 substitution. *Science* **372**, 525-530, doi:10.1126/science.abf2303 (2021).

391

Fig. 1| Structural features of the SARS-CoV-2 spike and the panel of nanobodies.

a,b, Side (**a**) and top (**b**) views of the 1-up+2-down conformation of the SARS-CoV-2 spike trimer⁵⁰ (PDB entry: 7KRR); domains of each protomer numbered I (up), II (down), and III (down). The NTD, the RBD, and the C-terminal domain (CTD) of the SARS-CoV-2 S1 domain are coloured cyan, blue, and orange, respectively; mutations and deletions studied here are indicated; clefts between the down-RBD and the NTD of the neighbouring protomer are highlighted with red circles. **c**, Graphs showing existence ratios (two different scales drawn black and grey)—*P* values in log₁₀ are plotted (see Methods)—after panning with IgM; APOBEC3G (A3G)²⁶; the S2 domain (S2); the HCoV-OC43 (HCoV) spike²⁷; and the SARS-CoV-2 spike (CoV-2). **d**, Sequences of nine clones and that of Ty1 are compared³⁶: changed amino acids in the framework regions are red; the variable complementary-determining regions (CDRs) are shown in blue. **e**, Calculated binding kinetics between each nanobody and the SARS-CoV-2 spike from sensorgrams (Extended Data Fig. 2c,d) are summarized. The upper four clones were assayed in a high-salt buffer (“stabilized” spike); the others were assayed in an anionic buffer (“fluctuated” spike).

Fig. 2| Nanobodies for Western blotting assays, ELISAs, and lateral flow assays.

a, Nanobody-based Western blotting assays of SARS-CoV-2 spikes: lysates of HEK cells expressing GFP or C-terminally C9-tagged SARS-CoV-2 spike (D614G) variants (indicated with numbers) were blotted with the indicated nanobodies. **b-d**, Sandwich ELISA: **b**, signals of gradually diluted sample concentrations using P158 as the capture antibody and P86 or P543 (**b,c**) or P543-Fc (**d**) as the detection antibodies are graphed as a mean of the measurements from 2 wells (n=2). In (**c**), sequentially diluted lysates of HEK cells expressing the original SARS-CoV-2 spike (D614G) or beta variant (D614G, N501Y, E484K, and K417N) were assayed. **e**, A photograph of the results from lateral flow nitrocellulose membrane assays: P158 for the capture line (allow head) and P86 for the detection beads; 300 ng of the purified SARS-CoV-2 spike delta variant (D614G, L452R, and T478K) (+) or PBS (-) was spotted.

Fig. 3| Pseudotyped virus neutralization assay.

a, Means of infecting units of virus for the indicated SARS-CoV-2 spike variants are graphed (n=3). K562 cells expressing ACE2 and TMPRSS2 infected with HIV-1-based pseudotyped virus were assessed via luciferase assay. Relative infection units at the indicated concentrations of the nanobodies, which are marked individually, are plotted. **b**, Measured IC₅₀s for each variant are summarized.

Fig. 4| Cryo-EM density maps of SARS-CoV-2 spike-P86 and SARS-CoV-2 spike-P17 complexes.

a,b, Final sharpened maps of the **(a)** 2-up+P86 and **(b)** 1-up+P17 datasets. The map regions corresponding to one protomer in the spike protein are coloured differently in cyan, blue, and turquoise. The map regions corresponding to one P86 or P17 molecule bound to the RBD regions are coloured magenta, red, and orange, respectively. **c**, Close-up view around P86 bound to the down-RBD with our fitted model. Right panel shows close-up view around the interface among P86, the down-RBD, and the NTD of the neighbouring protomer. **d**, Close-up view around P17 bound to the down-RBD. The model of D614G spike trimer (PDB entry: 7KRR) was fitted on the maps⁵⁰. **e**, The residues located on the interface between the down-RBD and P86 or P17. **f**, Epitope mapping on the down-RBD. The epitopes of Class-2, Class-3, and P86 are shown in yellow, blue, and magenta, respectively. The colouring priority of the overlapping region is Class-3>Class-2>P86. The model of P86 is a ribbon structure. **g**, Structure comparison with other antibodies bound to the down-RBD. The structures are superposed on the single RBD region.

Methods

Ethics statement for animal care

Two young alpacas (*Vicugna pacos*) half-siblings—a 19-month-old male named “Putu” and a 19-month-old female named “Christy”—were immunized. Veterinarians of the KYODOKEN Institute for Animal Science Research and Development (Kyoto, Japan) bred, maintained health, recorded conditions, and performed the immunization studies by adhering to the published Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan. The KYODOKEN Institutional Animal Care and Use Committee approved the protocols for these studies (approval number 20200312) and monitored health conditions. The veterinarians immunized the alpacas with antigens and collected blood samples under anaesthesia.

Immunization and library generation

Immunized antigens were purified recombinant CoV-2 spike complexes: the extracellular domain of the original WH-1 protein (GenBank: QHD43416) with or without the D614G mutation that carried a maintained or mutated furin cleavage site—N⁶⁷⁹SPRRA or IL⁶⁸⁰. The protein mixture emulsified in complete Freund’s adjuvant was

465 subcutaneously injected into the two alpacas up to 9 times at 2-week intervals. Blood
466 samples were collected from the jugular vein; peripheral blood mononuclear cells
467 (PBMCs) were obtained with a sucrose density gradient using Ficoll⁵¹ (Nacalai Tesque,
468 Kyoto, Japan). The PBMC samples were washed with PBS and suspended in RNAlater
469 solution (Thermo Fisher Scientific K.K., Tokyo, Japan). Total RNA was isolated from
470 the PBMC samples (Direct-Zol RNA MiniPrep: Zymo Research, Irvine, CA).

471 Complementary DNA was synthesized from 1 µg of the total RNA as a
472 template with random hexamer primers and using SuperScript II reverse transcriptase
473 (Thermo). Coding regions of the heavy-chain variable domains were amplified using
474 LA Taq polymerase (TAKARA Bio Inc., Shiga, Japan) with two PAGE-purified primers
475 (CALL001: 5'-GTCCTGGCTGCTCTTCTACAAGG-3' and CALL002: 5'-
476 GGTACGTGCTGTTGAACTGTTCC-3'). The amplified coding gene fragments of
477 heavy-chain variable domains were separated on a 1.5% low-temperature melting
478 agarose gel (Lonza Group AG, Basel, Switzerland). Approximately 700 base pair bands
479 corresponding to the heavy-chain only immunoglobulin were extracted (QIAquick Gel
480 Extraction Kit: Qiagen K.K., Tokyo, Japan). Nested PCR was performed to amplify
481 coding genes of VHH domains using the VHH-PstI-For and VHH-BstEII-Rev primers
482 and subcloned into the pMES4 phagemid vector^{5,52} (GeneArt DNA Synthesis: Thermo).

Electroporation-competent *Escherichia. coli* TG1 cells (Agilent Technologies Japan, Ltd., Tokyo, Japan) were transformed with the ligated plasmids under chilled conditions (Bio-Rad Laboratories, Inc., Hercules, CA). Colony-forming units of the libraries were checked with limiting dilution to maintain $>10^7$ per microlitre. Colonies from 8 ml of cultured cells were harvested, pooled, and reserved in frozen glycerol stocks as parent libraries.

Plasmid construction for protein expression

The gene encoding the extracellular domain of the SARS-CoV-2 spike protein (residues 31-1213) was codon optimized and synthesized into the pcDNA3.1(+) vector (Thermo) with an N-terminally modified IL-2-derived signal peptide (ILco2: MRRMQLLLLLIALSLALVTNS)⁵³; proline substitutions at residues K986P and V987P; and the C-terminal T4-phage fibritin trimerization domain (foldon) following a 6×His-tag^{34,54-56}. The expression vector of the SARS-CoV-2 S2 domain (residues 744-1213) was constructed by removing an N-terminal part of the extracellular domain of the SARS-CoV-2 spike (residues 31-743) and subcloned into the pcDNA3.1(+) vector. The expression vectors of the full-length SARS-CoV-2 spike and the human serine protease TMPRSS2 with a C-terminal C9-tag (TETSQVAPA) were acquired from AddGene

501 (Summit Pharmaceutical International, Tokyo, Japan). The genes encoding the
502 extracellular domain of human ACE2 (residues 1-614) and the endemic human
503 coronavirus HCoV-OC43 (residues 1-1322) were codon optimized, synthesized with a
504 C-terminal 6×His-tag and subcloned into the pcDNA3.1(+) vector. The whole gene of
505 the human apolipoprotein B messenger-RNA-editing enzyme catalytic polypeptide-like
506 (APOBEC) 3G (A3G)²⁶ was also codon optimized, synthesized with a C-terminal
507 6×His-tag, and subcloned into the pcDNA3.1(+) vector.

508 For structural analyses, the sequence encoding the spike ectodomain (residues
509 1-1208) with proline substitutions, a “GSAS” substitution at the furin cleavage site
510 (residues 682-685)⁵⁷, and the C-terminal foldon trimerization motif followed by an
511 8×His-tag was cloned into the pcDNA3.1(+) expression vector (Invitrogen).
512 Furthermore, the D614G mutation in the spike protein was introduced by the inverse
513 PCR method. For cryo-EM, recombinant spike proteins were transiently expressed in
514 Expi293-F cells (Thermo) maintained in HE400AZ medium (Gmep, Inc., Fukuoka,
515 Japan). The expression vector was transfected using a Gxpress 293 Transfection Kit
516 (Gmep) according to the manufacturer’s protocol. The culture supernatants were
517 harvested five days post-transfection. The C-terminally 6×His-tagged spike proteins
518 were purified using a nickel Sepharose 6 FF column (Cytiva) and size exclusion

chromatography using a Superdex200increase 10/300 GL column (Cytiva) with buffer containing 50 mM HEPES (pH 7.0) and 200 mM NaCl.

Biopanning

The purified proteins were i) the extracellular domain of the SARS CoV-2 spike; ii) only the S2 domain of the SARS CoV-2 spike; iii) the extracellular domain of the seasonal cold coronavirus spike of HCoV-OC43; iv) A3G; and v) homemade IgM coupled to N-hydroxysuccinimide (NHS)-activated magnet beads (Dynabeads, Thermo). One round of biopanning was performed using different protein-coated magnet beads in 50 mM phosphate buffer (pH 7.4) containing 1% (n-dodecyl- β -D-maltopyranoside) (DDM: Nacalai), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS: Nacalai), 0.001% cholesterol hydrogen succinate (CHS: Tokyo Chemical Industry Co., Ltd. (TCI), Tokyo, Japan); 0.1% LMNG (anatrace, Maumee, OH); and 500 mM NaCl. After 3 washes with the same buffer, the remaining phages bound to the washed beads were eluted with a trypsin-ethylenediaminetetraacetic acid (EDTA: Nacalai) solution at room temperature for 30 min. The elution was neutralized with a PBS-diluted protein inhibitor cocktail (cOmplete, EDTA-free, protease inhibitor cocktail tablets: Roche Diagnostics GmbH,

Mannheim, Germany) and used to infect electroporation-competent cells. The infected cells were cultured and selected in LB broth Miller containing 100 µg ml⁻¹ ampicillin (Nacalai) at 37°C overnight. The selected phagemids were collected using a QIAprep Miniprep Kit (Qiagen).

Sequence analysis of nanobody libraries

The VHH-coding regions within parent libraries and 1-round target-enriched sublibraries were PCR amplified and purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK). Then, dual-indexed libraries were prepared and sequenced on an Illumina MiSeq (Illumina, San Diego, CA) using a MiSeq Reagent Kit v3 with paired-end 300 bp reads⁵⁸ (Bioengineering Lab. Co., Ltd., Kanagawa, Japan). Approximately 100,000 paired reads of each library were generated. The raw data of reads were trimmed of the adaptor sequence using cutadapt v1.18⁵⁹, and low-quality reads were subsequently removed using Trimmomatic v0.39⁶⁰. The remaining paired reads were merged using fastq-join⁶¹ and then translated to the amino acid sequences using EMBOSS v6.6.0.0⁶². Finally, unique amino acid sequences in each library were counted using a custom Python script combining seqkit v0.10.1⁶³ and usearch v.11⁶⁴. Enrichment scores of each clone were analysed by calculating the *P*-value of χ^2 tests

between the existing ratios among the different sublibraries. We chose nine clones whose enrichment scores in the SARS-CoV-2 spike biopanned sublibraries were higher than those in other biopanned sublibraries.

Nanobody expression

Each selected amino-acid sequence was connected with a (GGGS)₄ linker as a tandem dimer; coding genes of these and of the previously reported SARS72 dimer, mNb6 dimer, and Ty1 monomer were codon-optimized and synthesized (Eurofins Genomics Inc, Tokyo, Japan). The synthesized genes were subcloned in the pMES4 vector to express N-terminal PelB signal peptide-conjugated and C-terminal 6×His-tagged nanobodies into the bacterial periplasm. These gene constructs were transformed into BL21(DE3) *E. coli* cells (BioDynamics Laboratory Inc., Tokyo, Japan) and plated on LB agar with ampicillin, which were incubated at 37°C overnight. Colonies were picked and cultured at 37°C to reach an OD of 0.6 AU; the cells were cultured at 37°C for 3 h or at 28°C overnight with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside: Nacalai). Cultured cells were collected by centrifugation. Nanobodies were eluted from the periplasm by soaking in a high osmotic buffer containing 200 mM Tris, 0.5 mM EDTA, and 500 mM sucrose (pH 8.0) at 4°C for 1 h. They were incubated with 2× volumes of a

diluted buffer containing 50 mM Tris, 0.125 mM EDTA, and 125 mM sucrose (pH 8.0) with a trace amount of benzonase nuclease (Merck KGaA, Darmstadt, Germany) at 4°C for 45 min, and the supernatants were centrifuged (20,000×g, 4°C for 10 min). The supernatants were sterilized with the addition of gentamicin (Thermo) and passed through a 0.22-μm filter (Sartorius AG, Göttingen, Germany). The filtered supernatant was applied to a HisTrap HP nickel column (Cytiva) equipped on an ÄKTA purifier HPLC system (Cytiva) and washed; the bound His-tagged protein was eluted with 300 mM imidazole. The elution was concentrated with a VIVAspin 3,000-molecular weight cut off (MWCO) filter column (Sartorius) and applied to a Superdex75increase 10/300 GL gel-filtration column (Cytiva) equipped on an ÄKTA pure HPLC system (Cytiva) to obtain the dimer fractions and exclude cleaved monomers and imidazole. Purity was measured via Coomassie Brilliant Blue (CBB) staining.

Antibodies

Antibodies used for Western blotting and cell staining were anti-His (rabbit polyclonal PM032: Medical and Biological Laboratories Co., Ltd. (MBL), Nagoya, Japan), anti-His (rabbit monoclonal EPR20547: ab213204, Abcam), and anti-rhodopsin C9 (TETSQVAPA) (mouse monoclonal 1D4, sc-57432: Santa Cruz Biotechnology Inc.,

CA). Horseradish peroxidase (HRP)-linked secondary antibodies included anti-mouse IgG (sheep polyclonal, NA931: GE Healthcare, Buckinghamshire, UK), anti-rabbit IgG (sheep polyclonal, NA934: GE Healthcare), and anti-alpaca IgG⁶⁵ (goat polyclonal, 128-035-232: Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Alexa Fluor 488 goat anti-mouse IgG (rabbit polyclonal, P0449: Dako, Glostrup, Denmark), and Alexa Fluor 594 goat anti-rabbit IgG (Dako).

Western blotting and CBB staining

Samples were incubated at 37°C for 30 min (for SARS-CoV-2 spike variants) or boiled for 2 min (for nanobodies) with Laemmli's SDS sample buffer containing 2.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 13.3 mM dithiothreitol (DTT). Samples were electrophoretically separated on 5–20% or 15–25% gradient polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P. Millipore, Billerica, MA). Blotted membranes were incubated overnight at 4°C with the C9 antibody or the C-terminally 6×His-tagged homodimer of nanobodies—the dilution ratios of P158, P334, and P543 were 1:5000, 1:1000, and 1:2500, respectively—in Tris-buffered saline (TBS, pH 7.4) containing 0.005% Tween 20 (TBST) and 5% skim milk. In the case of nanobody-based

blotting, after 3 washes with TBST, the membranes were incubated with 1:5000-diluted anti-His-tag antibody (MBL) in TBST containing 5% skim milk at room temperature for 1 h. The membranes were soaked with 1:5000-diluted HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (GE Healthcare) in TBST containing 5% skim milk for 30 min at room temperature. After 3 washes with TBST, reactive protein bands were visualized using an ECL Plus system (Cytiva). For CBB R-250 staining, a Rapid Stain CBB Kit (Nacalai) was used according to the manufacturer's protocol.

Column chromatography for protein purification

HEK cells expressing the SARS-CoV-2 S2 domain or the extracellular domain of the HCoV-OC43 spike were cultured in serum-free Opti-MEM (modified Eagle's medium: Thermo) containing 1% penicillin and streptomycin. After 48 h, the culture supernatants were centrifuged, filtered, and concentrated with VIVAspin 20 size exclusion columns (30,000-MWCO). Pellets of HEK cells expressing the extracellular domain of the SARS-CoV-2 spike were suspended in 50 mM phosphate buffer (pH 7.4) containing 1% DDM, 0.1% CHAPS, 0.001% CHS, 0.1% LMNG, and 500 mM NaCl. The suspension was passed through a 21-gauge needle (Terumo Co., Tokyo, Japan) several times, trace amounts of benzonase nuclease (Merck) were added, and the lysates were incubated at

627 4°C overnight. The cell lysate was cleared by centrifugation and filtration. The C-
628 terminal 6×His-tagged spike protein was purified through a HisTrap HP 1 ml column
629 equipped on an ÄKTA pure HPLC system (Cytiva) under step-by-step elution
630 conditions using running (20 mM imidazole) and eluting (500 mM imidazole)
631 phosphate buffers containing 20 mM phosphate, 500 mM NaCl, 0.5% DDM, 0.1%
632 CHAPS, 0.001% CHS, and 0.1% LMNG (for spike proteins from the cell lysate) (pH
633 7.4). Elution fractions of 6×His-tagged proteins were identified via Western blotting.
634 Anti-6×His-tagged antibody-positive fractions were gathered and concentrated via
635 VIVAspin 6 size exclusion columns (30,000-MWCO) to reach a volume under 0.5 ml.
636 Size exclusion chromatography experiments were performed on a Superose6increase
637 10/300 GL column (Cytiva) with an ÄKTA pure HPLC system (Cytiva). Sample
638 volumes were approximately 0.5 ml; injected samples were separated in PBS or PBS-
639 LMNG (for SARS-CoV-2 spike protein) in a chilled chamber with a flow rate of 0.1 ml
640 min⁻¹. Chromatograms were monitored at 280 nm using a UV spectrophotometer.
641 Elution fractions were identified via Western blotting, gathered, and concentrated via
642 VIVAspin 6 size exclusion columns (30,000-MWCO). Protein concentrations were
643 measured with a NanoDrop 2000c spectrophotometer (1 mA.U. at 280 nm was
644 equivalent to 1 mg ml⁻¹) (Thermo).

645

646 **Cell culture and transfection**

647 HEK and K562 cells were grown in Dulbecco's modified Eagle's medium (DMEM:
648 Invitrogen) supplemented with 10% foetal bovine serum (FBS) and antibiotics (1%
649 penicillin and streptomycin). The cells were cultured in a humidified incubator with 5%
650 CO₂ at 37°C. The human ACE2 and TMPRSS2 genes were transduced into K562 cells
651 with a lentivirus. The lentiviral vector pWPI-IRES-Bla-Ak-ACE2-TMPRSS2 was
652 acquired from AddGene (plasmid #154983).

653

654 **Measuring titres and nanobody-based sandwich ELISA**

655 Two micrograms of the recombinant extracellular domain of the SARS-CoV-2 spike or
656 10 µg of the homodimer of nanobodies was diluted with 10 ml of 0.1 M carbohydrate
657 buffer (pH 9.8). Each well of a MaxiSorp 96-well plate (Thermo) was coated with 100 µl
658 of the diluent at 4°C overnight. To measure the titres of the immunized two alpacas, the
659 wells were washed 3 times with high-salt PBS-LMNG (500 mM NaCl and 0.001%
660 LMNG) and blocked with the high-salt PBS-LMNG containing 1% FBS at room
661 temperature for 1 h. For screening conditions with the nanobody-based sandwich ELISA
662 for the SARS-CoV-2 spike, the wells were washed with PBST (0.05% Tween 20, pH 7.4)

and were blocked at room temperature for 1 h with five kinds of blocking solutions: 1×Carbo-Free blocking solution (Vector Laboratories, Inc., Burlingame, CA); 5% bovine serum albumin (BSA) (Sigma-Aldrich) in PBST; 5% skim milk in PBST; 3% casein (Merck) in 20 mM TBS (pH 11.0); or 5% polyvinylpyrrolidone (PVP) average molecular weight 10,000 (Sigma-Aldrich) in PBST. For measuring titres, 10 µl of a 0.1% dilution of serum from immunized alpacas in PBST was added to the well and incubated at room temperature for 1 h. After 3 washes with PBST, HRP-conjugated anti-alpaca VHH antibody (Jackson) at a dilution of 1:5000 was reacted at room temperature for 30 min. For screening of nanobody-based sandwich ELISA conditions, 100 µl of 1% (w/v) of the extracellular domain of SARS-CoV-2 spike (1 µg 100 µl⁻¹) in high-salt PBS-LMNG was added to each well and captured at room temperature for 1 h. The well was washed 3 times with the high-salt PBS-LMNG; each 30 ng of biotin-conjugated nanobody in 100 µl (0.03% w/v) of PBS-LMNG was soaked at room temperature for 30 min. After 3 washes with PBS-LMNG, 100 µl of HRP-conjugated streptavidin (TCI) at a dilution of 1:5000 in PBS-LMNG was incubated at room temperature for 30 min.

The amounts of remaining HRP conjugates after 3 washes with the buffer were measured with the addition of 100 µl of 50 mM phosphate-citrate buffer (pH 5.0) containing 0.4 µg of *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) to

develop at room temperature for 30 min, after which the reaction was stopped with the addition of 10 μ l of 5 M sulfuric acid (H_2SO_4). Each well was read at an optical density (OD) of 450 nm using a microplate reader (Bio-Rad).

Immunochromatography

Antigen test kits detecting the SARS-CoV-2 spike based on nitrocellulose lateral flow assays were developed by Yamato Scientific Co., Ltd. (Tokyo, Japan). Briefly, purified P158 (330 ng μ l⁻¹) was lined approximately 1 mm wide on an IAB90 nitrocellulose membrane (Advantech, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The membrane was soaked in 1 $\times$ Carbo-Free blocking solution at room temperature for 1 h and air dried. Purified P86 or P543 nanobodies were amine coupled to 30-nm diameter Estapore beads (Sigma-Aldrich) according to the manufacturer's protocol. Glass fibre paper (GF/DVA: Cytiva) was soaked in blocking solution containing 0.005% (w/v) nanobody-coupled Estapore beads for saturation and then dried under vacuum conditions. The lined nitrocellulose membrane and the bead-absorbed glass fibre paper were overlaid on a backing sheet (Cytiva). CF4 paper (Cytiva) was used for both the sample pad and the absorbance pad; they were overlaid on the prepared backing sheet as well. The four-layered sheet was cut 5 mm wide and housed in black cases: the cassettes were stored in

sealed packages with silica gels. When dried, 150 μ l of sample was spotted onto the sample pad; the kits were photographed under a 315 nm UV lamp for an arbitrary amount of time.

Kinetic assays via biolayer interferometry (BLI)

Real-time binding experiments were performed using an Octet Red96 instrument (fortèBIO, Pall Life Science, Portsmouth, NH). Each purified nanobody clone was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo) according to the manufacturer's protocol; uncoupled biotin was excluded with a size exclusion spin column (PD SpinTrap G-25; Cytiva) in PBS (pH 7.4). Assays were performed at 30°C with shaking at 1,000 rpm. Biotin-conjugated clones at 10 μ g ml⁻¹ were captured on a streptavidin-coated sensor chip (SA: fortèBIO) to reach the signals at 4 nm. One uncoated sensor chip was monitored as the baseline; another biotin-conjugated anti-IL-6-coated sensor chip (anti-IL-6 nanobody: COGNANO Inc.) was monitored as the background. The remaining 6 channels were immobilized with biotinylated anti-SARS-CoV-2 spike clones, and real-time binding kinetics to the purified extracellular domain of the SARS-CoV-2 spike trimer complex were measured in sequentially diluted concentrations at the same time (8 channels per assay). The concentrations of the SARS-CoV-2 spike loaded

varied between 1-32 $\mu\text{g ml}^{-1}$, corresponding to 1-32 nM or less, when an average of the molecular weight of the SARS-CoV-2 spike trimer complex was estimated to be approximately 1,000 kDa or more according to chromatograms of gel filtration column chromatography (Extended Data Fig. 1b). Assays were performed with high-salt phosphate buffer containing 500 mM NaCl and 0.001% LMNG (stabilized spike: for P17, P86, P334, and C116) or with hypotonic phosphate buffer containing 25 mM NaCl and 0.00005% LMNG (fluctuated spike: for P158, P543, C17, C49, and C246). After baseline equilibration for 180 s in each buffer, the association and dissociation were carried out for 600 s each. The data were double subtracted before fitting was performed with a 1:1 fitting model in fortèBIO data analysis software. The equilibrium dissociation constant (K_D), k_{dis} , and k_{on} values were determined with a global fit applied to all data.

Pseudotyped virus production

HIV-1-based SARS-CoV-2 spike pseudotyped virus was prepared as follows: LentiX-HEK293T cells were transfected using a polyethyleneimine transfection reagent (Cytiva) with plasmids encoding the C-terminally C9-tagged full-length SARS-CoV-2 spike variants (original, alpha, beta, and delta) and HIV-1 transfer vector encoding a luciferase reporter, according to the manufacturer's protocol. The mutations of each variant are as

follows: original (D614G); alpha (H69del, V70del, Y144del, N501Y, A570D, D614G, P681H, T716I, S982A, and D1118H); beta (L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, and A701Y); and delta (T19R, G142D, E156del, F157del, R158G, L452R, T478K, D614G, P681R, and D950N). Cells were incubated for 4-6 h at 37°C with medium that was then replaced with DMEM containing 10% FBS for the following 48-h culture. The supernatants were then harvested, filtered through a 0.45-µm membrane, concentrated with ultracentrifugation, and frozen at -80°C.

Pseudotyped virus neutralization assay

Fivefold sequentially diluted nanobodies were incubated with K562 cells expressing human ACE2 and TMPRSS2 for 1 h at 37°C. K562 cells were subsequently infected with SARS-CoV-2 pseudotyped viruses and cultured for 2 days. The cells were lysed, and luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega KK, Osaka, Japan) with a microplate spectrophotometer (ARVO X3: PerkinElmer Japan Co., Ltd., Kanagawa, Japan). The obtained relative luminescence units were normalized to those derived from cells infected with the SARS-CoV-2 pseudotyped virus in the absence of nanobodies.

Flow cytometry

The ability of nanobodies to bind to the cell surface of the SARS-CoV-2 spike was studied by fluorescence-activated cell sorting (FACS)⁶⁶. K562 cells expressing the SARS-CoV-2 spike were incubated with 1 $\mu\text{g ml}^{-1}$ purified nanobody on ice for 30 min. After washing, the cells were incubated with an anti-His antibody (Abcam) on ice for 30 min and then Alexa 647-conjugated anti-rabbit IgG (Dako). The cells were analysed with a Beckman-Coulter FC-500 Analyzer (Coulter Electronics, Hialeah, FL). The Ty1 and B9⁶⁷ nanobodies were used as controls. A region of positive signals for Ty1 was square-gated.

Microscopy analyses for cell staining

HEK cells were transiently transfected with plasmids encoding the C-terminally C9-tagged full-length SARS-CoV-2 spike variants using Lipofectamine 3000 (Thermo) according to the manufacturer's instructions. The next day, the cells were seeded on collagen type I-coated culture plates (IWAKI, AGC TECHNO GLASS CO., LTD., Shizuoka, Japan) and cultured for 24 h before being fixed with 2% paraformaldehyde (PFA) at 4°C overnight. After 3 washes with PBST (0.005% Tween), the cells were blocked with PBST containing 2% goat serum (blocking solution) at room temperature for 1 h. Each well was soaked with 100 μl of the blocking solution containing 30 ng of

purified nanobody, except for 6 ng of C116, at 4°C overnight. After washing with PBST, an appropriately diluted anti-His-tagged antibody and anti-C9-tagged antibody in blocking buffer were added and reacted at room temperature for 1 h. Finally, after washing, fluorescently conjugated anti-rabbit IgG (594 nm emission) and anti-mouse IgG (488 nm emission) antibodies (Alexa Fluor: Thermo) were diluted with blocking buffer and added to the wells, and the fixed cells were labelled at room temperature for 1 h before washing 3 times with PBST. Cellular nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). Stained cells were imaged with a 2-ms exposure time (594 nm emission), with a 10-ms exposure time (488 nm emission), or automatically adjusted exposure time (DAPI) using microscopy (IX71S1F-3: Olympus Corporation, Tokyo, Japan) with the cellSens Standard 1.11 application (Olympus). A 3×4 cm² printed rectangle corresponds to a 165×220 μm² observed field.

Expression and purification of monomeric P86 and P17

The monomeric C-terminally 6×His tagged nanobody genes were cloned into the pMES4 vector. The complete amino acid sequences are as follows (signal peptide sequence is underlined). P86: MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLVQAGGSLR LSCVASGRTEFSSLNIVWFRQAPGKERKFVAAINDRNTAYAESVKGRFTISRDNK

789 NTVHLQMNSLKPEDTAVYYCHSADVNGGMDYWGKGTQVTVSSHHHHHH.

790 P17: MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLVQAGGSLRLSCAASGR

791 TSSVYNMAWFRQTPGKEREVFAAITGNGGTTLYADSVKGRLTISRGNNAKNTVSL

792 QMNVLKPDDTAVYYCAAGGWGKERNYAYWGQGTQVTVSSHHHHHH.

793 Bacterial BL21(DE3) *E. coli* cells were transformed with the plasmids and grown on an

794 LB ampicillin-supplemented plate; colonies were picked and inoculated into 5 ml of LB

795 medium containing 200 µg ml⁻¹ ampicillin; and the cells were cultured in a shaking

796 incubator overnight at 37°C. The cultures were transferred to 1 L of LB medium

797 containing 200 µg ml⁻¹ ampicillin. At an optical density below 0.6, cells were cultured

798 for 3 h at 37°C with 1 mM IPTG.

799 The bacterial pellet was collected by centrifuging at 6,000×g for 30 min and

800 suspended in 45 ml of lysis buffer containing 20 mM Tris (pH 8.0), 0.68 mM EDTA, 500

801 mM sucrose, and a trace amount of benzonase nuclease (Merck). The solutions were

802 mixed on a TR-118 tube rotator (AS ONE Corporation, Osaka, Japan); 90 mL of 20 mM

803 Tris (pH 8.0) was added, and the mixtures were rotated for 45 min at 4°C. The resulting

804 solutions were centrifuged at 20,000×g for 10 min at 4°C. The supernatant was filtered

805 and loaded onto a Ni-NTA column (Cytiva) equilibrated with 20 mM Tris buffer (pH 8.0).

806 The column was washed several times with 20 mM Tris buffer (pH 8.0) containing 40

mM imidazole for P86 or 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 40 mM imidazole for P17. Then, the nanobody was eluted with 20 mM Tris buffer (pH 8.0) containing 250 mM imidazole for P86 or 150 mM NaCl and 350 mM imidazole for P17.

The fractions containing nanobodies were collected and further purified using a HiLoad 16/60 Superdex 75 gel filtration column (Cytiva) equilibrated with 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl for P86 or with 20 mM HEPES buffer (pH 8.0) containing 150 mM NaCl for P17. The peak fractions were collected and concentrated. The final concentrations of the P86 and P17 sample were 51 mg ml⁻¹ and 3.0 mg ml⁻¹, respectively, based on the use of the absorption coefficient at 280 nm.

Cryo-EM specimen preparation and data collection

An epoxidized graphene grid (EG-grid) was used to increase the number of protein particles. The trimer complex of the SARS-CoV-2 spike (D614G) with the furin-resistant mutation (“GSAS”) at a concentration of 0.1 mg ml⁻¹ was mixed with a 5-time molar excess of P86 or P17 monomer and incubated on ice for 10 min, and 3 µl of the spike-nanobody complex solution was applied to the EG-grid. After incubation at room temperature for 5 min, the grids were blotted with a force of -3 and a time of 2 s in a Vitrobot Mark IV chamber (Thermo) equilibrated at 4°C and 100% humidity and then

immediately plunged into liquid ethane. Excessive ethane was removed with filter paper, and the grids were stored in liquid nitrogen. All cryo-EM image datasets were acquired using a JEM-Z300FSC (CRYO ARM 300: JEOL, Tokyo, Japan) operated at 300 kV with a K3 direct electron detector (Gatan, Inc., Pleasanton, CA) in CDS mode⁶⁸. The Ω -type in-column energy filter was operated with a slit width of 20 eV for zero-loss imaging. The nominal magnification was 60,000 $\times$, corresponding to 0.870 Å per pixel. Defocus varied between $-0.5\ \mu\text{m}$ and $-2.0\ \mu\text{m}$. Each movie was fractionated into 60 frames (0.0505 s each, total exposure: 3.04 s) with a total dose of 60 $\text{e}^-/\text{\AA}^2$.

Cryo-EM image processing and refinement

The images were processed using RELION 3.1⁶⁹. Movies were motion corrected using MotionCor2⁷⁰, and the contrast transfer functions (CTFs) were estimated using CTFFIND 4.1⁷¹. Micrographs whose CTF max resolutions were beyond 5 Å were selected. Three-dimensional (3D) template-based autopicking was performed for all images, and the particles were extracted with 4 $\times$ binning, which were subjected to two rounds of 2D classification. An initial model was generated and used as a reference for the following 3D classification in the P86 dataset. In the P17 dataset, a density map of spike trimers (our previous dataset) was used as a reference. Reference-based 3D classification (into 4

classes) without applying symmetry was conducted, and the selected particles were re-extracted without binning. 3D autorefinement without applying symmetry, soft mask generation, postprocessing, CTF refinement, Bayesian polishing, and another round of 3D autorefinement were performed. Then, focused 3D classification without alignment was performed to separate up- and down-states in one RBD. The selected particles were subjected to another round of 3D autorefinement, postprocessing, CTF refinement, 3D autorefinement. C3 symmetry was applied during the final round of 3D autorefinement for the 3-up+P86 dataset. The data were imported and further processed with non-uniform refinement in cryoSPARC v3.2.0⁷². The final map resolutions (FSC=0.143) were 3.03 Å and 2.70 Å in the 2-up and 3-up states in the P86 dataset and 3.20 Å and 3.29 Å in the 1-up and 2-up states in the P17 dataset, respectively. For the 2-up+P86 dataset, we tried local refinement to visualize the density of P86 bound to the down-RBD, but the resolution was limited to 5.13 Å (FSC=0.143).

The model was built for the 2-up+P86 dataset. The SARS-CoV-2 spike trimer D614G mutant (PDB entry: 7KRR)⁵⁰ and the crystal structure of P86 were used initial models. After the models were manually fitted into the density using UCSF Chimera v1.15⁷³ and modified in Coot v0.8.9.2⁷⁴, real space refinement was performed in PHENIX v1.19.1⁷⁵. The model was validated using MolProbity⁷⁶, and this cycle was repeated

several times. Figures were prepared using UCSF Chimera⁷³, ChimeraX⁷⁷, and PyMOL v2.5.0 (Schrödinger, LLC, New York, NY). The parameters are summarized in Extended Data Table 1.

Crystallization of P86

Crystallization was performed by the sitting drop vapour diffusion method. A mosquito crystallization machine (TTP LabTech Ltd., Melbourn, Hertfordshire, UK) was used to prepare drops on 96-well VIOLAMO plates (AS ONE). The reservoir solution was 60 µl in volume, and 0.1 µl of protein solution was mixed with 0.1 µl of reservoir solution. Crystals appeared under the condition of 51 mg ml⁻¹ protein, 0.2 M ammonium sulfate, and 30% (w/v) polyethylene glycol 4,000 at 20°C. A crystal cluster was crushed, and a peeled single crystal was harvested by LithoLoop (Protein Wave, Nara, Japan). Before the crystal was frozen in liquid nitrogen, it was soaked in the crystallization solution supplemented with 5% (v/v) ethylene glycol.

X-ray data collection, processing, structure solution, and refinement

An X-ray diffraction experiment was performed on the BL44XU beamline of SPring-8 (Hyogo, Japan). Diffraction images were collected at 100 K using an EIGER X 16M

detector (Dectris, Philadelphia, PA, USA). The beam size was 50.0×50.0 μm^2 (h×w). A 0.8-mm Al attenuator was used to weaken the X-ray. The crystal-to-detector distance was 160 mm. The exposure time per frame and the oscillation angle were 0.1 s and 0.1°, respectively. A total of 1,800 images were collected. The dataset was processed using XDS⁷⁸ and scaled by Aimless⁷⁹. Molecular replacement phase determination was performed by MOLREP⁸⁰ with a nanobody structure (PDB code ID: 5IVO)⁸¹ as a search model. Initial model building was performed by PhenixAutoBuild implemented in PHENIX⁷⁵. Manual model building was performed using Coot⁷⁴. The program refmac5⁸² in the ccp4 suite⁸³ and the program Phenix-refine⁷⁵ were used for structural refinement. The stereochemical quality of the final model was checked by Molprobity⁷⁶. Data collection and refinement statistics are summarized in Extended Data Table 2.

Material availability: COGNANO Inc. will agree to the use of any materials and methods except for therapeutic use as long as appropriately referring to this paper or the company name; materials will be delivered through NITTOBO MEDICAL Corporation Ltd., Tokyo, Japan; and lateral flow assay (antigen test) kits will be developed and delivered through Yamato Scientific, Co., Ltd., Tokyo, Japan. Responsible requests for materials for research use should be directed to A.T.-K.

897

898 **Data availability**

899 Density maps are available at the Electron microscopy Data Bank (EMDB) and Protein
900 Data Bank (PDB) with accession codes EMD-32078 (2-up+P86), EMD-32079 (3-
901 up+P86), EMD-32080 (1-up+P17), EMD-32081 (2-up+P17) and PDB-7VQ0 (2-up+P86).
902 Additional cryo-EM data supporting this study are available from Ke.N. on reasonable
903 request. The coordinate and structure factor files are deposited at the PDB (PDB code ID:
904 7VPY). Raw data are available at Integrated Resource for Reproducibility in
905 Macromolecular Crystallography (<https://proteindiffraction.org/>).

906

907 51 Valenzuela Nieto, G. *et al.* Potent neutralization of clinical isolates of SARS-
908 CoV-2 D614 and G614 variants by a monomeric, sub-nanomolar affinity
909 nanobody. *Sci Rep* **11**, 3318, doi:10.1038/s41598-021-82833-w (2021).

910 52 Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. Escherichia coli
911 secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043,
912 doi:10.1126/science.3285471 (1988).

913 53 Zhang, L., Leng, Q. & Mixson, A. J. Alteration in the IL-2 signal peptide affects
914 secretion of proteins in vitro and in vivo. *J Gene Med* **7**, 354-365,

915 doi:10.1002/jgm.677 (2005).

916 54 McLellan, J. S. *et al.* Structure-based design of a fusion glycoprotein vaccine for
917 respiratory syncytial virus. *Science* **342**, 592-598, doi:10.1126/science.1243283
918 (2013).

919 55 Boudko, S. P. *et al.* Crystal structure of human collagen XVIII trimerization
920 domain: A novel collagen trimerization Fold. *J Mol Biol* **392**, 787-802,
921 doi:10.1016/j.jmb.2009.07.057 (2009).

922 56 Sanders, R. W. *et al.* Stabilization of the soluble, cleaved, trimeric form of the
923 envelope glycoprotein complex of human immunodeficiency virus type 1. *J*
924 *Virol* **76**, 8875-8889, doi:10.1128/jvi.76.17.8875-8889.2002 (2002).

925 57 Sokal, A. *et al.* Maturation and persistence of the anti-SARS-CoV-2 memory B
926 cell response. *Cell* **184**, 1201-1213 e1214, doi:10.1016/j.cell.2021.01.050
927 (2021).

928 58 Yamazaki, H. *et al.* Endogenous APOBEC3B Overexpression Constitutively
929 Generates DNA Substitutions and Deletions in Myeloma Cells. *Sci Rep* **9**, 7122,
930 doi:10.1038/s41598-019-43575-y (2019).

931 59 Martin, M. Cutadapt removes adapter sequences from high-throughput
932 sequencing reads. *2011* **17**, 3, doi:10.14806/ej.17.1.200 (2011).

933 60 Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for
934 Illumina sequence data. *Bioinformatics* **30**, 2114-2120,
935 doi:10.1093/bioinformatics/btu170 (2014).

936 61 Aronesty, E. Comparison of Sequencing Utility Programs. *The Open*
937 *Bioinformatics Journal* **7**, 1-8, doi:10.2174/1875036201307010001 (2013).

938 62 Rice, P., Longden, I. & Bleasby, A. EMBOSS: the European Molecular Biology
939 Open Software Suite. *Trends in genetics : TIG* **16**, 276-277, doi:10.1016/s0168-
940 9525(00)02024-2 (2000).

941 63 Shen, W., Le, S., Li, Y. & Hu, F. SeqKit: A Cross-Platform and Ultrafast Toolkit
942 for FASTA/Q File Manipulation. *PLOS ONE* **11**, e0163962,
943 doi:10.1371/journal.pone.0163962 (2016).

944 64 Edgar, R. C. Search and clustering orders of magnitude faster than BLAST.
945 *Bioinformatics* **26**, 2460-2461, doi:10.1093/bioinformatics/btq461 (2010).

946 65 Esparza, T. J., Martin, N. P., Anderson, G. P., Goldman, E. R. & Brody, D. L.
947 High affinity nanobodies block SARS-CoV-2 spike receptor binding domain
948 interaction with human angiotensin converting enzyme. *Sci Rep* **10**, 22370,
949 doi:10.1038/s41598-020-79036-0 (2020).

950 66 Konishi, Y. *et al.* Intravital Imaging Identifies the VEGF-TXA2 Axis as a

951 Critical Promoter of PGE2 Secretion from Tumor Cells and Immune Evasion.
952 *Cancer Res* **81**, 4124-4132, doi:10.1158/0008-5472.CAN-20-4245 (2021).

953 67 McCoy, L. E. *et al.* Molecular evolution of broadly neutralizing Llama
954 antibodies to the CD4-binding site of HIV-1. *PLoS Pathog* **10**, e1004552,
955 doi:10.1371/journal.ppat.1004552 (2014).

956 68 Yamaguchi, T. *et al.* Structure of the molecular bushing of the bacterial flagellar
957 motor. *Nat Commun* **12**, 4469, doi:10.1038/s41467-021-24715-3 (2021).

958 69 Zivanov, J., Nakane, T. & Scheres, S. H. W. Estimation of high-order aberrations
959 and anisotropic magnification from cryo-EM data sets in RELION-3.1. *IUCrJ* **7**,
960 253-267, doi:10.1107/S2052252520000081 (2020).

961 70 Zheng, S. Q. *et al.* MotionCor2: anisotropic correction of beam-induced motion
962 for improved cryo-electron microscopy. *Nat Methods* **14**, 331-332,
963 doi:10.1038/nmeth.4193 (2017).

964 71 Rohou, A. & Grigorieff, N. CTFFIND4: Fast and accurate defocus estimation
965 from electron micrographs. *J Struct Biol* **192**, 216-221,
966 doi:10.1016/j.jsb.2015.08.008 (2015).

967 72 Punjani, A., Zhang, H. & Fleet, D. J. Non-uniform refinement: adaptive
968 regularization improves single-particle cryo-EM reconstruction. *Nat Methods*

- 969 **17**, 1214-1221, doi:10.1038/s41592-020-00990-8 (2020).
- 970 73 Pettersen, E. F. *et al.* UCSF Chimera--a visualization system for exploratory
971 research and analysis. *J Comput Chem* **25**, 1605-1612, doi:10.1002/jcc.20084
972 (2004).
- 973 74 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development
974 of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501,
975 doi:10.1107/S0907444910007493 (2010).
- 976 75 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for
977 macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-
978 221, doi:10.1107/S0907444909052925 (2010).
- 979 76 Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular
980 crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12-21,
981 doi:10.1107/S0907444909042073 (2010).
- 982 77 Pettersen, E. F. *et al.* UCSF ChimeraX: Structure visualization for researchers,
983 educators, and developers. *Protein Sci* **30**, 70-82, doi:10.1002/pro.3943 (2021).
- 984 78 Kabsch, W. Xds. *Acta Crystallogr D Biol Crystallogr* **66**, 125-132,
985 doi:10.1107/S0907444909047337 (2010).
- 986 79 Evans, P. R. & Murshudov, G. N. How good are my data and what is the

987 resolution? *Acta Crystallogr D Biol Crystallogr* **69**, 1204-1214,
988 doi:10.1107/S0907444913000061 (2013).

989 80 Vagin, A. & Teplyakov, A. Molecular replacement with MOLREP. *Acta*
990 *Crystallogr D Biol Crystallogr* **66**, 22-25, doi:10.1107/S0907444909042589
991 (2010).

992 81 Braun, M. B. *et al.* Peptides in headlock--a novel high-affinity and versatile
993 peptide-binding nanobody for proteomics and microscopy. *Sci Rep* **6**, 19211,
994 doi:10.1038/srep19211 (2016).

995 82 Murshudov, G. N. *et al.* REFMAC5 for the refinement of macromolecular
996 crystal structures. *Acta Crystallogr D Biol Crystallogr* **67**, 355-367,
997 doi:10.1107/S0907444911001314 (2011).

998 83 Winn, M. D. *et al.* Overview of the CCP4 suite and current developments. *Acta*
999 *Crystallogr D Biol Crystallogr* **67**, 235-242, doi:10.1107/S0907444910045749
1000 (2011).

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Author contributions A.I. and A.T.-K. conceived the study. R.M. performed

biochemical experiments, generated large libraries, performed biopanning, and assembled microscopy data. H.Y. performed bioinformatic analyses. Yo.K. and Ya.K. performed virus assays. K.K., Ka.N., Y.Y., K.M., A.R., K.Y., and I.A. prepared and certified materials. J.F. prepared cryo-EM specimens and collected data. J.F. and F.M. processed the cryo-EM images. J.F., T.I., and K.N. interpreted structures. K.Y. performed crystallographic studies. R.M., J.F., A.I., Ke.N., and A.T.-K analysed the data. R.M., J.F., Yo.K., Ya.K., K.K., H.Y., and K.Y. drew figures. R.M., J.F., K.Y., I.A., and A.T.-K. wrote the first draft of the manuscript. J.F., K.S., Y.M., T.I., A.I., Ke.N., and A.T.-K. reviewed and commented on the manuscript. All authors approved the reviewed manuscript.

Competing interests Kyoto University, Osaka University, and COGNANO Inc. have filed a patent application (JP2021-170471) in connection with this research, on which R.M., J.F., A.T.-K., K.S., K.K., H.Y., A.I., F.M., Ke.N., K.Y., T.I., I.A., Y.M., Haruyasu Asahara, and Maiko Moriguchi are inventors. A.I. is a stockholder of COGNANO Inc., which has patents and ownership of antibody sequences (JP2021-089414) and an in-house method of identifying antibodies (PCT/JP2019/021353) described in this study on which A.I. is an inventor. R.M., H.Y., and K.K. are employees of COGNANO Inc. The other authors declare no competing interests.

Extended Data Fig. 1| Purification of the extracellular SARS-CoV-2 spike complex.

a, A chromatogram of the first purification step of the SARS-CoV-2 spike from HEK cell lysate using a nickel column: absorbance units (AU) of UV_{280 nm}, amounts (ml) of buffer, concentrations (M) of imidazole, and the elution peak containing the SARS-CoV-2 spike.

b, Chromatograms of the size-exclusion gel filtration of the recombinant SARS-CoV-2 spike complex under different conditions (concentrations of NaCl and LMNG). The first chromatogram is overlaid with those for marker proteins (arrowed). Two arrows indicate the elution peak of the SARS-CoV-2 spike. **c**, Western blotting analysis of the purified SARS-CoV-2 spike using an anti-His antibody and Coomassie Brilliant Blue (CBB) staining. **d**, Serum titres of the immunized alpacas—named P: Puta and C: Christy—for the SARS-CoV-2 spike were measured via ELISA and graphed: mean of the measurements for 2 wells.

Extended Data Fig. 2| Purification and kinetic assays. a, Chromatograms showing the

final purification step of the indicated clones: subpeaks confirmed as cleaved monomers are marked (m); the amounts of purified clones in dimers from 1 L-culture of BL21 bacteria are shown in insets. The dimer peaks observed for P158 are named A and B. **b**,

The purified clones were analysed using CBB staining (m; cleaved monomer). Both the A and B peaks of P158 showed no differences via SDS-PAGE analysis. **c, d**, Sensorgrams of real-time kinetics assays using biolayer interferometry (BLI), showing five different concentrations of the purified SARS-CoV-2 spike in a high-salt buffer (**c**) or an anionic buffer (**d**).

Extended Data Fig. 3| Microscopy and flow cytometric analyses of anti-spike nanobodies. a, Photographs of stained HEK cells expressing the C9-tagged SARS-CoV-2 spike. **b**, White squared regions show zoomed-in views: arrows indicate intracellularly stained SARS-CoV-2 spike (C49 and C246), and arrowheads indicate example background signals (C49). **c**, Flow cytometric analyses of K562 cells expressing the original SARS-CoV-2 spike—signals with Ty1 squared and B9: an anti-HIV-1 nanobody.

Extended Data Fig. 4| Microscopy analyses. a,b, Photographs of stained HEK cells expressing the SARS-CoV-2 spike variants carrying mutations in the RBD, original (D614G); alpha (N501Y); beta (K417N, E484K, and N501Y); and delta (L452R), with generated (**a**) and previously reported (**b**) nanobodies. **c**, Zoomed-in views of regions highlighted with white squares in (**b**): C246 intracellularly stained all spike variants

without background—indicated by white arrows.

Extended Data Fig. 5| ELISA condition screenings. **a**, P158 was coated on an ELISA plate as a capture antibody; five kinds of blocking solutions (grey)—Carbo-Free (CF), skim milk (Milk), bovine serum albumin (BSA), casein, polyvinylpyrrolidone (PVP), and nonblocking saline (PBST)—were tested. Signals from 2 µg of the purified SARS-CoV-2 spike (+) or blocking buffer only (–) were compared among eight clones used as detection antibodies. Measured optical densities (OD=450 nm) and signal ratios (relative changes in OD_{450 nm} with (+) compared to without the spike protein (–)) are gradually coloured from green (low) to red (high). **b**, Summary of the top five detection antibodies and appropriate blocking conditions for the P158-based sandwich ELISA: ratios >3.0 are shown in black.

Extended Data Fig. 6| Microscopy analyses. Photographs of stained HEK cells expressing the SARS-CoV-2 spike (D614G) variants carrying mutations in the S1 domain: **a,b**, alpha (N501Y, Y144del, Y145del, and A570D); Scotland (N439Y, V69del, and H70del); Danish mink (Y453F, V69del, and H70del); beta (K417N, E484K, N501Y, L18F, D80A, D215G, L242del, A243del, L244del, and R246I); gamma (K417T, E484K,

N501Y, L18F, T20N, P26S, D138Y, and R190S); and delta (L452R and T478K). **c**, Results of microscopy analyses are summarized: +) positive; +-) weak; or -) negative. The SARS-CoV-2 spike variants of interest (VOIs)—Scotland and Danish mink—are shown in parentheses.

Extended Data Fig. 7| Cryo-EM density maps of spike-nanobody complexes. a-d, Upper panels show final sharpened maps of 2-up+P86 (**a**), 3-up+P86 (**b**), 1-up+P17 (**c**), and 2-up+P17 (**d**) datasets from side views (left) and top views (right) coloured by local resolutions (3.0–7.0 Å). Lower panels show the Fourier shell correlation (FSC) curves for the corresponding final maps of the datasets.

Extended Data Fig. 8| Crystal structure of P86 and a binding model of spike-P86 complex. a, Two P86 monomers in the asymmetric unit. Chain A and Chain B are almost identical, with a root mean square deviation for C α atoms of 0.20 Å. **b**, Close-up view of three CDR regions in Chain A. **c**, Sharpened map from local refinement (5.13 Å resolution at FSC=0.143) around P86 bound to the down-RBD with our fitted model. C-terminal (C-ter.) and N-terminal (N-ter.) ends are indicated. **d**, Close-up view around P86 bound to the up-RBD with our fitted model structure. The model of ACE2 bound to the spike

trimer (PDB entry: 7KMS) is superposed on the up-RBD shown in blue. **e**, Structure comparison between P86 alone (crystal structure) and P86 complexed with spike trimer (cryo-EM). **f**, Epitope mapping on the down-RBD. The epitopes of Class-1 to Class-4 compared to P86. **g**, A fitting model of P86 and the SARS-CoV-2 spike trimer. The down-RBD, the neighbouring NTD, and P86 are coloured white, cyan, and pink, respectively; other domains and protomers of the SARS-CoV-2 spike trimer are in reduced transparency.

Extended Data Table 1| Cryo-EM data collection and processing.

Extended Data Table 2| Crystallographic data collection and refinement statistics.

Fig. 1| Structural features of the SARS-CoV-2 spike and the panel of nanobodies.

a,b, Side (**a**) and top (**b**) views of the 1-up+2-down conformation of the SARS-CoV-2 spike trimer⁵⁰ (PDB entry: 7KRR); domains of each protomer numbered I (up), II (down), and III (down). The NTD, the RBD, and the C-terminal domain (CTD) of the SARS-CoV-2 S1 domain are coloured cyan, blue, and orange, respectively; mutations and deletions studied here are indicated; clefts between the down-RBD and the NTD of the neighbouring protomer are highlighted with red circles. **c**, Graphs showing existence ratios (two different scales drawn black and grey)—*P* values in log₁₀ are plotted (see Methods)—after panning with IgM; APOBEC3G (A3G)²⁶; the S2 domain (S2); the HCoV-OC43 (HCoV) spike²⁷; and the SARS-CoV-2 spike (CoV-2). **d**, Sequences of nine clones and that of Ty1 are compared³⁶; changed amino acids in the framework

Fig. 1| Structural features of the SARS-CoV-2 spike and the panel of nanobodies.

a,b, Side (a) and top (b) views of the 1-up+2-down conformation of the SARS-CoV-2 spike trimer³⁰ (PDB entry: 7KRR); domains of each protomer numbered I (up), II (down), and III (down). The NTD, the RBD, and the C-terminal domain (CTD) of the SARS-CoV-2 S1 domain are coloured cyan, blue, and orange, respectively; mutations and deletions studied here are indicated; clefts between the down-RBD and the NTD of the neighbouring protomer are highlighted with red circles. **c**, Graphs showing existence ratios (two different scales drawn black and grey)— P values in $\log_{10}$ are plotted (see Methods)—after panning with IgM; APOBEC3G (A3G)²⁶; the S2 domain (S2); the HCoV-OC43 (HCoV) spike²⁷; and the SARS-CoV-2 spike (CoV-2). **d**, Sequences of nine clones and that of Ty1 are compared³⁶; changed amino acids in the framework regions are red; the variable complementary-determining regions (CDRs) are shown in blue. **e**, Calculated binding kinetics between each nanobody and the SARS-CoV-2 spike from sensorgrams (Extended Data Fig. 2c,d) are summarized. The upper four clones were assayed in a high-salt buffer (“stabilized” spike); the others were assayed in an anionic buffer (“fluctuated” spike).

Figure 1d, e

Fig. 2 | Nanobodies for Western blotting assays, ELISAs, and lateral flow assays.

a, Nanobody-based Western blotting assays of SARS-CoV-2 spikes: lysates of HEK cells expressing GFP or C-terminally C9-tagged SARS-CoV-2 spike (D614G) variants (indicated with numbers) were blotted with the indicated nanobodies. **b-d**, Sandwich ELISA: **b**, signals of gradually diluted sample concentrations using P158 as the capture antibody and P86 or P543 (**b,c**) or P543-Fc (**d**) as the detection antibodies are graphed as a mean of the measurements from 2 wells ($n=2$). In (**c**), sequentially diluted lysates of HEK cells expressing the original SARS-CoV-2 spike (D614G) or beta variant (D614G, N501Y, E484K, and K417N) were assayed. **e**, A photograph of the results from lateral flow nitrocellulose membrane assays: P158 for the capture line (allow head) and P86 for the detection beads; 300 ng of the purified SARS-CoV-2 spike delta variant (D614G, L452R, and T478K) (+) or PBS (-) was spotted.

Figure 2

Figure 3

Fig. 3| Pseudotyped virus neutralization assay.

a, Means of infecting units of virus for the indicated SARS-CoV-2 spike variants are graphed (n=3). K562 cells expressing ACE2 and TMPRSS2 infected with HIV-1-based pseudotyped virus were assessed via luciferase assay. Relative infection units at the indicated concentrations of the nanobodies, which are marked individually, are plotted. **b**, Measured IC50s for each variant are summarized.

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Fig. 4| Cryo-EM density maps of SARS-CoV-2 spike-P86 and SARS-CoV-2 spike-P17 complexes.

a,b, Final sharpened maps of the **(a)** 2-up+P86 and **(b)** 1-up+P17 datasets. The map regions corresponding to one protomer in the spike protein are coloured differently in cyan, blue, and turquoise. The map regions corresponding to one P86 or P17 molecule bound to the RBD regions are coloured magenta, red, and orange, respectively. **c,** Close-up view around P86 bound to the down-RBD with our fitted model. Right panel shows close-up view around the interface among P86, the down-RBD, and the NTD of the neighbouring protomer. **d,** Close-up view around P17 bound to the down-RBD. The model of D614G spike trimer (PDB entry: 7KRR) was fitted on the maps³⁰. **e,** The residues located on the interface between the down-RBD and P86 or P17. **f,** Epitope mapping on the down-RBD. The epitopes of Class-2, Class-3, and P86 are shown in yellow, blue, and magenta, respectively. The colouring priority of the overlapping region is Class-3>Class-2>P86. The model of P86 is a ribbon structure. **g,** Structure comparison with other antibodies bound to the down-RBD. The structures are superposed on the single RBD region.

Extended Data Figure 1

Extended Data Fig. 1 | Purification of the extracellular SARS-CoV-2 spike complex. **a**, A chromatogram of the first purification step of the SARS-CoV-2 spike from HEK cell lysate using a nickel column: absorbance units (AU) of UV_{280nm}, amounts (ml) of buffer, concentrations (M) of imidazole, and the elution peak containing the SARS-CoV-2 spike. **b**, Chromatograms of the size-exclusion gel filtration of the recombinant SARS-CoV-2 spike complex under different conditions (concentrations of NaCl and LMNG). The first chromatogram is overlaid with those for marker proteins (arrowed). Two arrows indicate the elution peak of the SARS-CoV-2 spike. **c**, Western blotting analysis of the purified SARS-CoV-2 spike using an anti-His antibody and Coomassie Brilliant Blue (CBB) staining. **d**, Serum titres of the immunized alpacas—named P: Puta and C: Christy—for the SARS-CoV-2 spike were measured via ELISA and graphed: mean of the measurements for 2 wells.

Extended Data Fig. 2| Purification and kinetic assays. **a**, Chromatograms showing the final purification step of the indicated clones: subpeaks confirmed as cleaved monomers are marked (m); the amounts of purified clones in dimers from 1 L-culture of BL21 bacteria are shown in insets. The dimer peaks observed for P158 are named A and B. **b**, The purified clones were analysed using CBB staining (m; cleaved monomer). Both the A and B peaks of P158 showed no differences via SDS-PAGE analysis. **c**, **d**, Sensorgrams of real-

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Extended Data Fig. 2| Purification and kinetic assays. **a**, Chromatograms showing the final purification step of the indicated clones: subpeaks confirmed as cleaved monomers are marked (m); the amounts of purified clones in dimers from 1 L-culture of BL21 bacteria are shown in insets. The dimer peaks observed for P158 are named A and B. **b**, The purified clones were analysed using CBB staining (m; cleaved monomer). Both the A and B peaks of P158 showed no differences via SDS-PAGE analysis. **c**, **d**, Sensorgrams of real-time kinetics assays using biolayer interferometry (BLI), showing five different concentrations of the purified SARS-CoV-2 spike in a high-salt buffer (c) or an anionic buffer (d).

Extended Data Figure 2c, 2d

Extended Data Fig. 3 | Microscopy and flow cytometric analyses of anti-spike nanobodies. **a**, Photographs of stained HEK cells expressing the C9-tagged SARS-CoV-2 spike. **b**, White squared regions show zoomed-in views: arrows indicate intracellularly stained SARS-CoV-2 spike (C49 and C246), and arrowheads indicate example background signals (C49). **c**, Flow cytometric analyses of K562 cells expressing the original SARS-CoV-2 spike—signals with Ty1 squared and B9: an anti-HIV-1 nanobody.

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Extended Data Fig. 4| Microscopy analyses. a,b, Photographs of stained HEK cells expressing the SARS-CoV-2 spike variants carrying mutations in the RBD, original (D614G); alpha (N501Y); beta (K417N, E484K, and N501Y); and delta (L452R), with generated (**a**) and previously reported (**b**) nanobodies. **c,** Zoomed-in views of regions highlighted with white squares in (**b**): C246 intracellularly stained all spike variants without background—indicated by white arrows.

Extended Data Figure 4a

Extended Data Fig. 4| Microscopy analyses. **a,b**, Photographs of stained HEK cells expressing the SARS-CoV-2 spike variants carrying mutations in the RBD, original (D614G); alpha (N501Y); beta (K417N, E484K, and N501Y); and delta (L452R), with generated (**a**) and previously reported (**b**) nanobodies. **c**, Zoomed-in views of regions highlighted with white squares in (**b**): C246 intracellularly stained all spike variants without background—indicated by white arrows.

Extended Data Figure 4b, 4c

a **P158-coated plate**

Detection	P17	C17	P158	P334	P543	C116	P86	Ty1	Blocking
-	0.04	0.07	0.11	0.06	0.06	0.03	0.04	0.05	Carbo-Free (CF)
+	0.28	0.26	0.31	0.14	0.37	0.11	0.15	0.05	
-	0.02	0.04	0.04	0.03	0.06	0.01	0.04	0.02	Skim-Milk (Milk)
+	0.12	0.11	0.15	0.02	0.29	0.02	0.08	0.03	
-	0.07	0.09	0.26	0.08	0.11	0.07	0.09	0.06	Albumin (BSA)
+	0.70	0.68	0.18	0.15	0.77	0.10	0.33	0.09	
-	0.03	0.06	0.06	0.12	0.07	0.03	0.03	0.01	Casein
+	0.10	0.11	0.27	0.03	0.13	0.04	0.07	0.03	
-	0.14	0.18	0.21	0.12	0.13	0.07	0.10	0.22	PVP (Polyvinylpyrrolidone)
+	0.22	0.24	0.19	0.12	0.26	0.07	0.16	0.14	
-	0.07	0.20	0.26	0.11	0.26	0.09	0.16	0.11	PBST (0.005% Tween20)
+	0.64	0.71	0.31	0.12	0.59	0.06	0.31	0.11	

Signal ratio

P17	C17	P158	P334	P543	C116	P86	Ty1	
6.4	3.9	2.9	2.2	6.6	3.6	3.7	0.9	CF
5.9	2.9	3.7	0.7	5.2	1.5	2.3	1.9	Milk
10.6	8.0	0.7	1.8	6.8	1.4	3.5	1.7	BSA
3.8	2.0	4.7	0.3	1.8	1.2	1.9	2.1	Casein
1.5	1.3	0.9	1.1	2.0	0.9	1.6	0.6	PVP
9.4	3.6	1.2	1.1	2.3	0.6	2.0	0.9	PBST

b **Summary of ELISA conditions**

Capture: P158	Blocking				
	CF	Milk	BSA	Casein	PBST
P543	6.6	5.2	6.8	<2.0	2.3
P86	3.7	2.3	3.5	<2.0	2.0
P17	6.4	5.9	10.6	3.8	9.4
C17	3.9	2.9	8.0	2.0	3.6
P158	2.9	3.7	<2.0	4.7	<2.0

**Extended
Data
Figure 5**

Extended Data Fig. 5| ELISA condition screenings. a, P158 was coated on an ELISA plate as a capture antibody; five kinds of blocking solutions (grey)—Carbo-Free (CF), skim milk (Milk), bovine serum albumin (BSA), casein, polyvinylpyrrolidone (PVP), and nonblocking saline (PBST)—were tested. Signals from 2 µg of the purified SARS-CoV-2 spike (+) or blocking buffer only (-) were compared among eight clones used as detection antibodies. Measured optical densities (OD=450 nm) and signal ratios (relative changes in OD_{450nm} with (+) compared to without the spike protein (-)) are gradually coloured from green (low) to red (high). b, Summary of the top five detection antibodies and appropriate blocking conditions for the P158-based sandwich ELISA: ratios >3.0 are shown in black.

Extended Data Fig. 6| Microscopy analyses. Photographs of stained HEK cells expressing the SARS-CoV-2 spike (D614G) variants carrying mutations in the S1 domain: **a,b**, alpha (N501Y, Y144del, Y145del, and A570D); Scotland (N439Y, V69del, and H70del); Danish mink (Y453F, V69del, and H70del); beta (K417N, E484K, N501Y, L18F, D80A, D215G, L242del, A243del, L244del, and R246I); gamma (K417T, E484K, N501Y, L18F, T20N, P26S, D138Y, and R190S); and delta (L452R and T478K). **c**, Results of microscopy

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Extended Data Fig. 6| Microscopy analyses. Photographs of stained HEK cells expressing the SARS-CoV-2 spike (D614G) variants carrying mutations in the S1 domain: a,b, alpha (N501Y, Y144del, Y145del, and A570D); Scotland (N439Y, V69del, and H70del); Danish mink (Y453F, V69del, and H70del); beta (K417N, E484K, N501Y, L18F, D80A, D215G, L242del, A243del, L244del, and R246I); gamma (K417T, E484K, N501Y, L18F, T20N, P26S, D138Y, and R190S); and delta (L452R and T478K). c, Results of microscopy

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C

Staining cells expressing the SARS-CoV-2 spike variants (D614G)

Spike of VOCs (VOIs)	Alpha (Scotland)	(Mink)	Beta	Gamma	Delta	
Mutations in RBD	N501Y	N439Y	Y453F	K417N /E484K /N501Y	K417T /E484K /N501Y	L452R /T478K
Mutations and deletions in NTD	ΔY144 /ΔY145 /A570D	ΔH69 /ΔV70	ΔH69 /ΔV70	L18F/D80A /D215G/ ΔLAL244 /R246I	L18F/T20N /P26S /D138Y /R190S	
C17	+	+	+	—	—	+
P17	+	+	+	—	—	+
P86	+	+	+	+	+	+
C116	+	+	+	+—	+—	+—
P158	+	+	+	+	+	+
P334	+	+	+	+	+	+
P543	+	+	+	+	+	+

Extended Data Fig. 6| Microscopy analyses. Photographs of stained HEK cells expressing the SARS-CoV-2 spike (D614G) variants carrying mutations in the S1 domain: **a,b**, alpha (N501Y, Y144del, Y145del, and A570D); Scotland (N439Y, V69del, and H70del); Danish mink (Y453F, V69del, and H70del); beta (K417N, E484K, N501Y, L18F, D80A, D215G, L242del, A243del, L244del, and R246I); gamma (K417T, E484K, N501Y, L18F, T20N, P26S, D138Y, and R190S); and delta (L452R and T478K). **c**, Results of microscopy analyses are summarized: +) positive; +—) weak; or —) negative. The SARS-CoV-2 spike variants of interest (VOIs)—Scotland and Danish mink—are shown in parentheses.

Extended Data Figure 6

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Extended Data Fig. 7 | Cryo-EM density maps of spike-nanobody complexes. a-d, Upper panels show final sharpened maps of 2-up+P86 (a), 3-up+P86 (b), 1-up+P17 (c), and 2-up+P17 (d) datasets from side views (left) and top views (right) coloured by local resolutions (3.0–7.0 Å). Lower panels show the Fourier shell correlation (FSC) curves for the corresponding final maps of the datasets.

Extended Data Figure 7

Extended Data Fig. 8 | Crystal structure of P86 and a binding model of spike-P86 complex. **a**, Two P86 monomers in the asymmetric unit. Chain A and Chain B are almost identical, with a root mean square deviation for C_{α} atoms of 0.20 Å. **b**, Close-up view of three CDR regions in Chain A. **c**, Sharpened map from local refinement (5.13 Å resolution at FSC=0.143) around P86 bound to the down-RBD with our fitted model. C-terminal (C-ter.) and N-terminal (N-ter.) ends are indicated. **d**, Close-up view around P86 bound to the up-RBD with our fitted model structure. The model of ACE2 bound to the spike trimer (PDB entry: 7KMS) is superposed on the up-RBD shown in blue. **e**, Structure comparison between P86 alone (crystal structure) and P86 complexed with spike trimer (cryo-EM). **f**, Epitope mapping on the down-RBD. The epitopes of Class-1 to Class-4 compared to P86. **g**, A fitting model of P86 and the SARS-CoV-2 spike trimer. The down-RBD, the

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Extended Data Figure 8

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1146 Extended Data Table 1 | Cryo-EM data collection and processing.

Dataset	2-up+P86 (EMDB-32078) (PDB ID: 7VQ0)	3-up+P86 (EMDB-32079)	1-up+P17 (EMDB-32080)	2-up+P17 (EMDB-32081)
Data collection and processing				
Magnification	60,000	60,000	60,000	60,000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	60	60	60	60
Defocus range (μm)	-0.5 to -2.0	-0.5 to -2.0	-0.5 to -2.0	-0.5 to -2.0
Pixel size (Å)	0.870	0.870	0.874	0.874
Symmetry imposed	C1	C3	C1	C1
Imported movies (no.)	4,175	4,175	2,124	2,124
Initial particle images (no.)	878,975	878,975	766,652	766,652
Final particle images (no.)	38,004	44,292	67,529	48,715
Map resolution (Å)	3.03	2.70	3.20	3.29
FSC threshold	0.143	0.143	0.143	0.143
Refinement				
Model vs. Map CC (volume)	0.79			
Protein residues	3624			
R.m.s. deviations				
Bond lengths (Å)	0.003			
Bond angles (°)	0.516			
Validation				
MolProbity score	1.81			
Clashscore	9.09			
Rotamer outliers (%)	0.03			
Ramachandran plot				
Favored (%)	95.30			
Allowed (%)	4.61			
Disallowed (%)	0.08			

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1152 **Extended Data Table 2 | Crystallographic data collection and refinement statistics.**

Data Collection	
X-ray source	SPring-8 BL44XU
Wavelength (Å)	0.9000
Space group	C2
Unit cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	105.08, 43.17, 56.03
Unit cell <i>α</i> , <i>β</i> , <i>γ</i> (°)	90.00, 110.76, 90.00
Resolution range (Å)	49.13–1.60 (1.63–1.60)*
Total No. of reflections / No. of unique reflections	103,494 (5,156) / 30,851 (1,493)
Completeness (%)	99.1 (99.5)
Redundancy	3.4 (3.5)
$\langle I/\sigma(I) \rangle$	11.9 (2.8)
<i>R</i> _{meas} (all I+ & I-)	0.067 (0.668)
<i>R</i> _{meas} (within I+/I-)	0.065 (0.628)
CC _{1/2}	0.998 (0.835)
Refinement	
Resolution range (Å)	30.80–1.60 (1.65–1.60)
Completeness (%)	98.8 (99.0)
No. of reflections, working set	30,833 (3,054)
No. of reflections, test set	1,510 (138)
<i>R</i> _{work} / <i>R</i> _{free}	0.177/0.213 (0.236/0.245)
No. of non-H atoms	2167
Protein	1885
Ion/Ligand	54
Water	232
R.m.s. deviation bonds (Å), angles (°)	0.006, 0.87
Average <i>B</i> factors (Å ²)	23.61
Protein	22.29
Ion/Ligand	34.89
Water	31.84
Ramachandran favored / allowed / disallowed (%)	98.25 / 1.75 / 0
PDB code ID	7VPY

*Values in parentheses refer to the highest resolution shell.