

A potent SARS-CoV-2 neutralising nanobody shows therapeutic efficacy in the Syrian golden hamster model of COVID-19

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Table 1: PCR primer sequences

Table 2: DNA sequences of C1, C5, F2 and H3 nanobodies

a

CloneID	CDR1-IMGT	CDR2-IMGT	CDR3-IMGT	Inhibition%
F2	GRTFHSYV	ISWSSTPT	AADRGESYYYYTRPTEYEF	76
H7	GRTFHSYV	ISWSSTLT	AADRGESYYYYTRPTEYEF	74
C1	GFTNDFYS	LSVSDNTP	AAGRFAGRDTWPSSYDY	62
C5	GVTLGRHA	IRTFDGIT	ALGVTAACSDNPYF	62
H3	GRTFSTYS	MRWTGSST	AITTIVRAYYTEYTEADFGS	56
H5	GRTFSMAR	INWSSGSI	AVQFGIRGYLDGYDY	47
H2	GRTLSMYP	ISWSGDNT	AARTRAHMYSTTYPYASEYDY	22
F8	GVTLGRHA	IRTFDGIT	ALRVTGACSDNPYF	21
G4	GRTFSSIV	IDWSGGGT	AADRGHYYYYTRPTEYDY	20
B5	GGTFRPYT	ISRLTGTT	AATTNTAVASTTSVYGY	18
D1	ERTSISSV	IRPGIGNT	AIAMSLNNLGAGLDTRVYDY	16
H8	GRTITAYT	ISKSSDST	AAGSWYGRDSDPAGYDY	15
G6	GRALNSLI	IVWSDEFT	AGRYGNLITQNQNEYQY	12
A5	GRTFSRHT	IRWLGGG	YCAAAPAGFVGPTMERYPYEY	5
E1	GRTFDTTR	IFRNTGTT	AAGRFSAAPLTRSTAFES	-20
H4	GSTLDNYS	FSRRYGAP	ATRSGPYCTTSVSDFD	-21

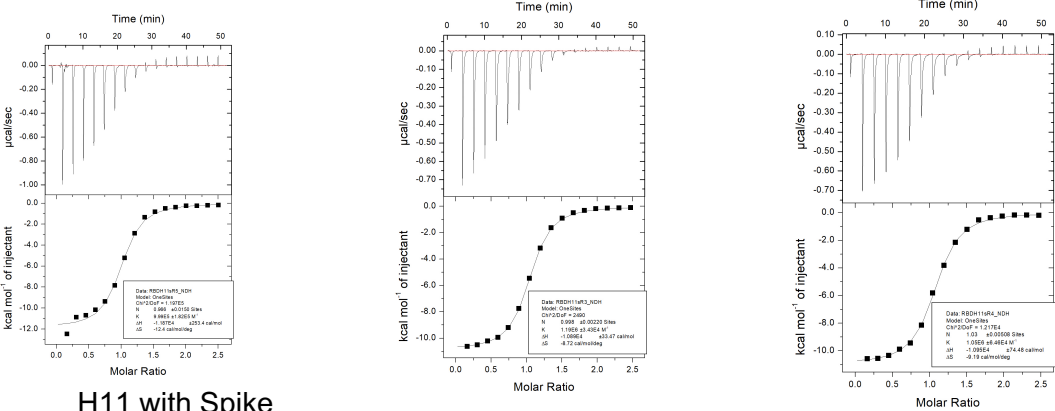
b



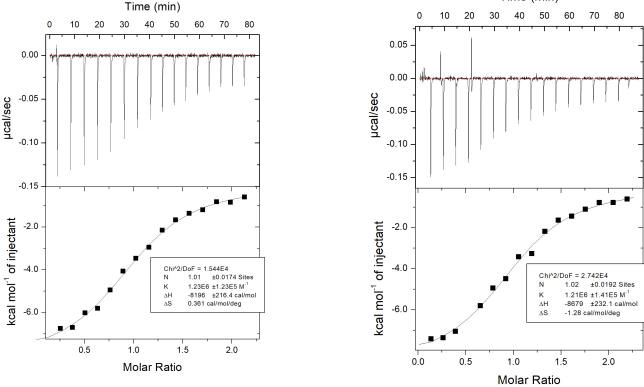
Supplementary Fig. 1 Nanobody sequences

(a) The CDR sequences of the 15 unique CDR sequences identified from 93 picked VHH clones ranked according to % inhibition of binding to immobilised RBD by soluble RBD. The selected clones are highlighted (b) amino acid alignment of alignment of selected nanobodies (C1, C5, F2 and H3).

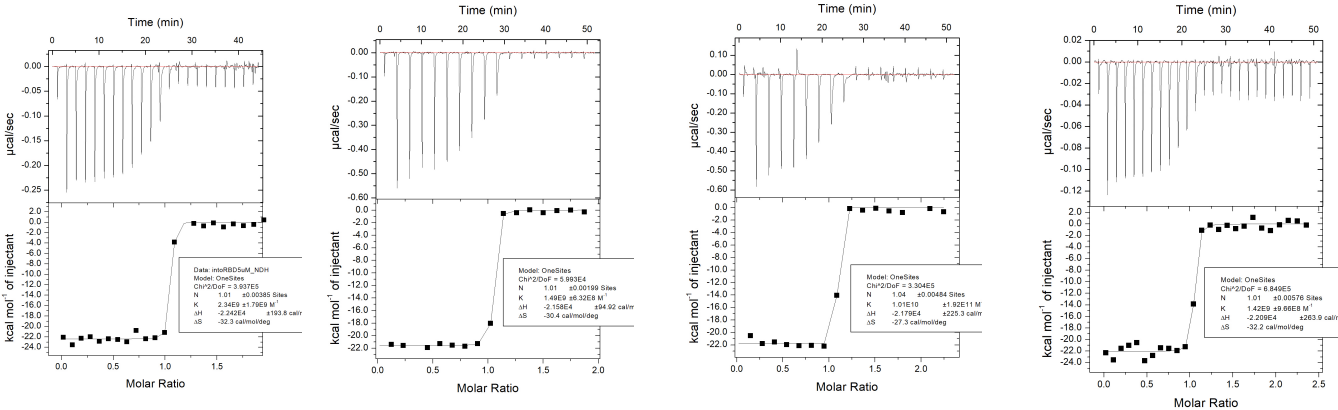
a H11 into RBD



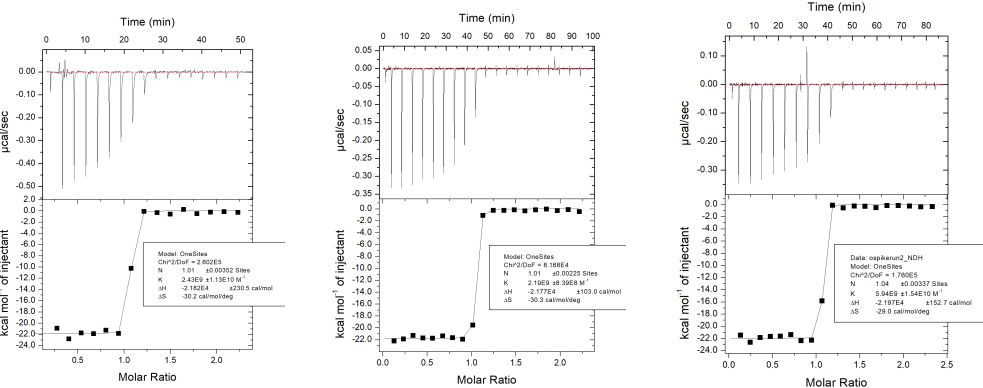
b H11 with Spike



c C5 into RBD

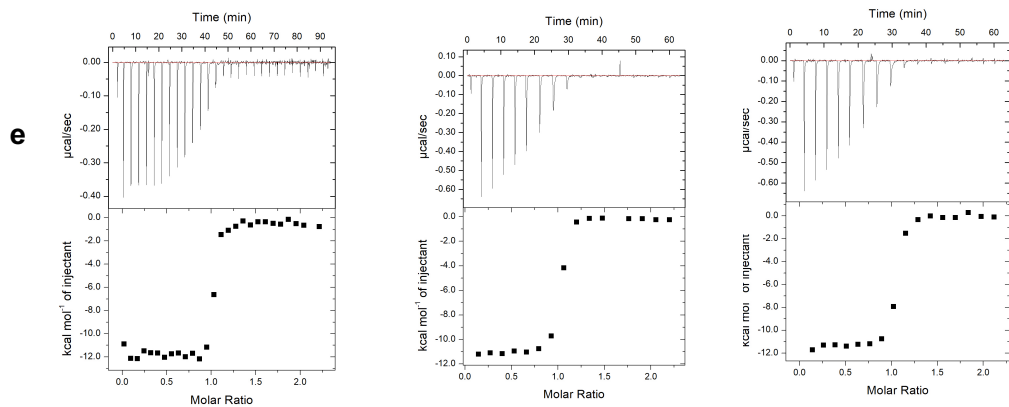


d C5 with spike

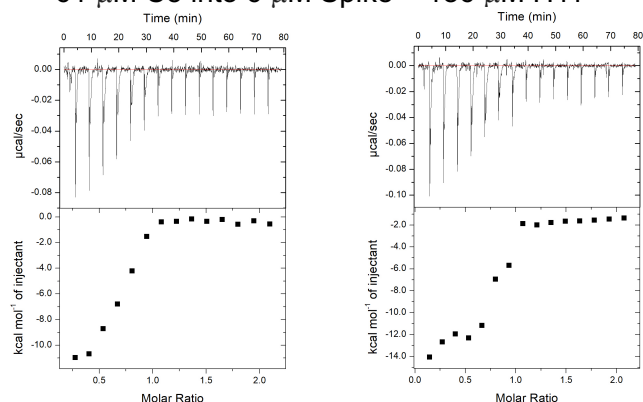


Supplementary Fig. 2

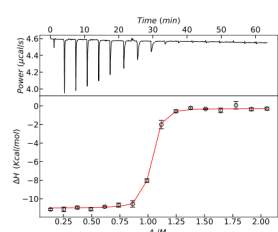
200 μM C5 with 20 μM RBD + 100 μM H11



61 μM C5 into 6 μM Spike + 186 μM H11

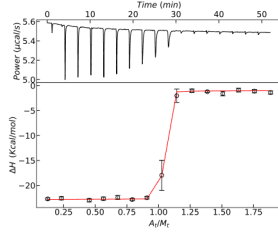


200 μM C5 into 20 μM RBD + 100 μM H11

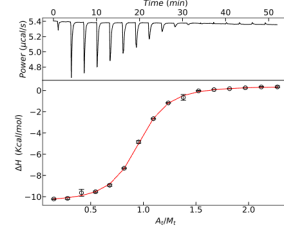


Kd (C5) = 213 pM; Kd (H11) = 854 nM

C5 into RBD

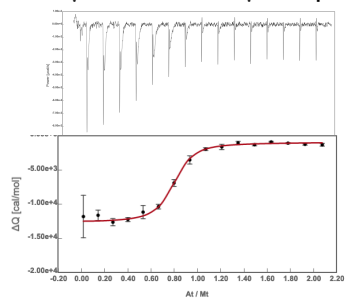


H11 into RBD



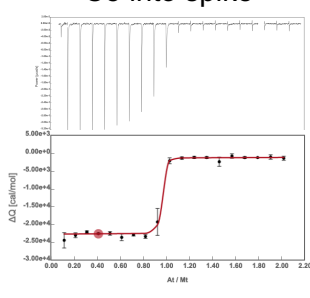
$K_{aC5} = 4.68\text{e}+9 \pm 1.6\text{e}+8$, $\Delta H_{C5} = -21.6 \text{ e}+3 \pm 1.17\text{e}+1$
 $K_{aH11} = 1.12\text{e}+6 \pm 9.6\text{e}+3$, $\Delta H_{H11} = -11.04\text{e}+3 \pm 6.95\text{e}+1$

61 μM C5 into 6 μM Spike + 186 μM H11

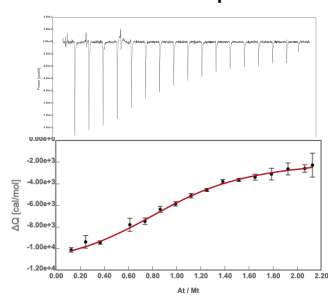


Kd (C5) = 345 pM, Kd (H11) = 1170 nM

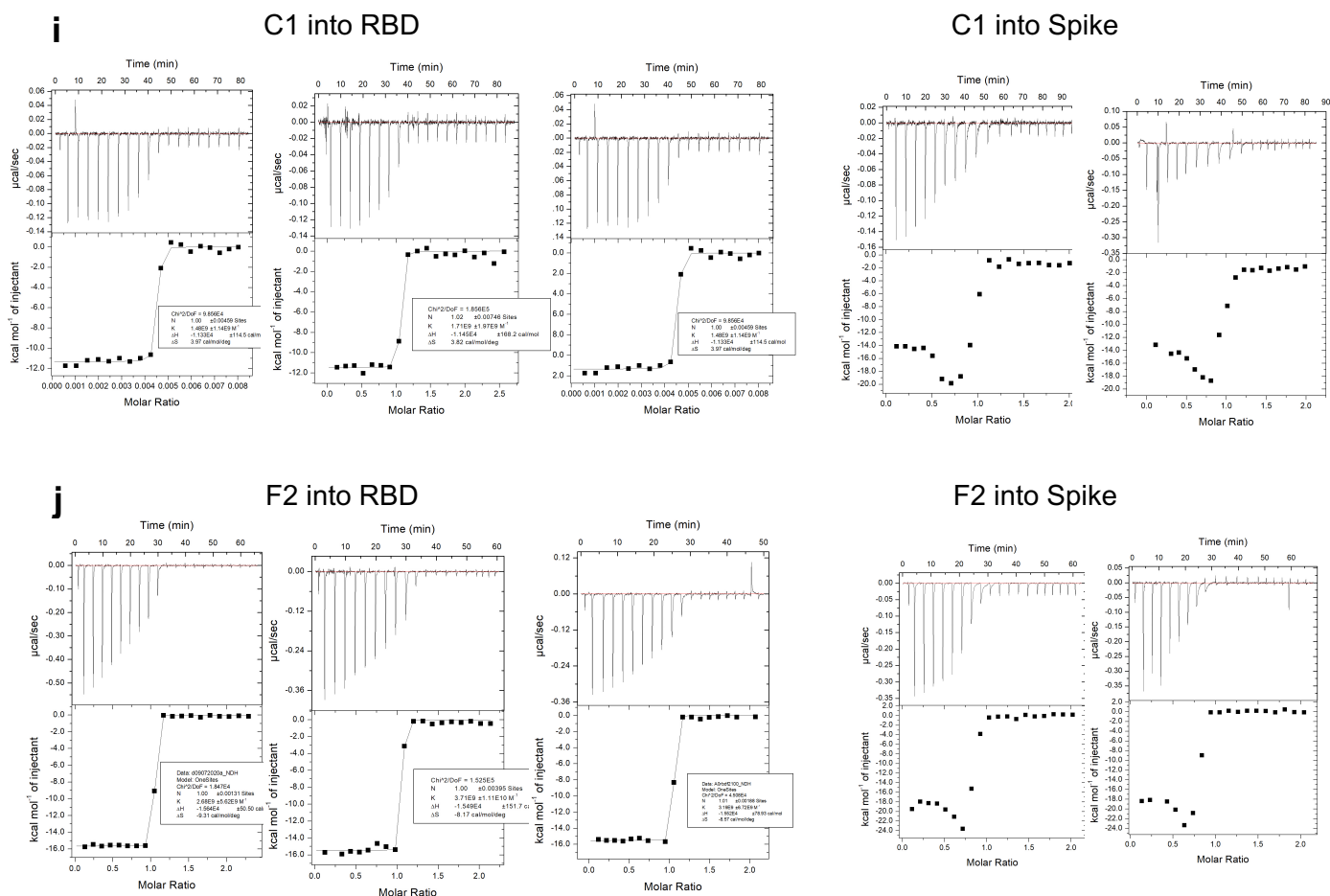
C5 into spike



H11 into Spike

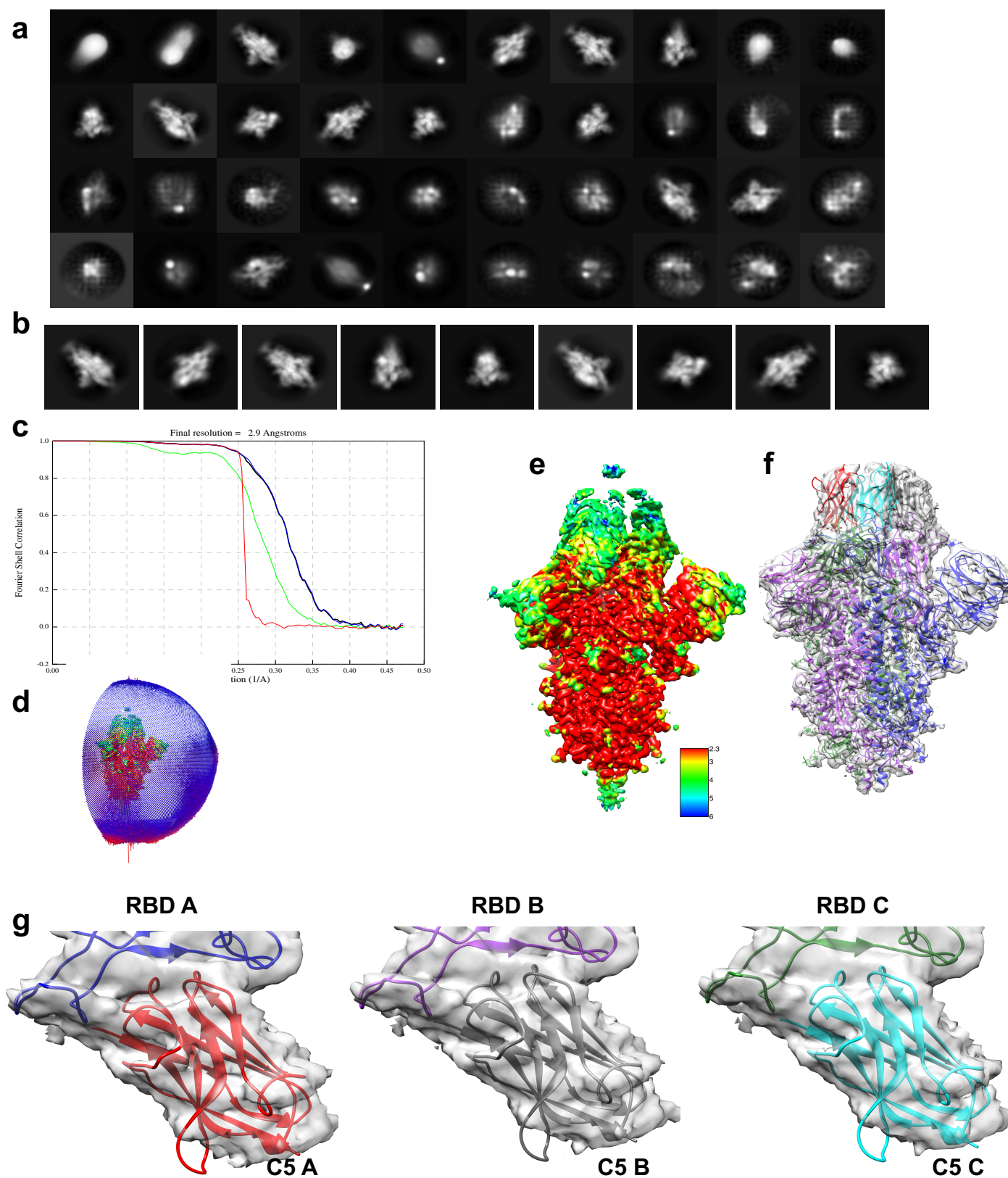


$K_{aC5} = 2.897\text{e}+9 \pm 4.57\text{e}+7$, $\Delta H_{C5} = -21.2 \text{ e}+3 \pm 2.3\text{e}+1$
 $K_{aH11} = 8.485\text{e}+5 \pm 1.33\text{e}+4$, $\Delta H_{H11} = -9.8\text{e}+3 \pm 4.2\text{e}+2$



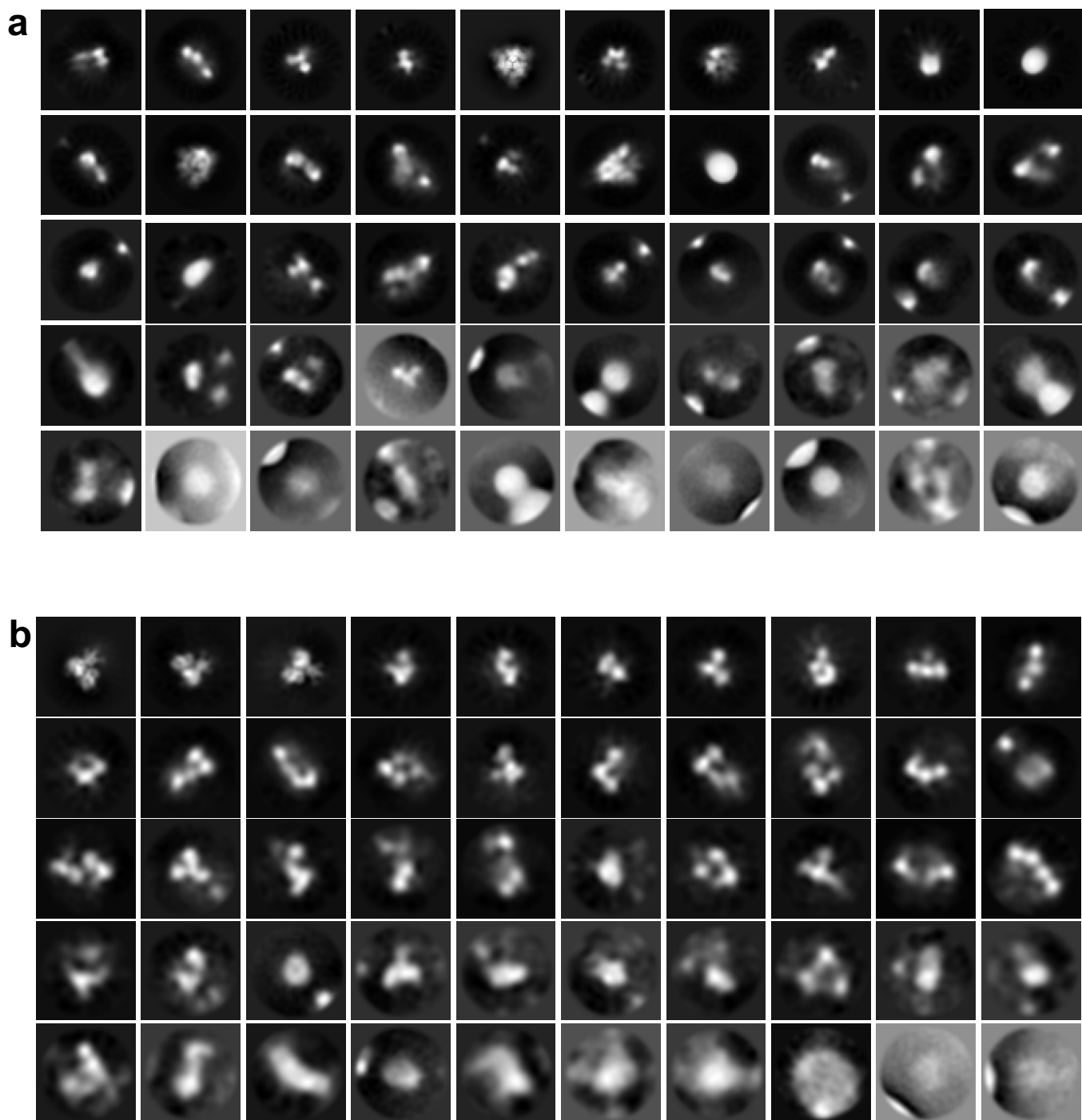
Supplementary Fig. 2 Analysis of nanobody binding by Isothermal calorimetry

(a) Titration of the H11 (472, 465, 460 μM) nanobody into RBD (47, 42, 38 μM) (three independent measurements), **(b)** Titration of the H11 nanobody (67 μM) into spike protein (6.8 μM) (two independent measurements), **(c)** Titration of the C5 nanobody (50, 89, 110 and 25 μM) into RBD (4.6, 9.7, 10 and 2.2 μM) (four independent measurements), the very tight binding make fitting thermodynamic parameters prone to large error, **(d)** Titration of the C5 nanobody (83, 67, 67 μM) into spike protein (7.6, 6.1, 5.8 μM) (three independent measurements), the very tight binding make fitting thermodynamic parameters prone to large error **(e)** Nanobody C5 (200, 194, 193 μM) was titrated into RBD (18, 19, 18 μM) where H11 (100 μM) was already present (three repeats). No binding curve was fitted, **(f)** Nanobody C5 (61, 68 μM) was titrated into spike protein (6, 6 μM) where H11 (180, 104 μM) was already present (two repeats). No binding curve was fitted. **(g)** AFFINImeter⁶⁰ simultaneous fitting (red line) of thermodynamic parameters for C5 into RBD in presence of H11, C5 into RBD and H11 into RBD. **(h)** AFFINImeter simultaneous fitting (red line) of thermodynamic parameters for C5 into spike protein in presence of H11, C5 into Spike and H11 into spike protein. **(i)** Three independent experiments of C1 (105, 49, 49 μM) titrated into RBD (9.9, 4.7, 3.9 μM respectively). The very tight binding means the calculation of K_D has large errors. Two experiments of C1 (64, 63 μM) titrated into spike protein (6.4, 6.4 μM respectively). The titration shows that complex conformational changes occur, and it was not possible to reliably fit a binding constant. **(j)** Three independent experiments of F2 (150, 100, 80 μM) titrated into RBD (13, 10, 7.8 μM respectively). The very tight binding means the calculation of K_D has large errors. Two experiments of F2 (86 μM) titrated into spike protein (8.6 μM respectively). The titration shows that complex conformational changes occur, and it was not possible to reliably fit a binding constant.



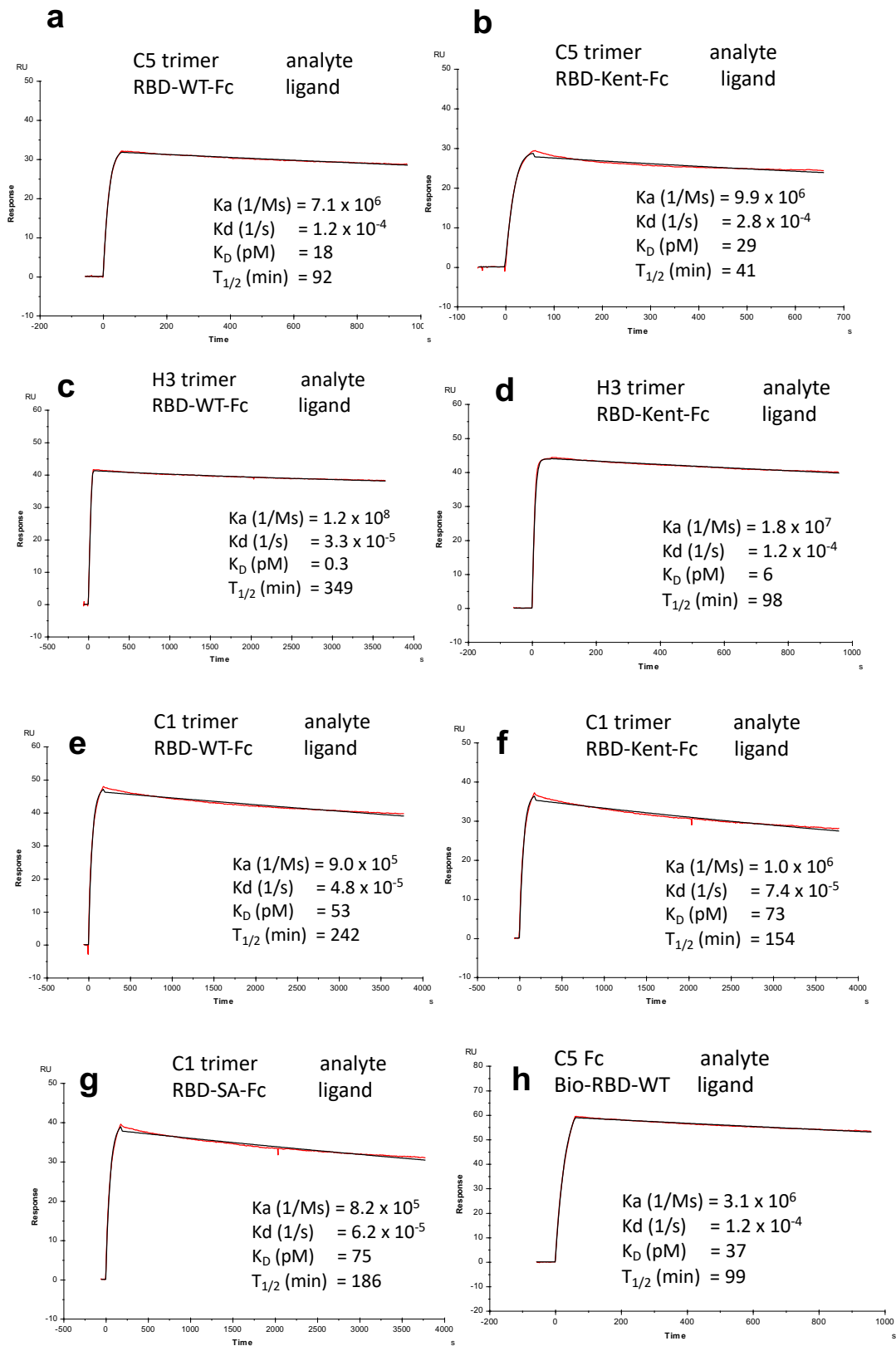
Supplementary Fig. 4 Cryo-EM of the Spike-nanobody C5 complex

(a) Unbiased 2D class averages of the Sars-CoV-2 spike-nanobody C5 complex, (b) 2D class averages selected for further processing. (c) Estimated map resolution using FSC criteria, (d) Particle orientation distribution for the final map. (e) Final cryo-EM map (coloured according to local resolution). (f) Ribbon diagram of the complex (cryo-EM map shown in grey and contoured at 5 σ in chimera). (g) Cryo-EM density with ribbon for each of the three C5 nanobodies bound to RBD, contoured at 5 σ in chimera. The amplitudes in the cryo-EM map used in panels f-g were scaled based on the refined coordinates using LocScale64 .



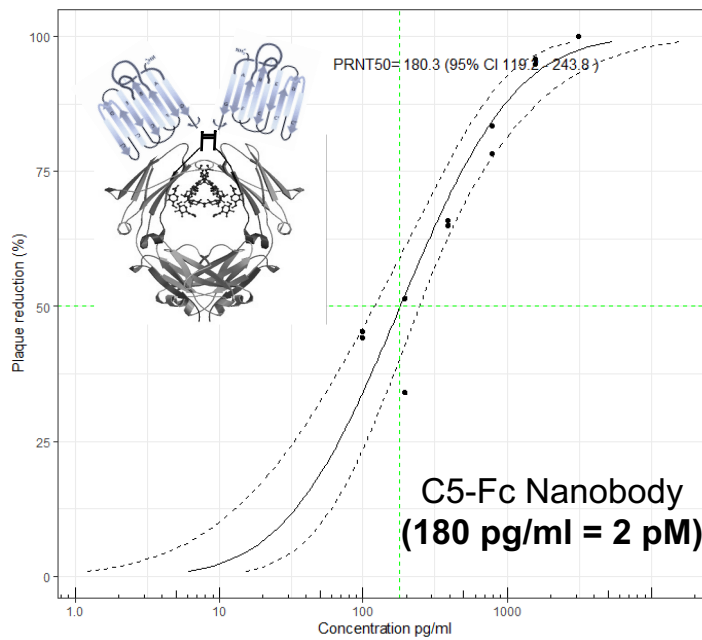
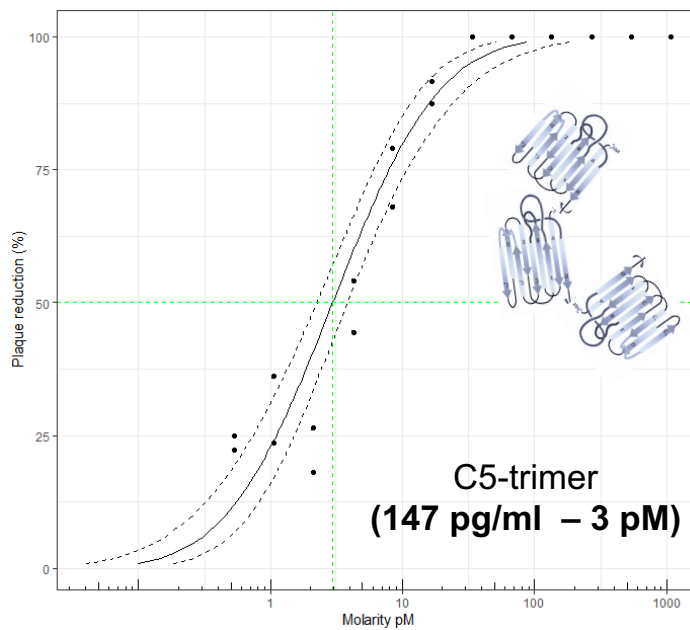
Supplementary Fig. 5 Cryo-EM of two different Spike-nanobody complexes

(a) Unbiased 2D class averages of the Sars-CoV-2 spike-nanobody C1 complex. These show heterogeneous particles without any indication of trimeric spike arrangement. **(b)** Unbiased 2D class averages of the Sars-CoV-2 spike-nanobody F2 complex. These show heterogeneous particles without any indication of trimeric spike arrangement.

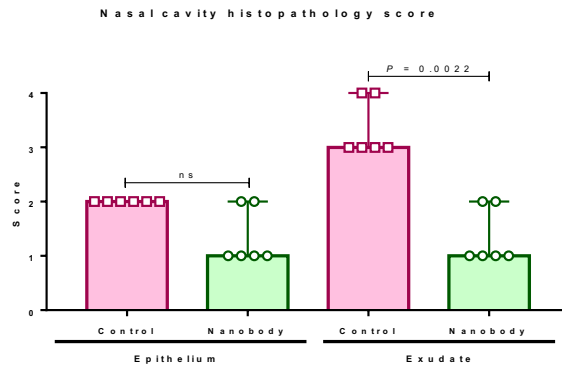
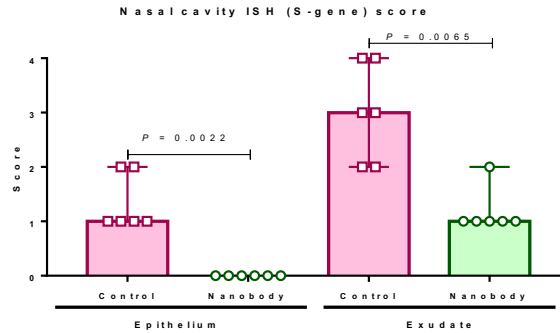
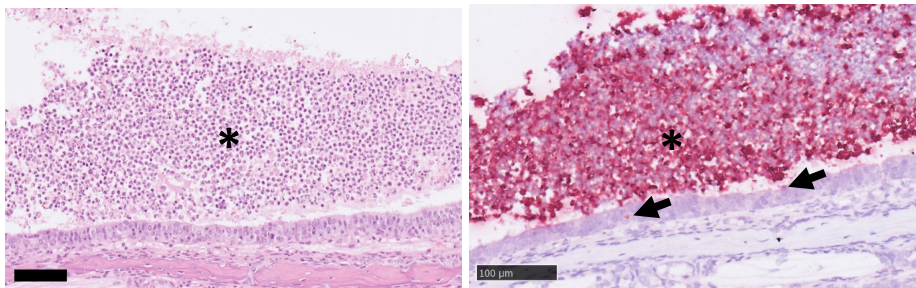


Supplementary Fig. 6 Nanobody binding kinetics

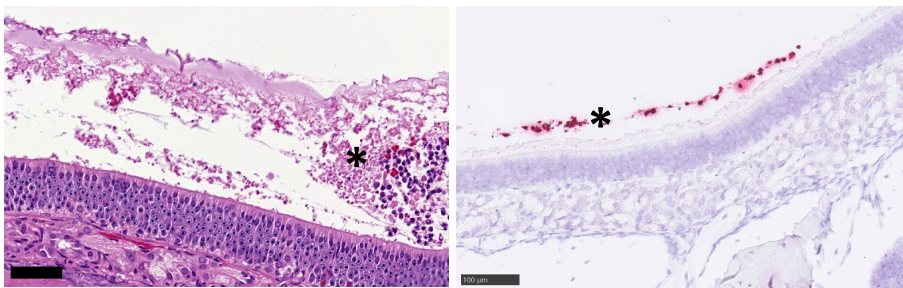
SPR Sensorgrams showing binding kinetics of (a-d) trimeric C5, H3, binding to WT (Victoria) and Kent RBDs (e-g) Trimeric C1 binding to Victoria, Kent and South Africa strain RBDs and (f) C5-Fc binding to WT (Victoria). A single injection of C1 / C5 / H3 trimer was performed over the huIgG1 Fc fusion of RBDs (a-g), and a single injection of C5-Fc over the biotinylated RBD-WT (f), to evaluate the kinetics of interaction.

a**b****Supplementary Fig. 7 Plaque Reduction Neutralisation assays**

The percentage reduction in plaques arising from virus is plotted against increasing (left to right) concentration of (a) C5-Fc (b) C5 trimer, shown schematically as insets. The confidence intervals are shown as dashed lines.

a**b****c**

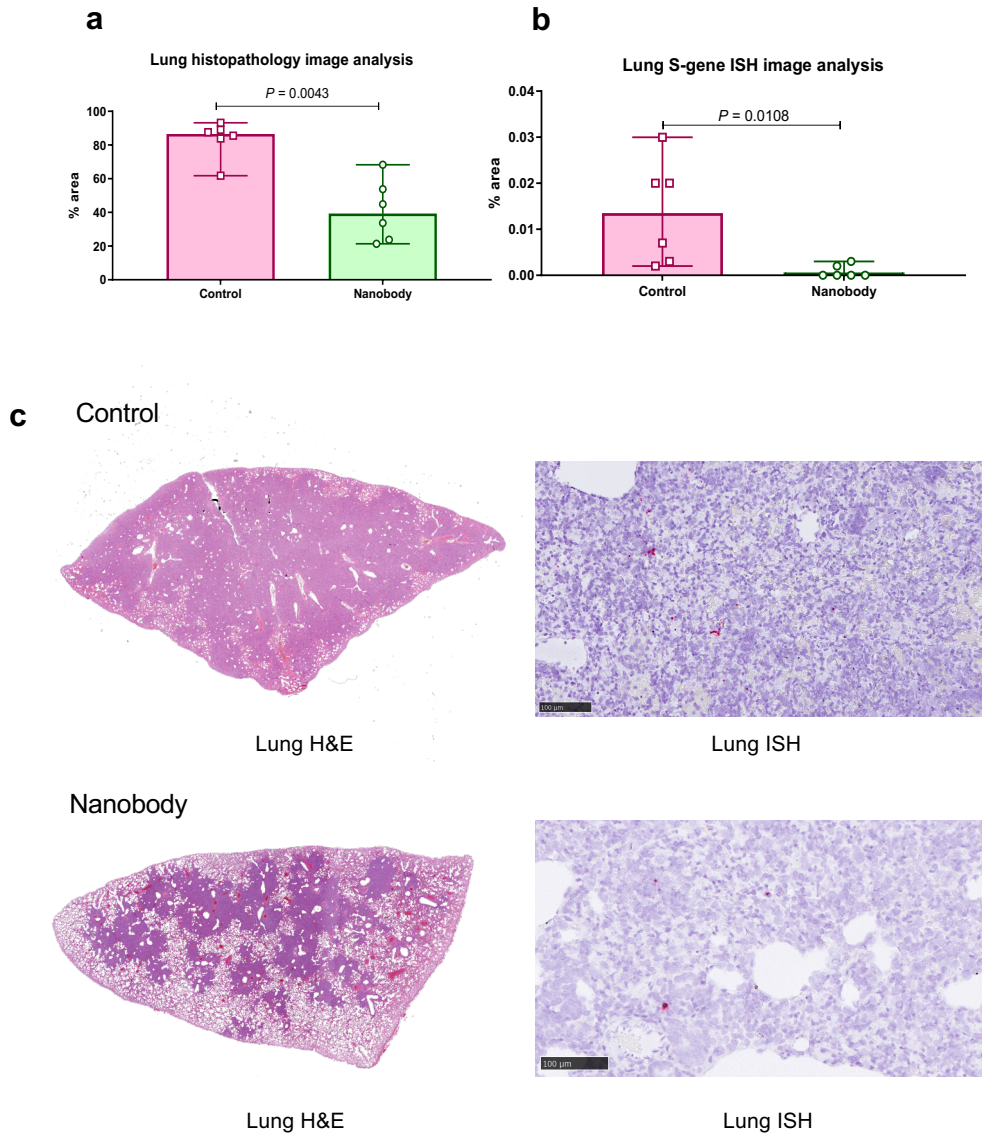
Control



Nanobody

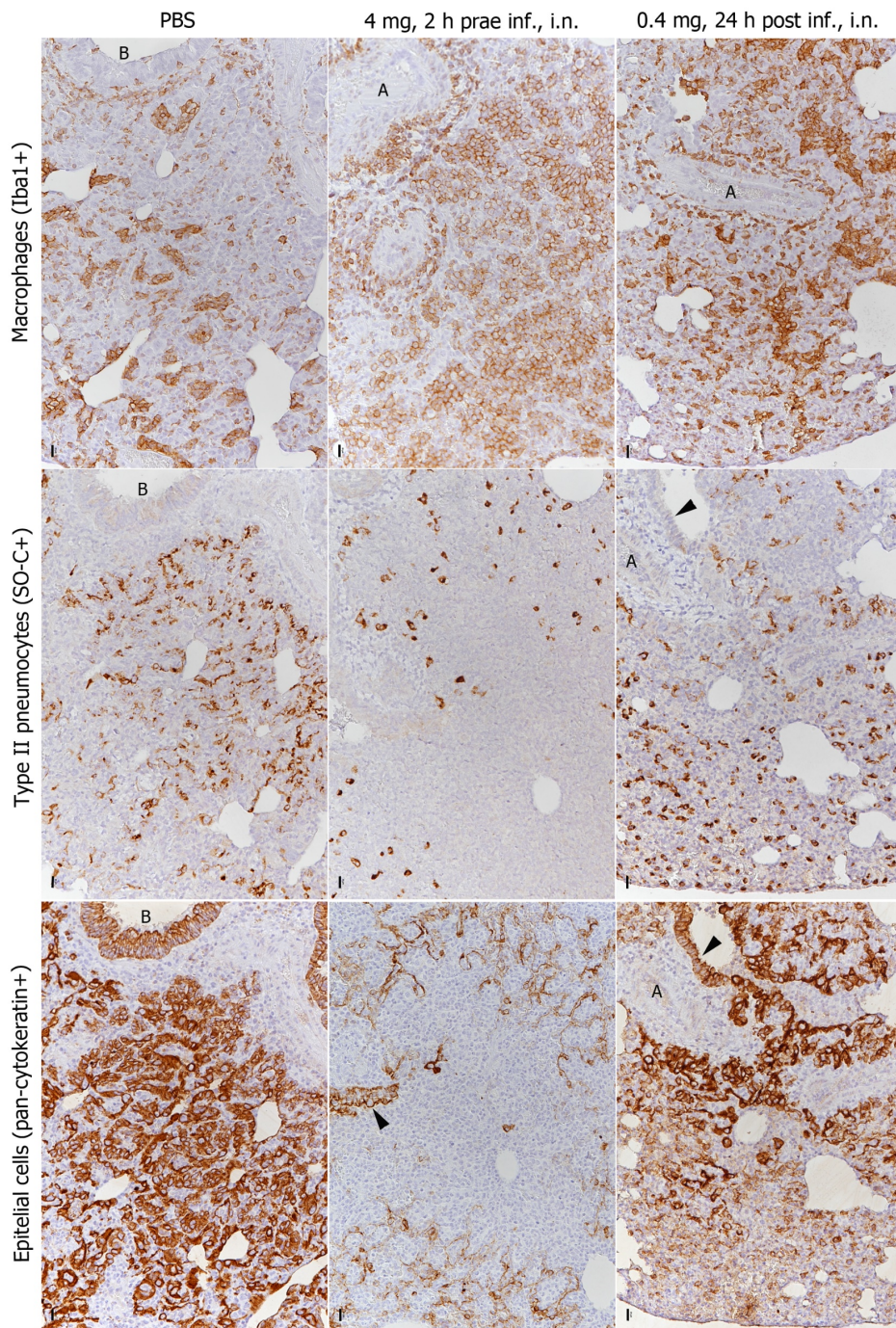
Supplementary Fig. 8 Histopathological semiquantitative analysis and presence of virus RNA (RNAScope ISH S-gene) in the nasal cavity of control and nanobody treated animals.

(a) A significant reduction in the presence of inflammatory exudates and the presence of virus RNA (*) was observed in the treatment group. (b) Minimal to mild necrosis of the epithelium was observed in both groups. (cc) representative images of nasal cavity stained with H&E and ISH (RNAScope for virus RNA). The presence of virus RNA in epithelial cells was only observed in control animals (arrows). Bar = 100 µm.



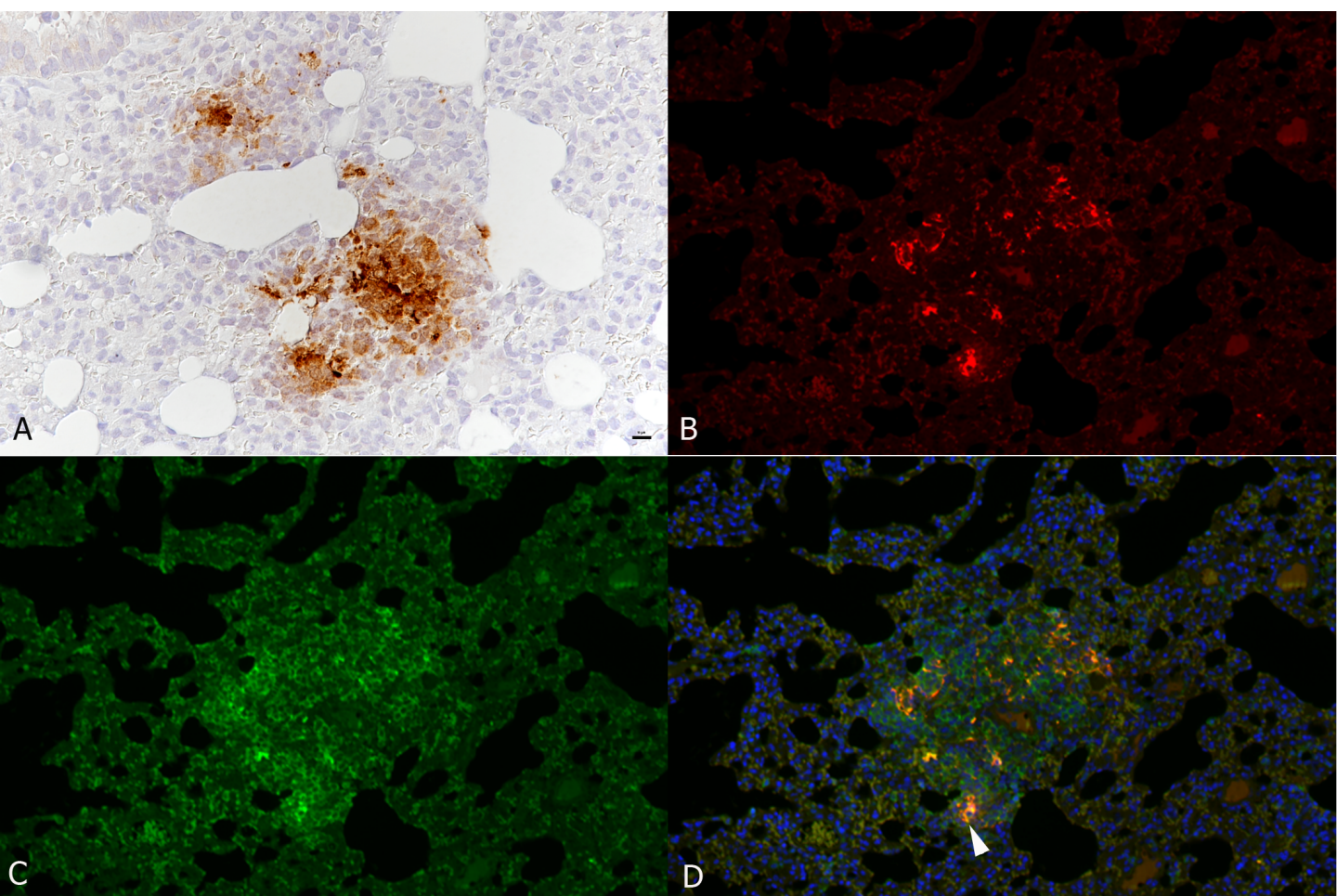
Supplementary Fig. 9 Histopathology and image analysis of lung in control and nanobody treated animals.

(a) lung histopathology image analysis showing the percentage of the lung area affected and (b) percentage of lung area with viral RNA-ISH staining with representative images of lung stained with HE showing larger areas of lung consolidation in the non-treated control group, and with RNAScope ISH (S-gene) showing larger areas of positive stained lung tissue in the control group. Box and bars show median and 95% C.I. Mann-Whitney's U test for median comparisons. (c) representative images of lung stained with H&E and ISH (RNAScope for virus RNA) Bar = 100 μ m.



Supplementary Fig. 10 Macrophage driven inflammatory response after C5 trimer neutralisation of SARS-CoV-2 in Syrian hamster model.

Lungs of hamsters infected intranasally with 10^4 PFU/100 μ l SARS-CoV-2 and euthanized at day 7 post infection. Animals had been untreated prior to infection (PBS) or treated with 4 mg/kg C5 intranasally at 2 h prae infection (h prae inf) or 0.4 mg/kg C5 intranasally at 24 h post inf (h post inf). Staining for macrophages (Iba1+) showed clusters of macrophages in the consolidated areas in the untreated (PBS) animals and a very intense macrophage infiltration in the treated animals. In untreated animals there are numerous activated and hyperplastic type II pneumocytes (SP-C+); these are less numerous in hamsters treated with the low C5 trimer dose and replaced by the macrophage dominated infiltrate in animals treated with the high dose at 2 h prae inf. Staining for cytokeratins shows that in untreated animals the consolidated areas are dominated by hyperplastic bronchiolar epithelium. The hyperplasia is less intense in hamsters treated with the low C5-trimer dose and limited to the periphery of the infiltrates in animals treated with the high dose at 2 h prae inf. B – bronchiole (arrowhead: normal bronchiolar epithelium); A – artery. Immunohistology, hematoxylin counterstain. Bars = 20 μ m.



Supplementary Fig. 11 Viral antigen sequestration in macrophages in the focal infiltrates after C5 trimer neutralisation of SARS-CoV-2 in Syrian hamster model.

Lung of hamster treated with 4mg/kg C5 intranasally at 2 h prae infection, infected intranasally with 10^4 PFU/100 μ l SARS-CoV-2 and euthanized at day 7 post infection. A. Small focal consolidated area with SARS-CoV-2 N expression in intact and degenerate cells. Immunohistology, hematoxylin counterstain. B. SARS-CoV-2 N expression (red fluorescence) in consecutive section. C. The consolidated area contains abundant macrophages (Iba1+; green fluorescence). D. Combined image, confirming viral antigen expression in macrophages (arrowhead) in the lesion.

Primer ID	Sequence
CALL_GSP	5'-CCTGCGGCTCCCGGGTCTGCCCTTTGGCC-3'
CALL_001	5'-GTCCTGGCTGCTCTTCTACAAGG-3'
CALL_002	5'-GGTACGTGCTGTTGAACTGTTCC -3'
VHH_For	5'-GTTATTACTCGCGGCCAGCCGGCCATGGCCGATGTGCAGCTGCAGGAGTCTGGRGGAGG-3'
VHH_Rev_IgG2	5'-GGTGATGGTGTGTGGCCTCCCGGGCCGGCCGCTGGTGTGGTTTTGGTGTCTT-3'
VHH_Rev_IgG3	5'-GGTGATGGTGTGTGGCCTCCCGGGCCGGCCGGAGCTGGGGTCTTCGCTGTG-3'
OmA_exp_F	5'-CTACCGTAGCGCAAGCTCAGGTGCAGCTGGTCGAGTCTGGGGGA-3'
OmA_exp_R	5'-GGTGATGGTGTGTTTTGTAGGAGACGGTGACCTGGGTCCCTGGCC-3'
TriNb_Neo_F1	5'-GCGTAGCTGAAACCGGCCAGGTGCAGCTGGTGGAGTCTGGG-3'
TriNb_R1	5'-GACTCCACCAGTGCACCTGGGAGCCAGAACCCTCCCTGAGGAGACGGTGACCTGG-3'
TriNb_F2	5'-CCCAGGTCACCGTCTCCTCAGGGAGCGGTTCTGGCTCCCAGGTGCAGCTGGTGGAG-3'
TriNb_R2	5'-GACTCCACCAGTGCACCTGCGACCCGCTACCTGAGCCTGAGGAGACGGTGACCTGG-3'
TriNb_F3	5'-CCCAGGTCACCGTCTCCTCAGGCTCAGGTAGCGGGTCGCAGGTGCAGCTGGTGGAG-3'
TriNb_Neo_R1	5'-GTGATGGTGTGTTTTGTAGGAGACGGTGACCTGGGTCCC-3'
TriNb_Neo_F2	5'-GCGTAGCTGAAACCGGCCAG-3'
TTGneo_RBD_F	gcgtagctgaaacggccgaatatcacaatctttgt
TTGneo_RBD_R	GTGATGGTGTGTTTATTGTACTTTTTTCGGTCCGCACAC
K417V_F	CAGATCGCGCCGGGCCAAACGGGCGTGATAGCTGACTATAATTATAAG
K417V_R	CTTATAATTATAGTCAGCTATCACGCCGTTTGGCCCGGCGCATCTG
E484K_F	ggttcaacccctgcaatggcgtaAGGGTTTTAACTGTTACTTCCCAC
E484K_R	GTGGGAAGTAACAGTTAAACCCCTTgacgccattgcagggggttgaacc
N501Y_F	Cagtcatacggattcaaccaacttacggggttgctatcagcgtaccgc
N501Y_R	gggtacggctgatagccaacccgtaagttggttgaaatccgtatgactG
PelB_F	5'-GTTATTACTCGCGGCCAGCCGGCCATGGCCAGGTGCAGCTGGTCGAGTCTGGG-3'
PelB_R	5'-GGTCACCGTCTCCTCACACCATCACCACCATCATTAATAAAGGCCAACACCATCACC-3'
AbVec-Fc_F	5'-CTAGTAGCAACTGCAACCGGTGTTCACTCTCAGGTGCAGCTGGTGGAGTCTGGG-3'
AbVec-Fc_R	5'-GATTTGGGCTCGGTGACGCTGAGGAGACGGTGACCTGGGTCCC-3'
RBD_Fc_R	5'-CAGAACTCCAGTTTATTTGTACTTTTTTCGGTCCGC-3'
TriNb_Neo_R2	5'-GTGATGGTGTGTTTTGAGG-3'
RBD_TTGneo_F	GCGTAGCTGAAACCGGCCGAATATCACAAATCTTTGTCC
RBD_TTGneo-BAP_R	GTCATTGAGCAAGCTATTTGTACTTTTTTCGGTCCGCACAC

Supplementary Table 1: PCR primer sequences

Nanobody	DNA sequence	Amino acid sequence
C1	CAGGTGCAGCTGGTGGAGTCTGGGGGAG GCTTGGTGCAGCCTGGGGGCTCTCTGAG ACTCTCCTGTGCAGCCTCTGGATTCACT AATGATTTTATAGCATCGCGTGGTTCC GCCAGGCCCCCAGGAAAGGAGCGTGAGGG GGTCTCATGGCTTAGTGTCAGTGATAAT ACCCCAACCTACGTAGACTCCGTGAAGG ACCGGTTCAACATCTCCAGACACAACGC CAACAACACCGTGTACCTGCAAATGAAC ATGCTGAAACCTGAGGACACGGCCATTT ACTATTGTGCAGCAGGACGCTTCGCGGG AAGGGATACTTGGCCCTCGTCCTATGAT TACTGGGGCCAGGGGACCCAGGTCACCG TCTCCTCA	QVQLVESGGGLVQPGGSLRLSCAA SGFTNDFYSIAWFRQAPGKEREGV SWLSVSDNTPTYVDSVKDRFTISR HNANNTVYLMNMLKPEDTAIYYC AAGRFAGRDTPSSYDYWGQGTQV TVSS
C5	CAGGTGCAGCTGGTGGAGTCTGGGGGAG GCTCGGTGCAGGCTGGGGGCTCTCTGAC ACTCTCCTGTGTCGCCTCTGGAGTCACT TTGGGACGTCATGCCATAGGCTGGTTCC GCCAGGCCCCCGGAAGGAGCGTGAGAG AGTCTCGTGTATTAGAACATTTGATGGC ATCACAAGTTATGTAGAGTCCACGAAGG GCCGATTACCATCTCCAGTAACAATGC CATGAACACGGTGTATCTGCAAATGAAT AGCCTAAACCTGAAGACACGGCCGTTTA TTTCTGTGCACTGGGAGTGAAGTGCAGCC TGTTTCAGATAATCCCTACTTCTGGGGCC AGGGGACCCAGGTCACCGTCTCCTCA	QVQLVESGGGSVQAGGSLTLSCVA SGVTLGRHAIGWFRQAPGKERERV SCIRTFDGITSYVESTKGRFTISS NNAMNTVYLMNLSLKPEDTAVYFC ALGVTAACSDNPYFWGQGTQVTVS S
F2	CAGGTGCAGCTGGTGGAGTCTGGGGGAG GATTGGTGCAGGCTGGGGGCTCTCTAAG CTCGCTTGTATAGCCTCTGGACGCACCT TCCATAGCTATGTTCATGGCCTGGTTCCG CCAGGCTCCAGGGAAGGAGCGTGAGTTT GTAGCAGCTATTAGTTGGAGTAGTACAC CGACATACTATGGAGAATCCGTGAAGGG CCGATTACCATCTCCAGAGACAACGCC AAGAACACGGTGTATCTGCAAATGAACC GCCTGAAACCTGAGGACACGGCCGTTTA TTTCTGTGCAGCAGACCGGGGTGAAAGT TACTACTACACTCGACCCACCGAGTATG AATTCTGGGGCCAGGGGACCCAGGTCAC CGTCTCCTCA	QVQLVESGGGLVQAGGSLRLACIA SGRTFHSYVMAWFRQAPGKEREFV AAISWSSTPTYYGESVKGRFTISR DNAKNTVYLMNRLKPEDTAVYFC AADRGESYYYTRPTEYEFWQGTQ VTVSS
H3	CAGGTGCAGCTGGTGGAGTCTGGGGGAG GATTGGTGAAGACTGGGGGCTCTCTGAG ACTCTCCTGTGCAGCCTCTGGCCGCACC TTCAGTACCTACAGCATGGGCTGGTTCC GCCAGGCTCCAGGGAAGGAGCGTGAGTT TGTAGCAGGTATGCGCTGGACGGGTAGT AGTACATTCTACTCAGACTCCGTGAAGG GCCGATTACCGTCTCCAGAAACAACGC CAAGGACACGGTGTATCTGCACATGAAC AGCCTGAAACCTGAGGACACGGCCGTTT ATTACTGTGCAATCACGACTATCGTAAG AGCTTACTATACTGAGTATACCGAAGCT GACTTTGGTTCTTGGGGCCAGGGGACCC AGGTCACCGTCTCCTCA	QVQLVESGGGLVKTGGSRLRLSCAA SGRTFSTYSMGWFRQAPGKEREFV AGMRWTGSSTFYSDSVKGRFTVSR NNAKDTVYLHMNSLKPEDTAVYYC AITTIVRAYYTEYTEADFGSWGQG TQVTVSS

Supplementary Table 3: DNA sequences of C1, C5, F2 and H3 nanobodies