

Appendix

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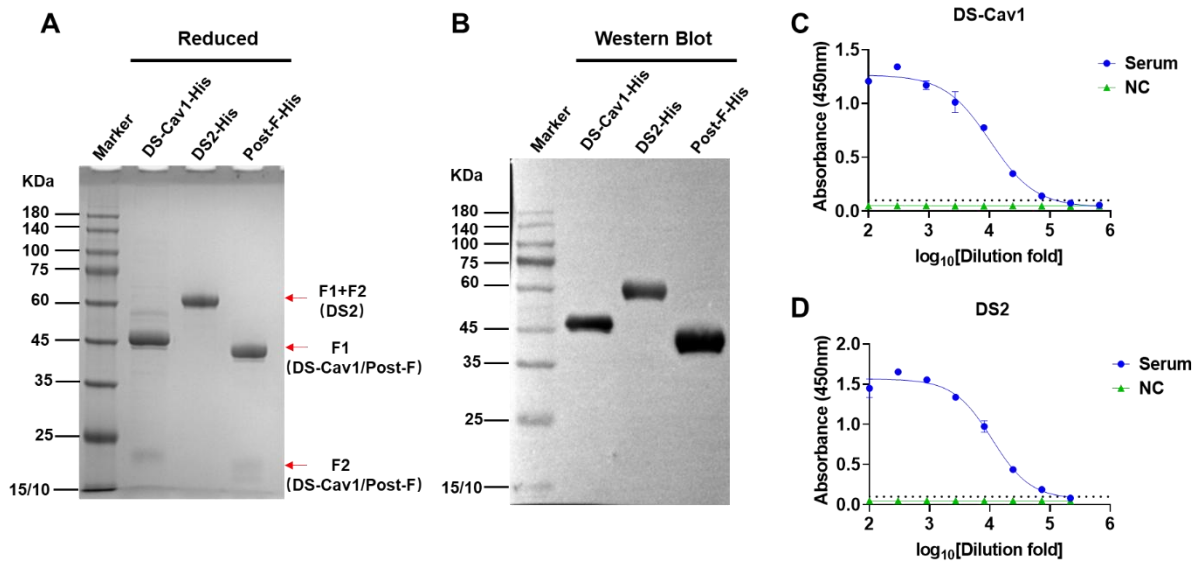
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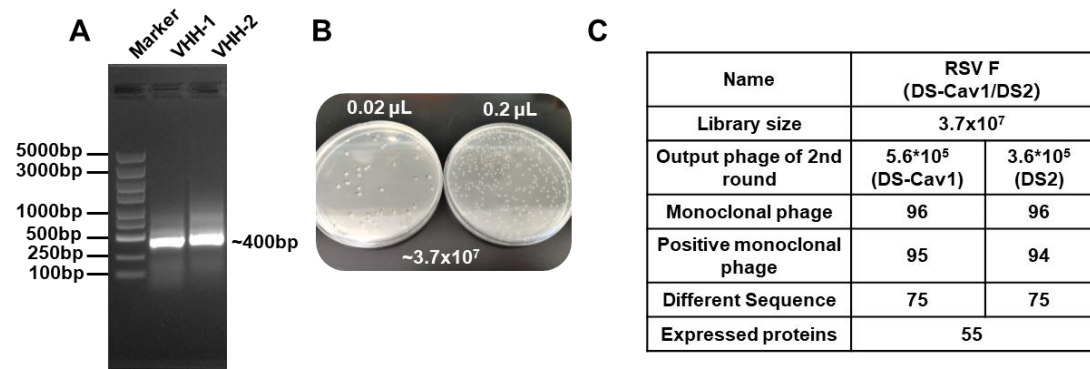
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Appendix Table S1. Cryo-EM data collection, refinement and validation. (Page 15)

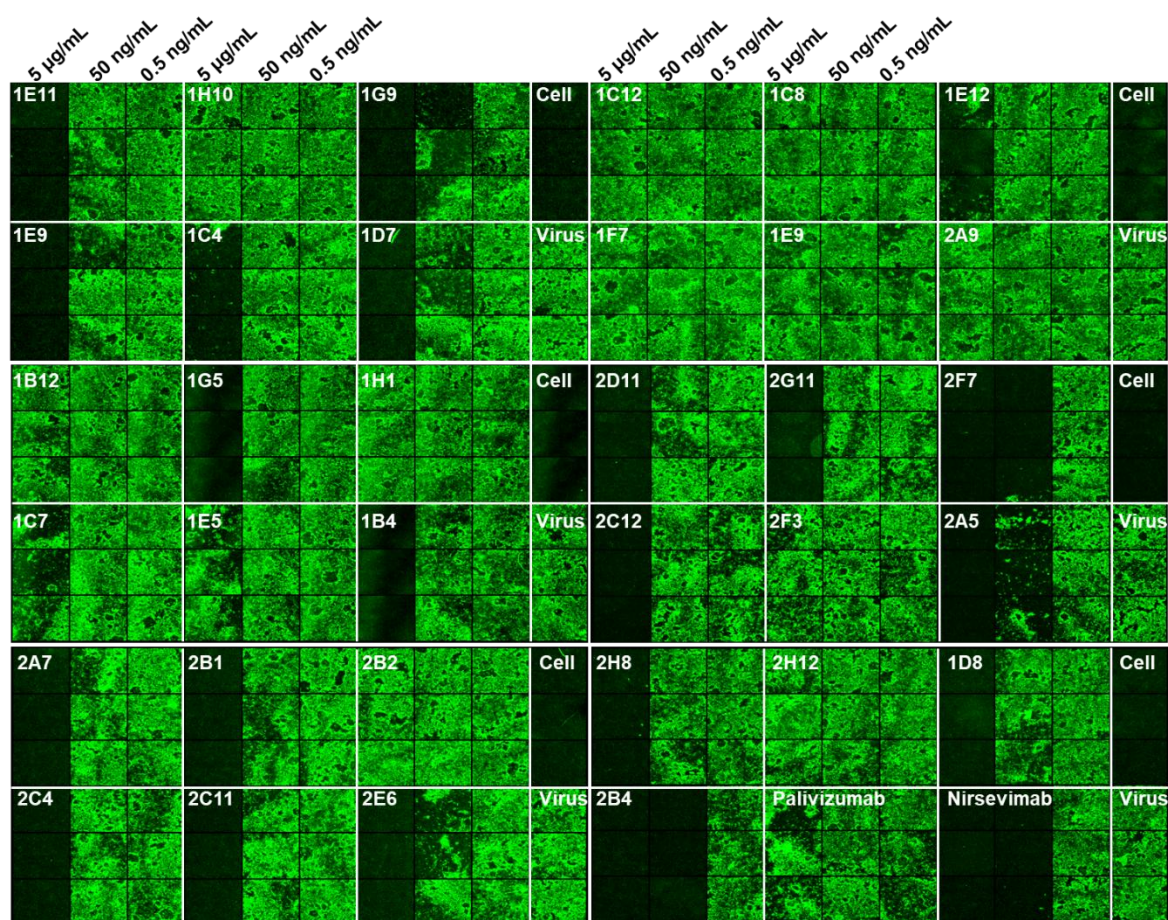
Appendix Table S2. Binding site of nanobodies on RSV F and sequence conservation. (Page 16)



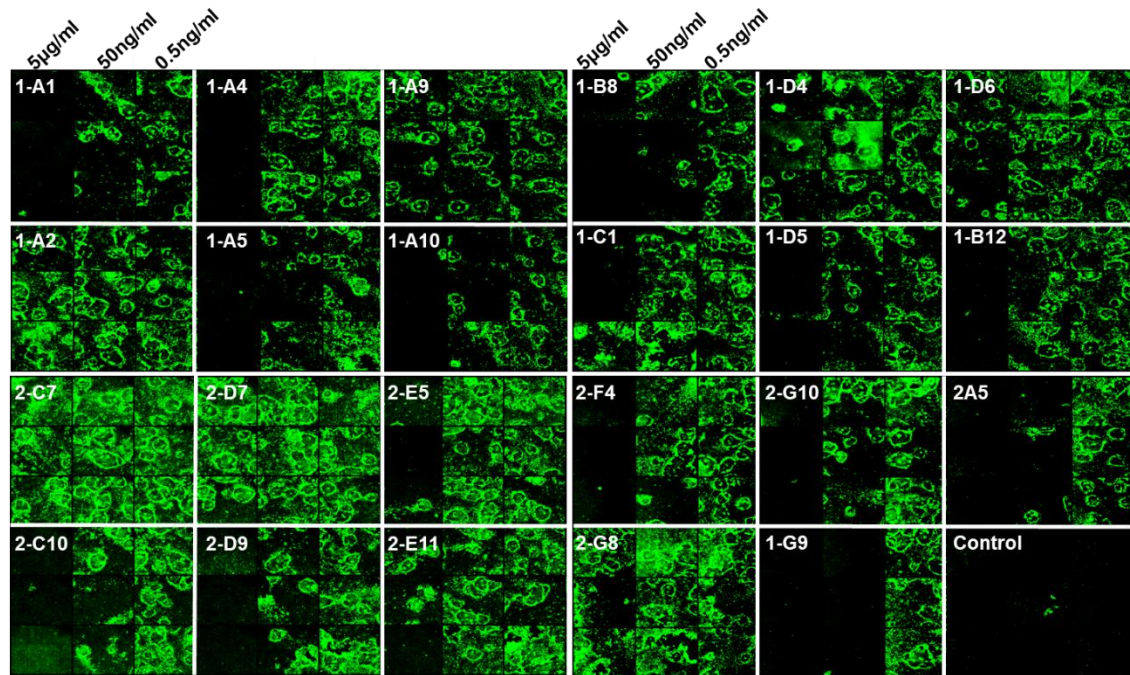
Appendix Figure S1. RSV F antigen preparation and Dromedary immunization. (A) Reduced SDS-PAGE analysis of the purified RSV pre-F (DS-Cav1 and DS2) and Post-F. The monomeric forms of DS1, DS2, and Post-F antigens showed molecular weights of 40-60 kDa. (B) Western Blot identification of purified DS-Cav1, DS2 and Post-F proteins using the Palivizumab as the primary antibody. (C-D) Following immunization dromedary, serum samples were collected to assess RSV pre-F-specific binding antibody titers by ELISA. NC, pre-immune serum. Data are average values of two replicates.



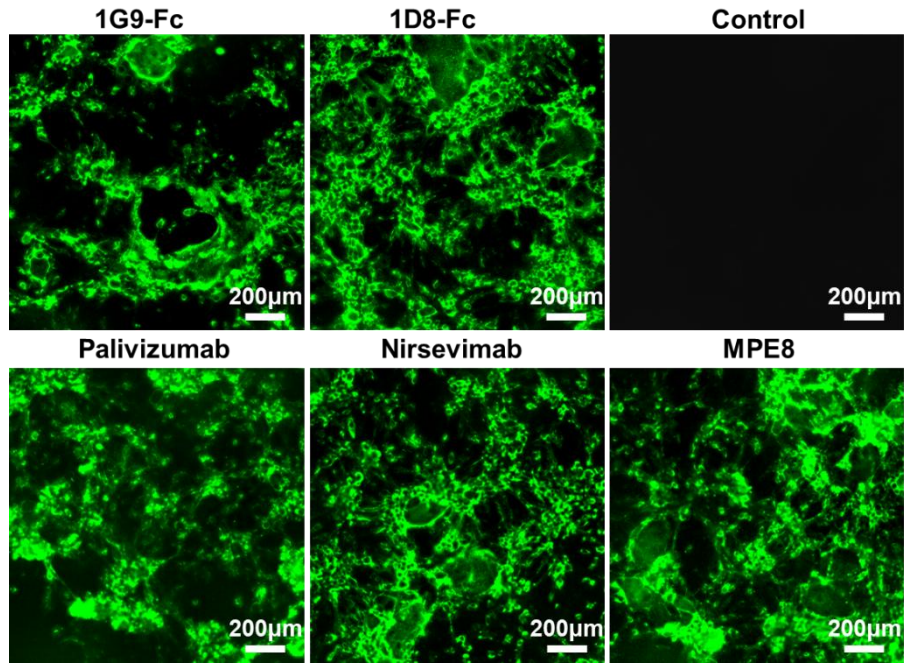
Appendix Figure S2. Construction of a phage display library. (A) Acquisition of VHH coding regions from immunized dromedary PBMCs through PCR. (B) The calculation result for phage library capacity. (C) Enrichment of phages after two rounds of panning on DS-Cav1 and DS2. The number of positive monoclonal clones ($OD_{450} > 0.5$) and comparison of sequence diversity.



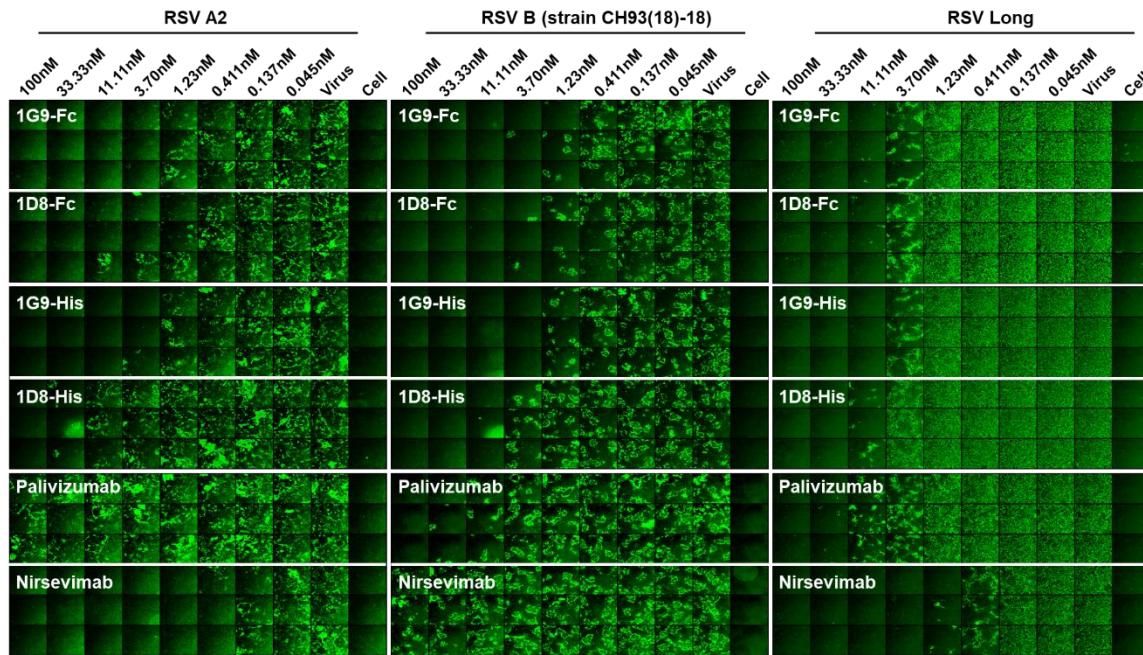
Appendix Figure S3. Immunofluorescence results of candidate Nb-Fc fusions neutralizing RSV A2 virus in vitro. Screening results for the neutralizing capacity of 34 nanobodies. White text in the upper left corner indicates the nanobody number. "Cell" represents the blank control group (no virus, no antibody). "Virus" represents the virus-only control group (virus added, no antibody). RSV F protein marker detected by antibody F-E2, the concentration of F-E2 used for detection is 1 µg/mL. Magnification: 10×. Palivizumab and nirsevimab were used as positive control neutralizing antibodies.



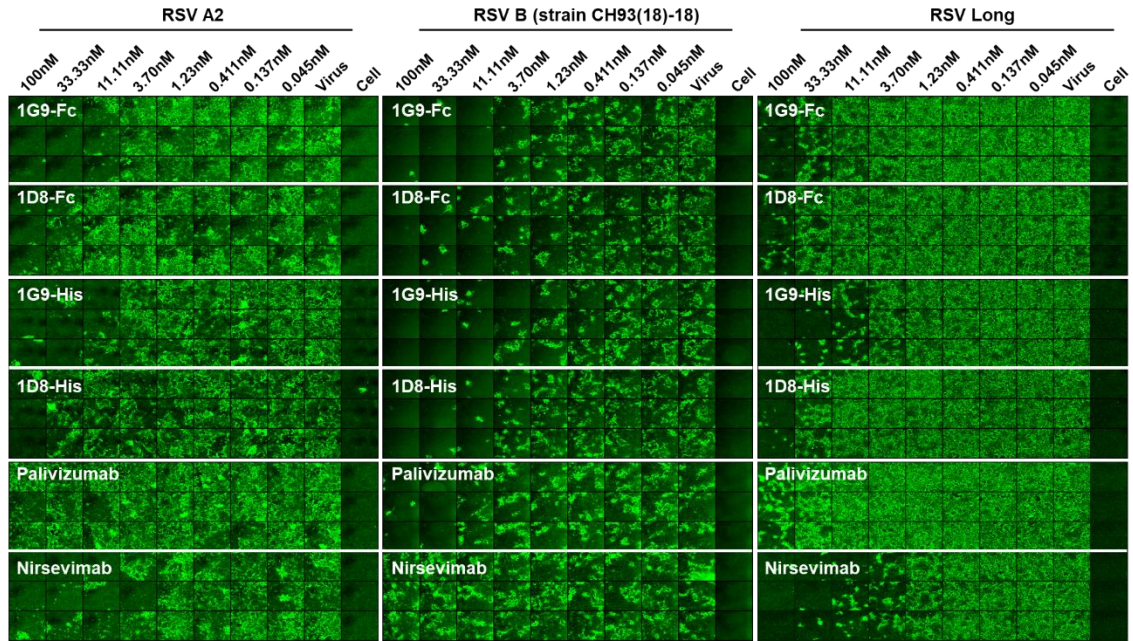
Appendix Figure S4. In vitro RSV A2 neutralization of the candidate Nb-Fc fusions. Screening results for the neutralizing capacity of 21 nanobodies. White text in the upper left corner indicates the nanobody number. "Control" represents the blank control group (no virus, no antibody). RSV F protein marker detected by antibody F-E2. the concentration of F-E2 used for detection is 1 µg/mL. Magnification: 10×.



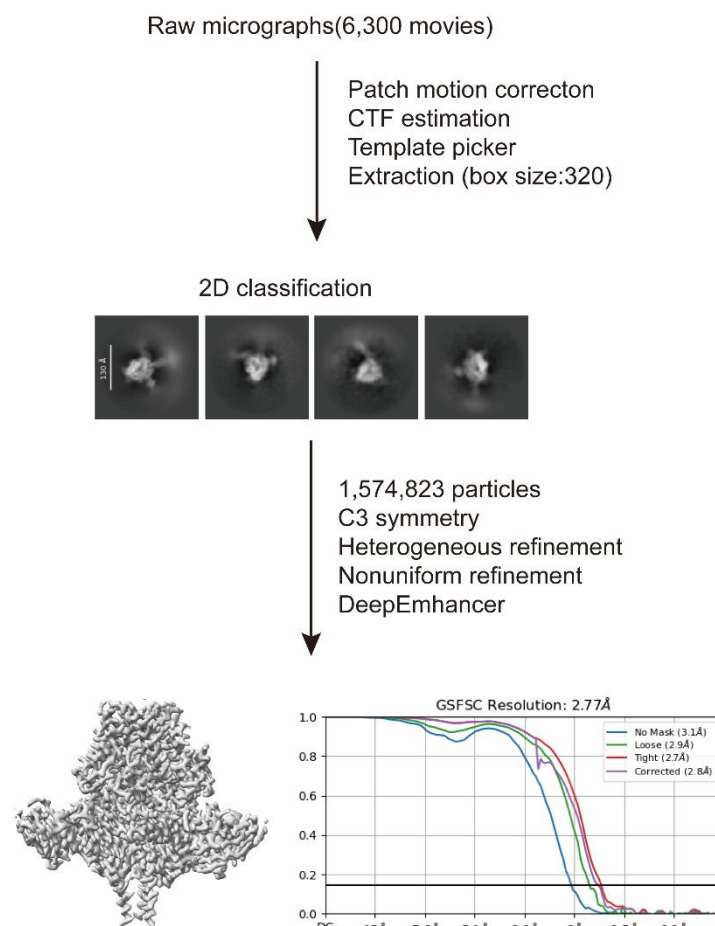
Appendix Figure S5. Binding activity of candidate Nb-Fc fusions was analyzed by immunofluorescence. Binding of the purified Nb-Fc fusions (1G9-Fc and 1D8-Fc) to native F protein (RSV A2) was detected by indirect immunofluorescence assay. Palivizumab, nirsevimab and MPE8 were used as positive control. The concentration of nanobody/antibody used for detection is 10 µg/mL. Scale bar: 200 µm. "Control" represents the blank control group (no virus, no antibody).



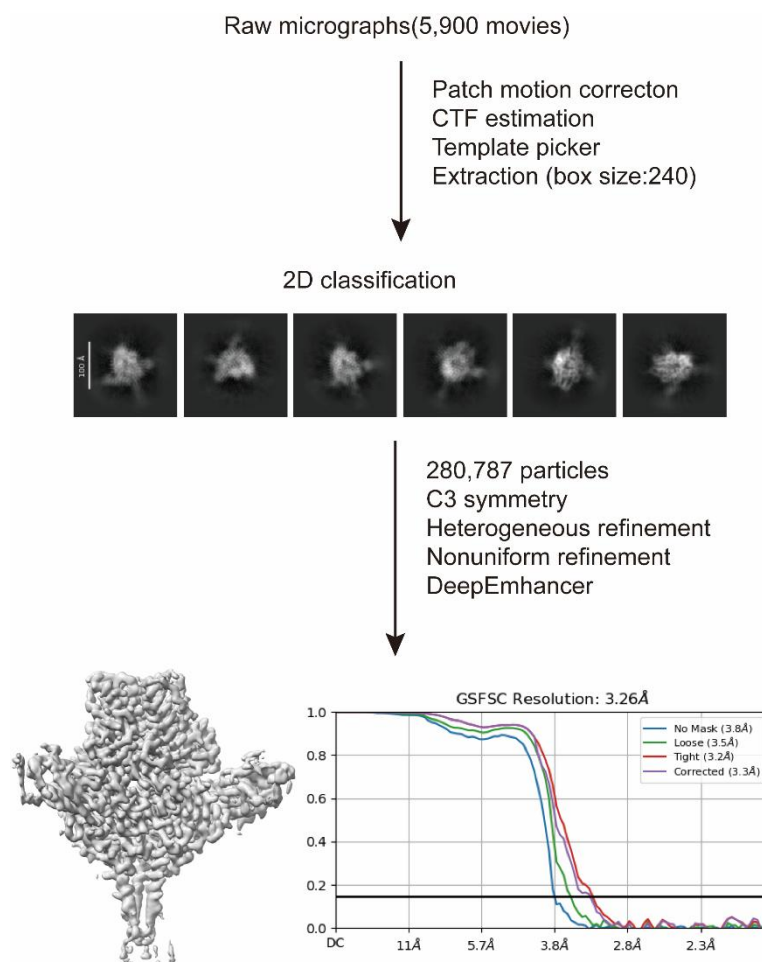
Appendix Figure S6. The neutralizing activity of nanobodies 1G9 and 1D8 against RSV A2, RSV B and RSV Long subtypes. The nanobodies were serially diluted and incubated with RSV for 1 h at 37°C. Then, mixtures were added to 96 plates and incubated for 2 h at 37°C. After changing fresh DMEM containing 2% FBS and 2% PS, Vero cells are grown at 37°C. After 72 h, cells were fixed, and RSV F was stained with primary antibody (F-E2, 1 µg/mL) and FITC-anti-human Fc (green, 1:2000). "Virus" represents the virus-only control group (virus added, no antibody). "Cell" represents the blank control group (no virus, no antibody).



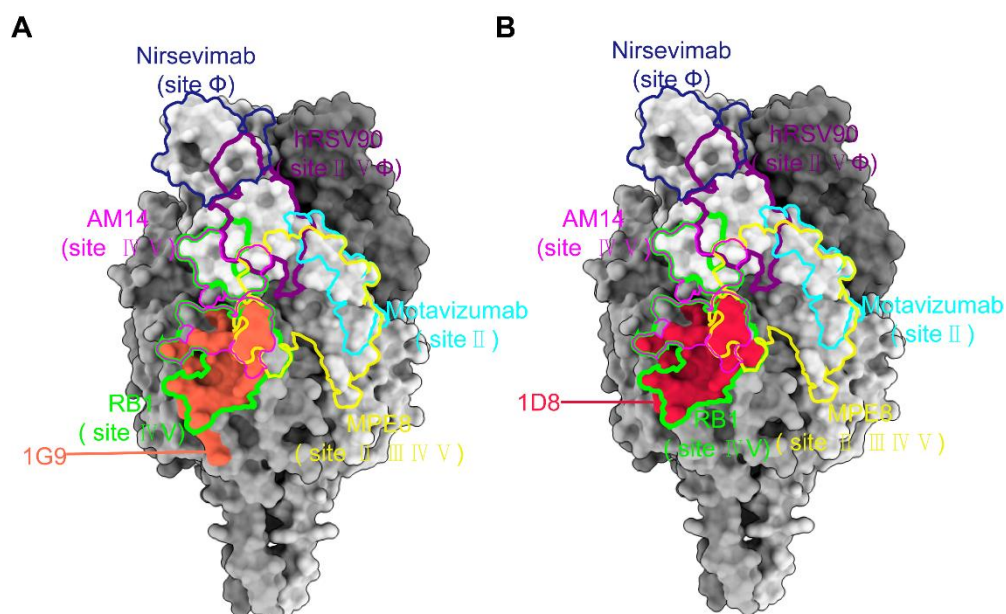
Appendix Figure S7. The fusion inhibition of nanobodies 1G9 and 1D8 against RSV A2, RSV B and RSV Long subtypes. (B) RSV A2, RSV B and RSV Long were pre-incubated in Vero cells for 1 h at 4°C to allow virus attachment. The nanobodies 1G9 and 1D8 were serially diluted in DMEM (2% FBS) and added to plates, and continuously incubated at 4°C. Finally, Vero cells were cultured at 37°C for 72 h. RSV F was stained with primary antibody (F-E2, 1 µg/mL) and FITC-anti-human Fc (green, 1:2000). "Virus" represents the virus-only control group (virus added, no antibody). "Cell" represents the blank control group (no virus, no antibody).



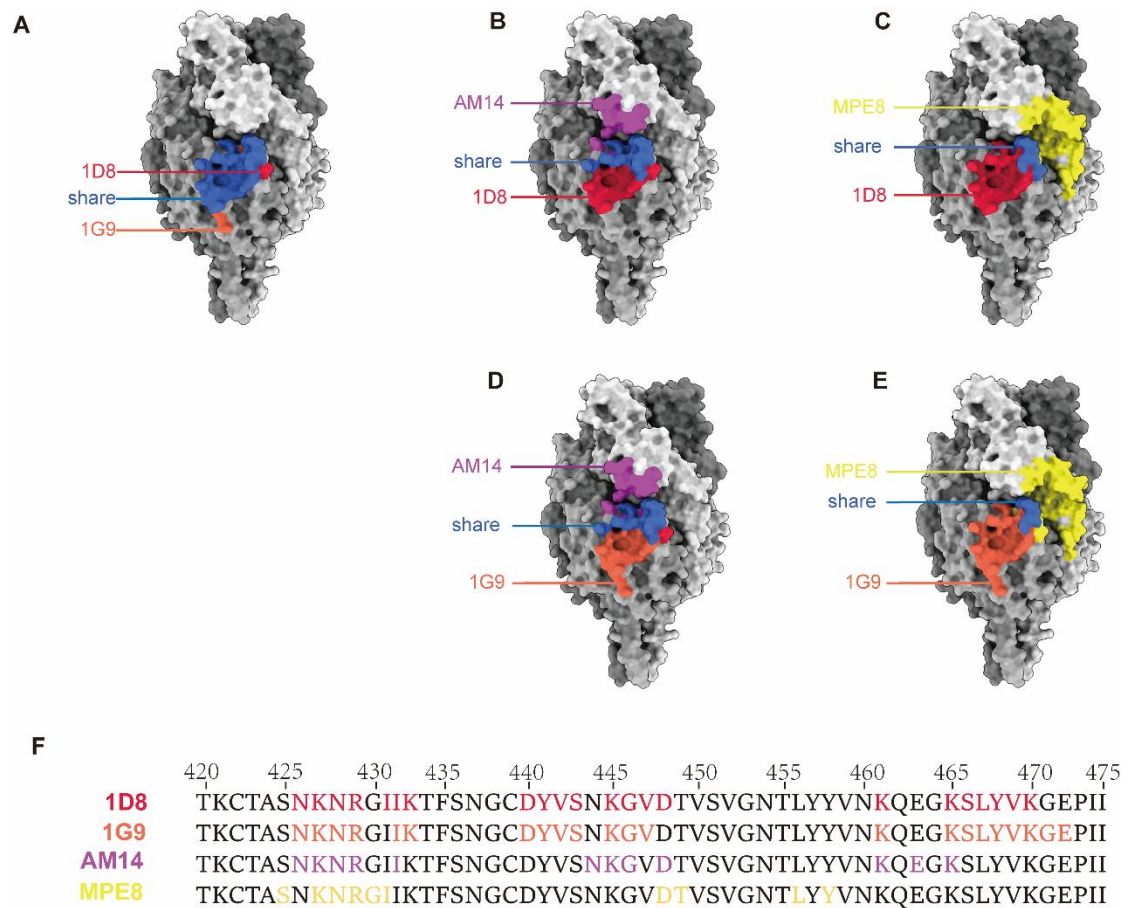
Appendix Figure S8. Single-particle cryo-EM image processing workflow and global and local resolution estimations for the RSV A2 DS-Cav1:1G9 complex. Representative 2D classification results, maps after DeepEnhancer sharpening, FSC curves are shown.



Appendix Figure S9. Single-particle cryo-EM image processing workflow and global and local resolution estimations for the RSV A2 DS-Cav1:1D8 complex. Representative 2D classification results, maps after DeepEnhancer sharpening, FSC curves are shown.



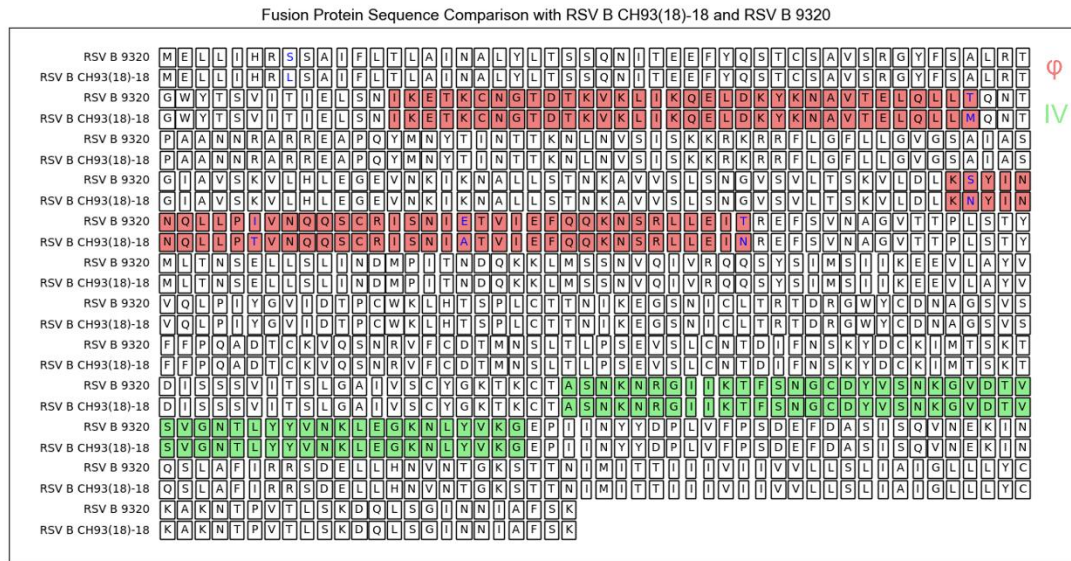
Appendix Figure S10. The comparison of the epitopes of 1G9 (A) and 1D8 (B) with those of six previously reported antibodies. Nirsevimab (site Φ , blue line), AM14 (magenta line), motavizumab (site II, cyan line), RB1 (site IV and V, lime line), hRSV90 (sites II, V, and Φ , purple line), and MPE8 (sites II, III, IV, and V, yellow line). The protomers of the F protein are colored light gray, gray, and dark gray, respectively. The epitopes of the nanobodies are highlighted in the same color as the nanobody they represent.



Appendix Figure S11. Epitope comparison of 1G9 and 1D8 with three other antibodies targeting antigenic site IV. (A-C) Epitope comparison of 1D8 (red) with 1G9 (orange), AM14 (purple, PDB: 7MMN), and MPE8 (yellow, PDB: 5U68). Residues shared across the interfaces are highlighted in blue. (D-E) Epitope comparison of 1G9 (orange) with AM14 (purple, PDB: 7MMN) and MPE8 (yellow, PDB: 5U68). Residues shared across the interfaces are highlighted in blue. (F) The interface of 1D8 (crimson), 1G9 (orange), AM14 (purple), and MPE8 (yellow) on RSV-F was analyzed using PDBePISA. Residues located within the structural pivot region are colored as described above.

	1	10	20	30	40	50	60	70	80	90	100	110	120	128																																														
169	-----																																																											
188	QL	QL	VE	SG	GG	AV	QS	GG	SL	RL	SC	RA	SD	TY	SY	SH	GW	RQ	AP	GR	ER	EG	VA	RI	FS	DG	ST	NY	AS	VE	GR	FT	IS	QD	NA	NT	LY	QD	NS	LP	ED	TA	MY	FC	AR	GG	---	NA	AG	LV	QY	TT	ER	DF	RY	AG	GG	TQ	VT	YS
Consensus	QL	QL	VE	SG	GG	AV	QS	GG	SL	RL	SC	RA	SD	TY	SY	SH	GW	RQ	AP	GR	ER	EG	VA	RI	FS	DG	ST	NY	AS	VE	GR	FT	IS	QD	NA	NT	LY	QD	NS	LP	ED	TA	MY	FC	AR	GG	---	NA	AG	LV	QY	TT	ER	DF	RY	AG	GG	TQ	VT	YS

Appendix Figure S12. The sequencing and alignment analysis of Nbs 1G9 and 1D8.



Appendix Figure S13. Genetic sequencing and alignment analysis of F gene in RSVB [CH93(18)-18] and RSV B9320. The F protein sequence of RSV B9320 was downloaded from GenBank (GenBank: AY353550.1), and the full-length sequence of RSVB [CH93(18)-18] was obtain by DNA sequence (Sangon Biotech (Shanghai) Co., Ltd.). We performed sequence alignment analysis by Python package and represented as color-coded boxes. The red background represents site Ø, the green background represents site IV, mutant amino acid in blue.

Appendix Table S1. Cryo-EM data collection, refinement and validation

	#1 RSV A2 DS Cav1:1G9 (EMD-63216) (PDB: 9LM5)	#2 RSV A2 DS Cav1:1D8 (EMD-63217) (PDB: 9LM6)
Data collection and processing		
Magnification	50,000	50,000
Voltage (keV)	300	300
Pixel size (Å)	0.95	0.95
Defocus	1.0-2.0	1.0-2.0
Symmetry imposed	C3	C3
Map Resolution (Å)	2.77	3.26
FSC threshold	0.143	0.143
Map sharpening B factor (Å ²)	-131.8	-197
Model composition		
Non-hydrogen atoms	13,479	13,350
Protein residues	1,755	1,731
Water	0	0
RMS deviation from ideality		
Bond lengths (Å)	0.005	0.005
Bond angles (°)	0.979	1.041
Validation		
MolProbity score	2.10	2.90
Sidechain outliers	4.70	13.00
Clash score	10.00	14.00
Rotamer outliers (%)	2.49	11.79
Ramachandran statistics		
Favored regions (%)	96.03	93.29
Allowed regions (%)	3.97	6.71
Outlier (%)	0	0

Appendix Table S2. Binding site of nanobodies on RSV F and sequence conservation

The frequency of conserved amino acid residues in the F protein of RSV A and B viruses within the nanobody binding site					
		RSV A (N = 5535)		RSV B (N = 4343)	
Location in F subunit	Amino acid position in F protein	Consensus residue	Conservation rate (%)	Consensus residue	Conservation rate (%)
F2	429	R	100	R	100
	430	G	100	G	100
	431	I	100	I	100
	432	I	99.91	I	99.98
	433	K	100	K	99.95
	*				
	443	S	99.96	S	99.98
	444	N	100	N	100
	445	K	99.96	K	99.98
	446	G	99.98	G	99.98
	*				
	465	K	99.91	K	99.95
	466	S	98.93	N	99.86
	467	L	99.73	L	99.82
	468	Y	100	Y	99.98
	469	V	99.98	V	99.93
	470	K	99.96	K	99.98