

Supplementary Information for:
Molecular mechanism of antibody neutralization of coxsackievirus A16

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Figures S1 –S9

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a

MAb	Heavy chain variable region				Light chain variable region		
	V	D	J	Identity (%)	V	J	Identity (%)
8C4	1-82*01	1-1*01	2*01	95.6	13-85*01	2*01	99.3
9B5	1-22*01	2-4*01	4*01	92.2	12-46*01	4*01	96.8
9G1	14-4*02	2-4*01	1*01	100	8-24*01	1*01	94.3

b

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                20          40
                |          |
            CDR1
8C4-VH  QVQLQQSGPELVKPGASVKISCKASGYAFSTSWMNWV I QRPQGQGLEWIGR 50
9B5-VH  E . . . . . M . . . T . . . T . TENT . H . . R . SH . KS . . . . . G 50

        60          80          100
        |          |          |
        CDR2
8C4-VH  IYPGDGDTNYNGKFKGKATLTADKSSSTAYMQLSSLTSVDSAVYFCARRD 100
9B5-VH  . . . KND . . K . . Q . . . . . V . . . . . C . E . R . . . . E . . . . Y . . . G . 100

        120
        |
        CDR3
8C4-VH  Y - GYF - - - DYWGQGTTLT VSS 117
9B5-VH  . EN . . YAM . . . . . SV . . . . 121

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c

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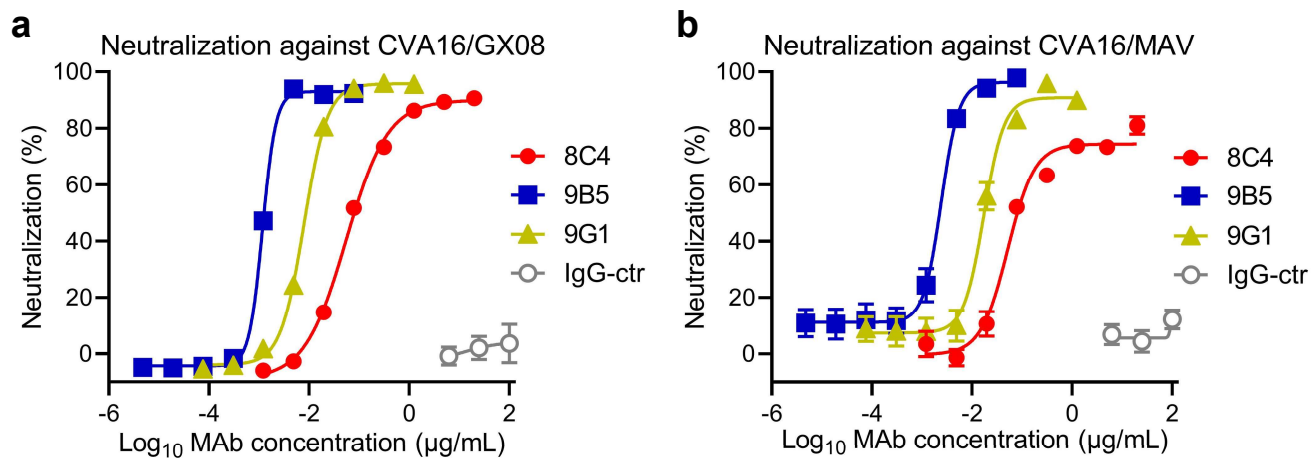
                20          40
                |          |
            CDR1
8C4-VL  DIQMTQSSSYLSVSLGGRVTITCKASDHINNWLAWYQQKPGNAPRLLISG 50
9B5-VL  . . . . . PAS . . . . V . ET . . . . . R . . EN . YSN . . . . . Q . KS . Q . . VYA 50

        60          80          100
        |          |          |
        CDR2          CDR3
8C4-VL  ATSLETGVPSRFSGSGSGKDYTLSTLSLQTEDVATYYCQQYWNSPYTFGG 100
9B5-VL  . . N . AD . . . . . TQ . S . K . N . . . S . . FG . . . . . F . DT . F . . . S 100

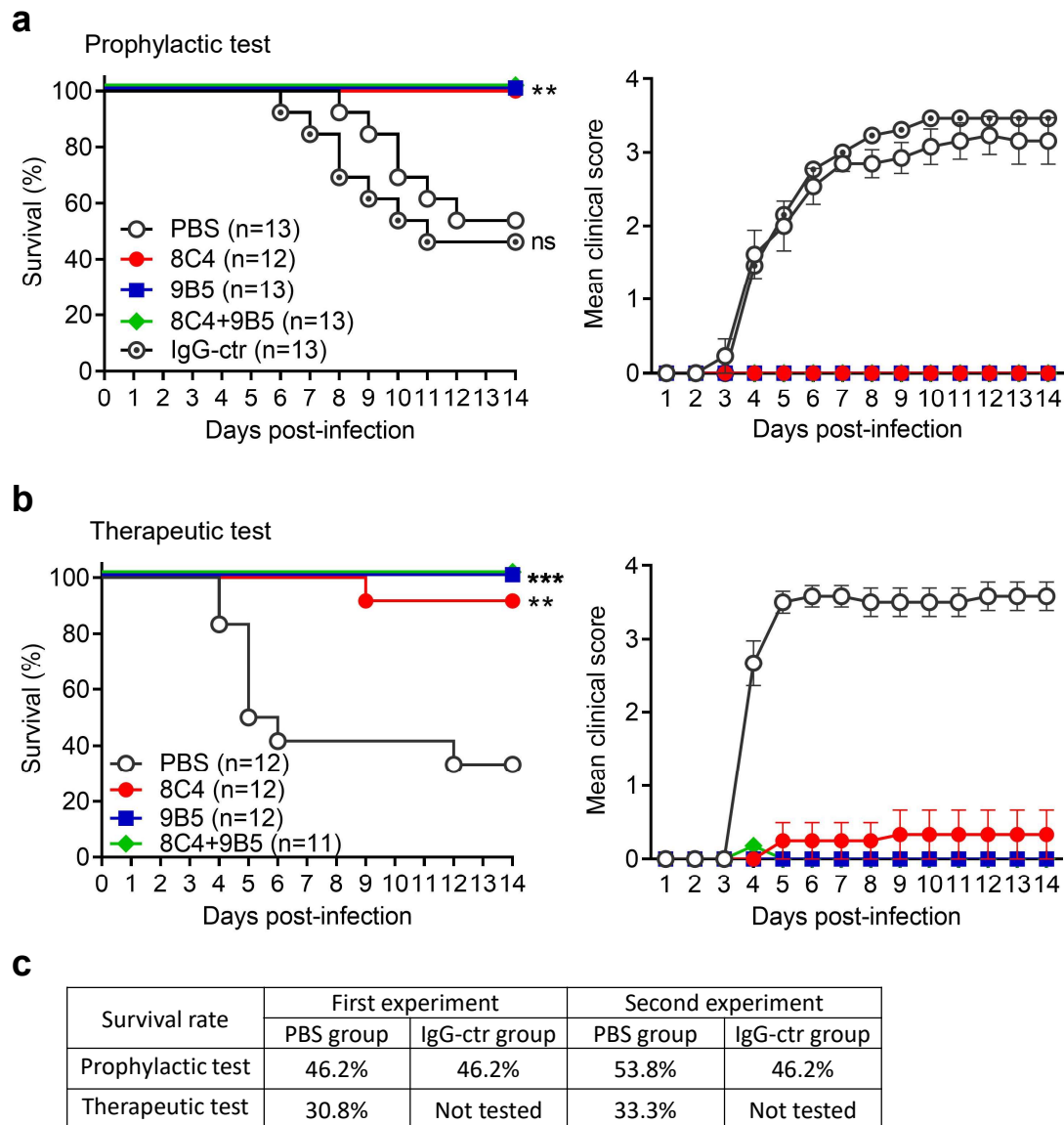
8C4-VL  GTKLEIK 107
9B5-VL  . . . . A . . 107

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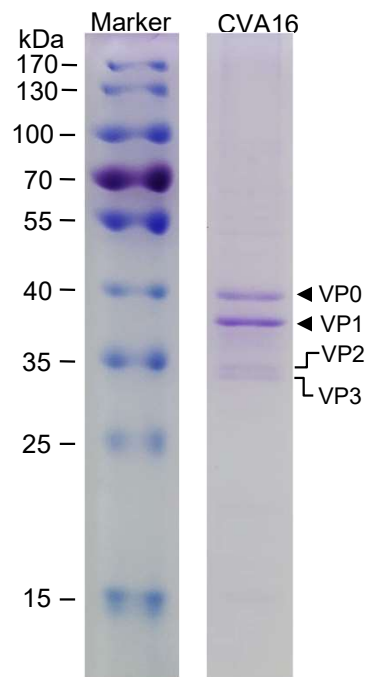
Supplementary Figure 1. Sequence analysis of the anti-CVA16 MAbs. **(a)** The closest mouse germline genes were identified using NCBI IgBLAST. The percentage identity of nucleotide sequences of variable regions was also shown. **(b-c)** Amino acid sequences of heavy-chain variable regions (VH) **(b)** and light-chain variable regions (VL) **(c)** of the 8C4 and 9B5 antibodies. Dots represent residues identical to those of antibody 8C4, and red dashes are gaps. Locations of complementarity determining regions (CDR) were obtained from NCBI IgBLAST.



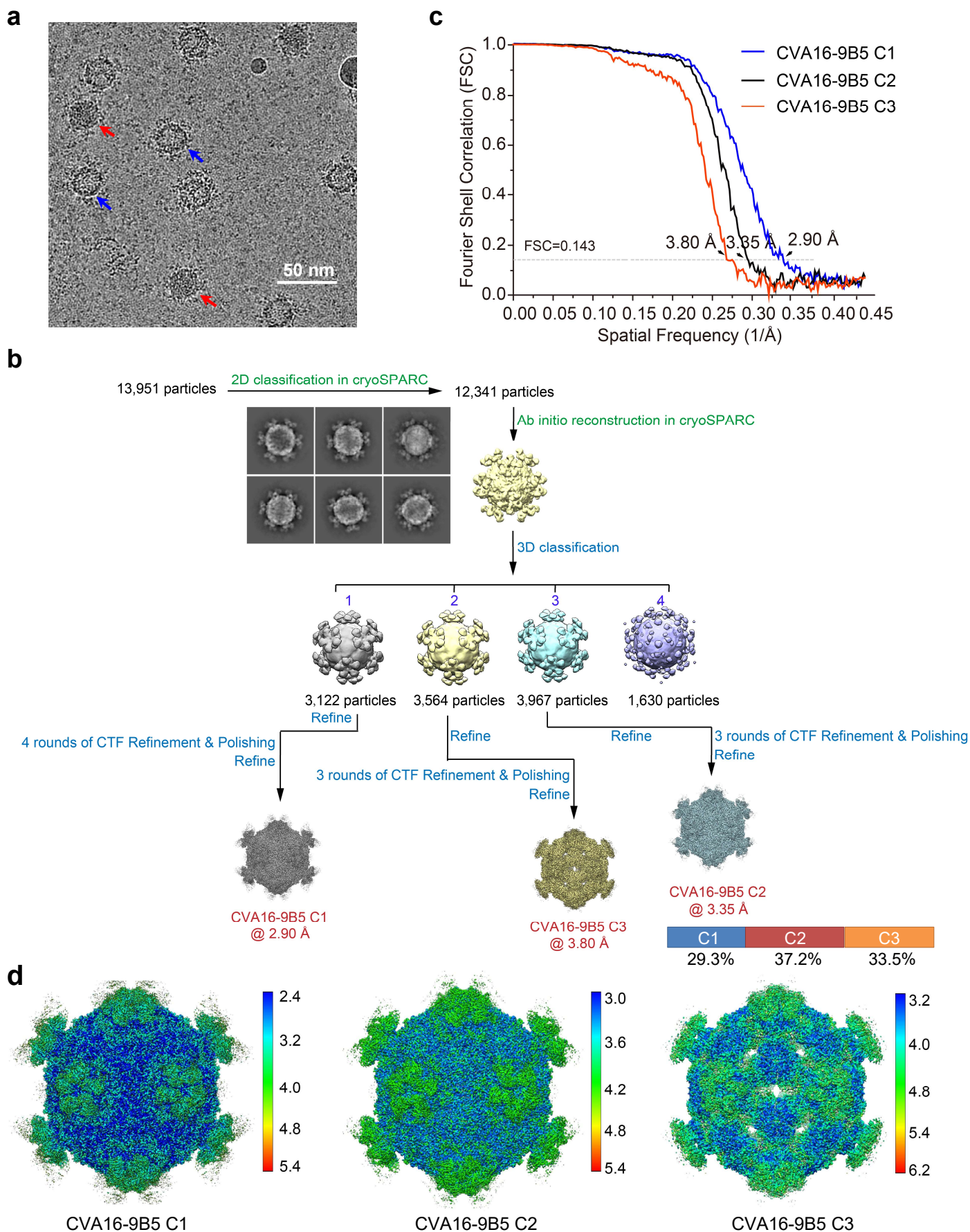
Supplementary Figure 2. Neutralization of the MAbs against CVA16 strains GX08 **(a)** and MAV **(b)** was measured by the cell viability assay. Related to Figure 1A. IgG-ctr, anti-SARS-CoV-2 MAb 2H2. Data are mean $\pm$ SEM of five replicate wells in 96-well cell culture plates.



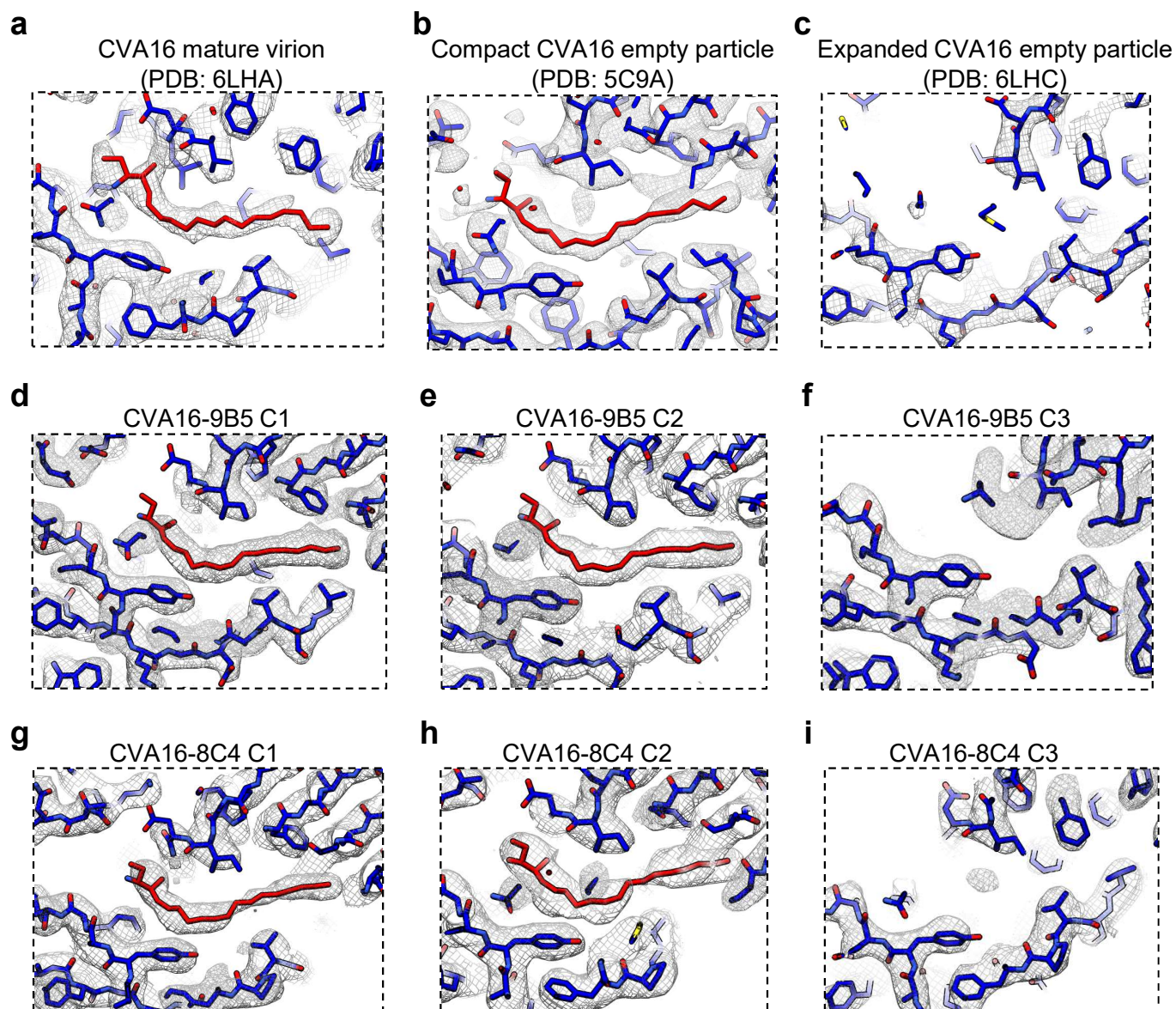
Supplementary Figure 3. In vivo prophylactic efficacy (**a**) and therapeutic efficacy (**b**) of MAbs 8C4, 9B5 and the 8C4+9B5 cocktail against CVA16 infection in mice. Results of two independent experiments are shown in Figure 2 and Supplementary Figure 3, respectively. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, limb paralysis; 4, death. The number of mice in each group were indicated in the bracket. Survival rates of MAb-treated mice were compared with the mice in the PBS-treated group. Statistical significance was determined by Log-rank (Mantel-Cox) test. ns, no significant difference ($p \geq 0.05$); **, $p < 0.01$; ***, $p < 0.001$. For panel **a**, p value between the PBS group and the 8C4 group is 0.0082; p value between the PBS group and the 9B5 group is 0.0060; p value between the PBS group and the 8C4+9B5 group is 0.0060. For panel **b**, p value between the PBS group and the 8C4 group is 0.0025; p value between the PBS group and the 9B5 group is 0.0006; p value between the PBS group and the 8C4+9B5 group is 0.0010. All error bars represent SEM. (**c**) The survival rates of mice in PBS and IgG-ctr groups.



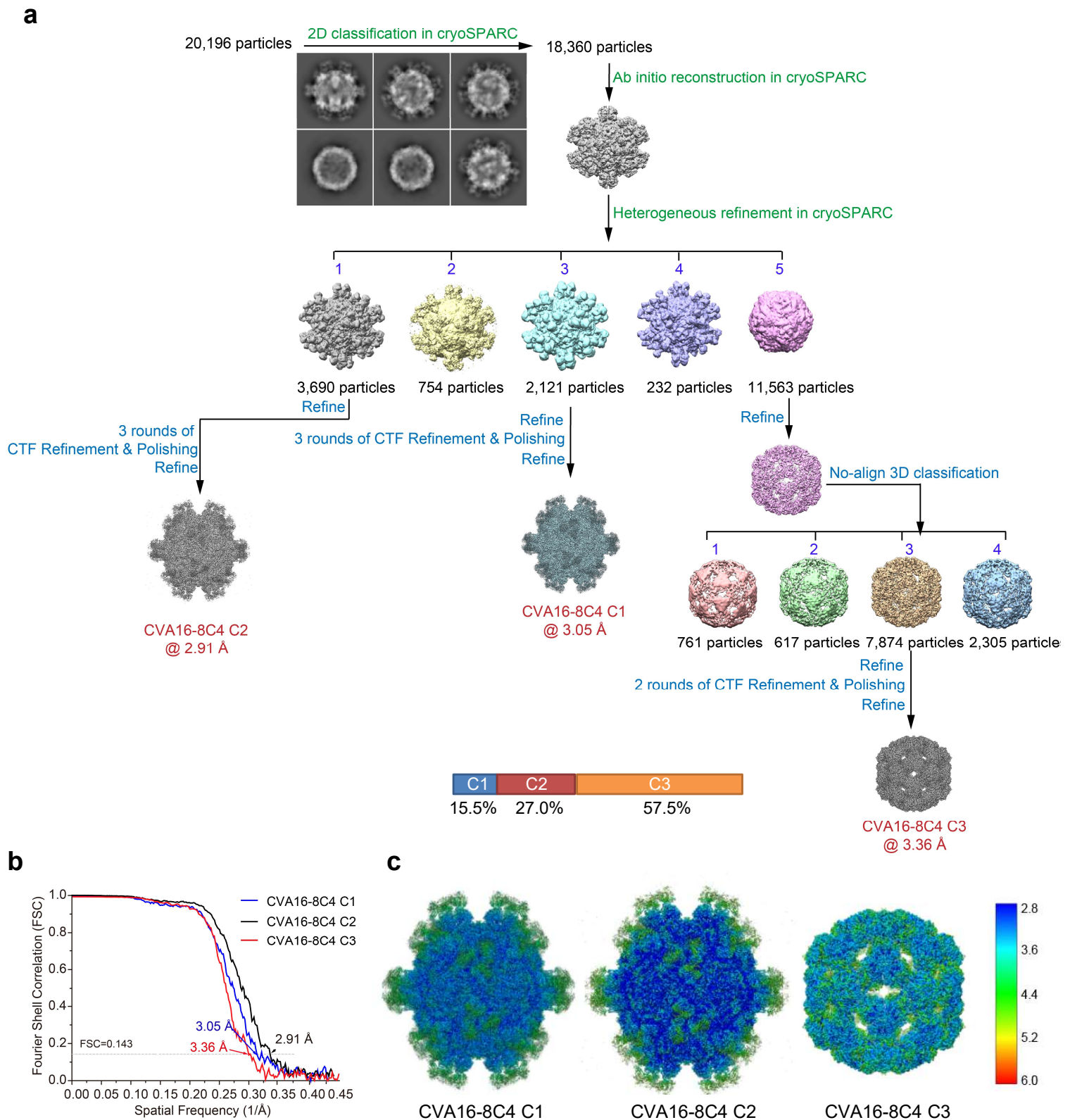
Supplementary Figure 4. SDS-PAGE analysis of purified CVA16. Note that the content of VP0 protein was far more than that of VP2 protein. Only one batch of CVA16 viral particles was prepared and purified.



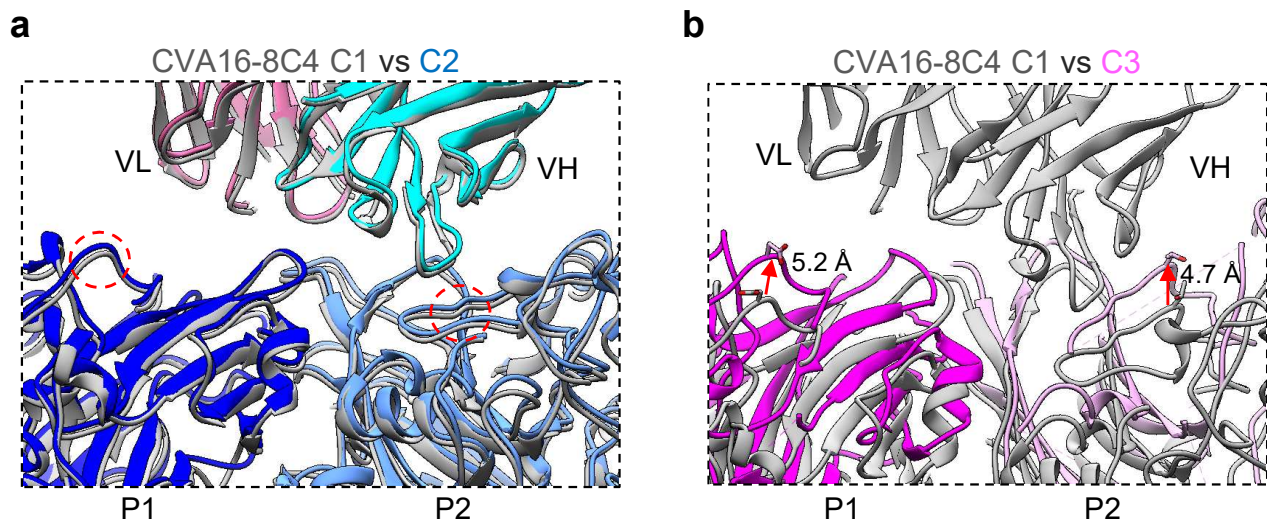
Supplementary Figure 5. Cryo-EM structural analysis of the CVA16–9B5 complex. **(a)** A representative cryo-EM image of the CVA16–9B5 complex. Red and blue arrows indicate full and empty CVA16 viral particles engaged by 9B5 Fabs, respectively. Two independent experiments were performed, with similar results. **(b)** Data processing procedure for the CVA16–9B5 dataset. Population distribution of CVA16–9B5 C1 to C3 is also shown. **(c)** Resolution estimation of the CVA16–9B5 C1 to C3 maps according to the gold-standard FSC criterion of 0.143. **(d)** Local resolution evaluations for the CVA16–9B5 C1 to C3 maps.



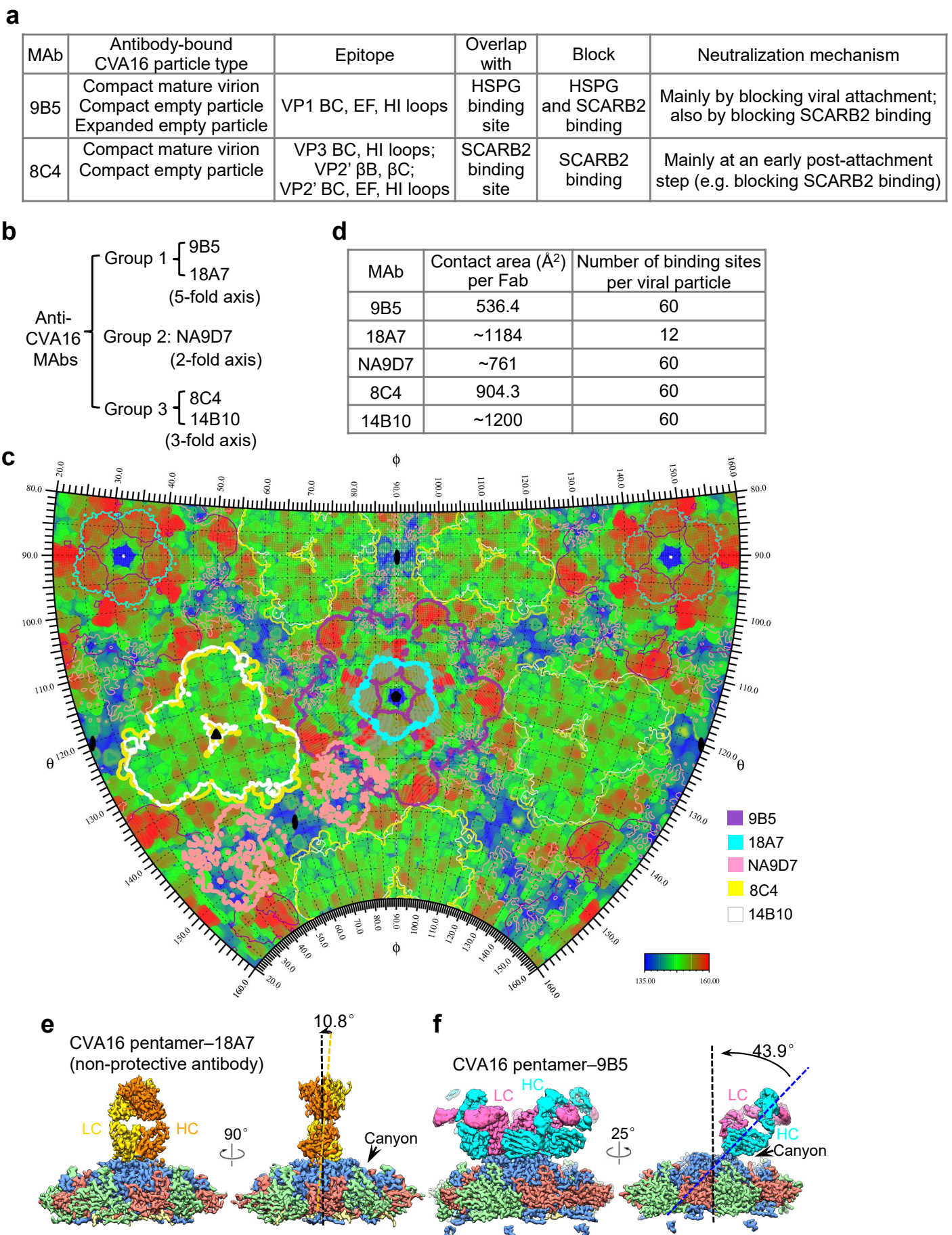
Supplementary Figure 6. Representative density maps of the VP1 pocket region in the CVA16 mature virion (**a**), compact empty particle (**b**), expanded empty particle (**c**), CVA16-9B5 C1 to C3 (**d-f**), and CVA16-8C4 C1 to C3 (**g-i**). Density is shown in gray mesh. The atomic models for VP1 and pocket factor are shown as blue and red sticks, respectively.



Supplementary Figure 7. Cryo-EM structural analysis of the CVA16–8C4 complex. **(a)** Data processing procedure for the CVA16–8C4 dataset. Population distribution of CVA16–8C4 C1 to C3 is also shown. **(b)** Resolution estimation of the CVA16–8C4 C1 to C3 maps according to the gold-standard FSC criterion of 0.143. **(c)** Local resolution evaluations for the CVA16–8C4 C1 to C3 maps.



Supplementary Figure 8. Structural basis of antibody 8C4 binding to CVA16 capsid in the C2 structure but not in C3. **(a)** Superposition of the CVA16-8C4 C1 (gray) and C2 (color) structures. The red circles indicate slight movements of the capsid in the C2 structure relative to the C1 structure. **(b)** Docking of the C3 structure (magenta) into the C1 structure (gray). The red arrow represents the movement direction of the capsid in the C3 structure relative to the C1 structure.



Supplementary Figure 9. Summary of information regarding the reported CVA16 MAbs with resolved structures. **(a)** Summary of epitopes and neutralization mechanisms of MAbs 8C4 and 9B5. **(b)** Grouping of anti-CVA16 MAbs. Antibody binding regions are shown in brackets. **(c)** Roadmap showing the footprints of the Fabs on the CVA16 virion surface. The 9B5, 18A7, NA9D7, 8C4, and 14B10 Fabs are indicated by purple, cyan, pink, yellow, and white contour lines, respectively. Surface area of CVA16 viral capsid covered by each Fab was also shown. **(d)** The surface area of contact between each Fab and CVA16 viral capsid.

(e) Cryo-EM densities of 5-fold vertex of CVA16 mature virion and a single bound 18A7 Fab. The angle between the long axis (gold dashed line) of the 18A7 Fab and the 5-fold axis (black dashed line) of viral capsid is shown. VP1, VP2, VP3, and VP4 are colored in cornflower blue, light green, salmon, and khaki, respectively. HC and LC of 18A7 are colored in orange and gold, respectively. (f) Cryo-EM densities of 5-fold vertex of CVA16 and five bound 9B5 Fabs. On the right panel, the densities of four 9B5 Fabs are removed for clarity. The angle between the long axis (blue dashed line) of the 9B5 Fab and the 5-fold axis (black dashed line) of viral capsid is shown.

Supplementary Table 1. A summary of all the CVA16 strains used in this study.

CVA16 strains	Genotype	Genbank ID
CVA16/SZ05 ^a	B1b	EU262658
CVA16/GX08	B1b	KC342228
CVA16/MAV	B1a	KC695830

^a used as the antigen for mouse immunization.

Supplementary Table 2. Cryo-EM data collection and refinement statistics.

	CVA16–9B5			CVA16–8C4		
Data collection and processing						
Magnification	64,000			64,000		
Voltage (kV)	300			300		
Detector	K3			K3		
Electron exposure (e ⁻ /Å ²)	38			50		
Defocus range (μm)	-0.8 to -2.5			-0.8 to 2.5		
Pixel size (Å)	1.1			1.1		
Symmetry imposed	I			I		
Structures	C1	C2	C3	C1	C2	C3
Initial particle images (no.)	13,951	13,951	13,951	20,196	20,196	20,196
Final particle images (no.)	3,122	3,967	3,564	2,121	3,690	7,874
Map resolution (Å)	2.90	3.35	3.80	3.05	2.91	3.36
FSC threshold						
Map resolution range (Å)	2.4-4.0	3.0-4.2	3.2-4.8	2.6-4.4	2.6-4.4	2.8-4.4
Refinement						
Initial model used (PDB code)	5C4W	5C9A	6LHC	5C4W	5C9A	6LHC
Model resolution (Å)	3.0	3.2	3.7	3.5	3.1	3.4
FSC threshold						
Map sharpening <i>B</i> factor (Å ²)	-60.7	-73.6	-88.4	-78.3	-69.3	-98.0
Model composition						
Non-hydrogen atoms	9,720	9,091	7,822	9,720	9,049	4,477
Protein residues	1,258	1,174	1,010	1,258	1167	572
Ligands	1	1		1	1	
<i>B</i> factors (Å ²)						
Protein	73.9	85.0	108.72	55.40	67.80	74.23
Ligand	31.2	42.3		33.81	33.81	
R.m.s. deviations						
Bond lengths (Å)	0.007	0.008	0.006	0.011	0.010	0.003
Bond angles (°)	0.919	0.976	0.952	0.761	0.755	0.709
Validation						
MolProbity score	1.62	1.72	1.65	1.54	1.40	1.40
Clashscore	4.70	5.98	6.08	4.77	3.76	3.70
Poor rotamers (%)	0.65	0.59	0.34	0.14	0.20	0.00
Ramachandran plot						
Favored (%)	94.45	94.14	95.43	95.78	96.45	96.38
Allowed (%)	5.47	5.26	4.57	4.22	3.55	3.62
Disallowed (%)	0.08	0.60	0.00	0.00	0.00	0.00

Supplementary Table 3. RMSD values between the capsid protomers of different CVA16 structures.

RMSD (Å)	CVA16– 9B5 C1	CVA16– 9B5 C2	CVA16– 9B5 C3	CVA16– 8C4 C1	CVA16– 8C4 C2	CVA16– 8C4 C3
Mature CVA16 virion (PDB: 5C4W)	1.051	1.204	2.310	1.098	1.212	2.297
Compact CVA16 empty particle (PDB: 5C9A)	1.212	1.162	2.313	1.194	1.178	2.264
Expanded CVA16 empty particle (PDB: 6LHC)	2.314	2.428	1.240	2.294	2.258	0.937

Supplementary Table 4. Interaction interface analysis of the CVA16–9B5 C1 structure (upper table) and conservation analysis of the contacting residues (lower table).

CVA16		Distance (Å)	9B5		Interaction
Residue	Location		Residue	Location	
G99 [N]	VP1 BC loop	3.02	N103 [OD1]	HCDR3	H-bond
G99 [O]	VP1 BC loop	3.07	Y104 [N]	HCDR3	H-bond
D104 [OD2]	VP1 BC loop	2.73	K59 [NZ]	HFR3	H-bond, salt bridge
D104 [OD1]	VP1 BC loop	3.62	K59 [NZ]	HFR3	Salt bridge
R166 [NH2]	VP1 EF loop	2.90	D93 [OD2]	LCDR3	H-bond, salt bridge
R166 [NH1]	VP1 EF loop	3.96	D93 [OD1]	LCDR3	Salt bridge
K242 [NZ]	VP1 HI loop	2.84	W92 [O]	LCDR3	H-bond

9B5-binding residues		Number of sequences	Conservation
VP1 (1127 sequences) ^a	G99	1122	99.6%
	D104	1105	98.0%
	R166	1127	100%
	K242	1126	99.9%

^a Complete or near-complete CVA16 capsid protein sequences were obtained from NCBI database and used for conservation analysis.

Supplementary Table 5. Surface area of CVA16 viral capsid covered by MAb 9B5 and 8C4 (in C1 structure) determined using PISA.

MAb	Interface area (Å ²)	
	Total	Contribution
9B5-VH	275.0	51.3%
9B5-VL	261.4	48.7%
9B5	536.4	100.0%
8C4-VH	508.1	56.2%
8C4-VL	396.2	43.8%
8C4	904.3	100.0%

Supplementary Table 6. Interaction interface analysis of the CVA16–8C4 C1 structure (upper table) and C2 structure (middle table) and conservation analysis of the contacting residues (lower table).

C1 structure	CVA16		Distance (Å)	8C4		Interaction
	Residue	Location		Residue	Location	
Protomer 1	S77 [O]	VP3 BC loop	3.17	R99 [NH2]	HCDR3	H-bond
	S77 [O]	VP3 BC loop	3.32	R99 [NH1]	HCDR3	H-bond
	T210 [OG1]	VP3 HI loop	3.07	N30 [OD1]	LCDR1	H-bond
Protomer 2	S230 [OG]	VP2 HI loop	3.28	W92 [O]	LCDR3	H-bond
	G227 [O]	VP2 HI loop	3.07	W92 [NE1]	LCDR3	H-bond
	A228 [O]	VP2 HI loop	3.43	W92 [NE1]	LCDR3	H-bond
	K69 [NZ]	VP2 βB	2.73	D55 [OD2]	HCDR2	H-bond, salt bridge
	W78 [NE1] ^a	VP2 βC	3.55	D57 [OD2]	HCDR2	H-bond
	V159 [N]	VP2 EF loop	3.49	D55 [O]	HCDR2	H-bond
	D74 [OD1]	VP2 BC loop	3.73	Y60 [N]	HFR3	H-bond
	D74 [OD1]	VP2 BC loop	3.60	N59 [ND2]	HFR3	H-bond
	D74 [OD2]	VP2 BC loop	3.72	N59 [ND2]	HFR3	H-bond
	E231 [OE1] ^a	VP2 HI loop	3.22	R99 [NH2]	HCDR3	H-bond, salt bridge

C2 structure	CVA16		Distance (Å)	8C4		Interaction
	Residue	Location		Residue	Location	
Protomer 1	S77 [O]	VP3 BC loop	3.61	R99 [NH2]	HCDR3	H-bond
	S77 [O]	VP3 BC loop	3.51	R99 [NH1]	HCDR3	H-bond
	T210 [OG1]	VP3 HI loop	2.83	N30 [ND2]	LCDR1	H-bond
Protomer 2	S230 [OG]	VP2 HI loop	2.82	W92 [O]	LCDR3	H-bond
	S230 [N]	VP2 HI loop	3.55	N93 [OD1]	LCDR3	H-bond
	G227 [O]	VP2 HI loop	3.28	W92 [NE1]	LCDR3	H-bond
	A228 [O]	VP2 HI loop	3.49	W92 [NE1]	LCDR3	H-bond
	K69 [NZ]	VP2 βB	2.70	D55 [OD2]	HCDR2	H-bond, salt bridge
	V159 [N]	VP2 EF loop	3.58	D55 [O]	HCDR2	H-bond
	D74 [OD1]	VP2 BC loop	2.64	Y60 [N]	HFR3	H-bond
	D74 [OD1]	VP2 BC loop	2.87	N59 [ND2]	HFR3	H-bond

8C4-binding residues		Number of sequences	Conservation
VP2 (399 sequences) ^b	K69 ^c	398	99.7%
	D74	397	99.5%
	W78	399	100%
	V159 ^c	397	99.5%
	G227	399	100%
	A228	399	100%
	S230	399	100%
	E231	399	100%
VP3 (191 sequences) ^b	S77	191	100%
	T210	191	100%

^a The interaction exists in the C1 structure, but not in the C2 structure.

^b Complete or near-complete CVA16 capsid protein sequences were obtained from NCBI database and used for conservation analysis.

^c The residues mutated in the neutralization escape mutants.