Supplemental Data

Epitope-focused immunogens against the CD4-binding site of HIV-1 envelope protein induce neutralizing antibodies against auto- and heterologous viruses

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Supplemental Methods

Cell lines, plasmids, antibodies and Env proteins

The yeast surface display system including the *Saccharomyces cerevisiae* EBY100 strain and pCTCON2 vector were originally obtained from Dr. K. Dane Wittrup and Annie Gai (Massachusetts Institute of Technology, USA). A modified version of pCTCON2 with T overhang (pCTCON2-T) was generated in our laboratory for the construction of a combinatorial antigen fragment library derived from HIV-1 envelope in China. The Sf9 cell line and pFastBac^{TM} Dual vector for the production of recombinant proteins were kindly provided by Dr. Xinquan Wang (Tsinghua University, China). The TZM-bl cell line for measuring neutralizing activity of the immune sera and monoclonal antibodies (mAbs) was obtained from the AIDS Research and Reference Program at National Institutes of Health (NIH) of USA. The Human Ramos B cell line for studying antibody mediated BCR clustering and signaling was a gift from Dr. Susan K. Pierce at NIH of USA. Gcamp5 gene was gifted by Dr. Chenqi Xu (Chinese Academy of Sciences, CAS, China). The genes encoding the variable regions of heavy and light chains of mAbs VRC01, 447-52D, 19b and 39F were kindly provided by Dr. John Mascola at NIH of USA and the remaining mAbs 12A12, VRC-CH31, 3BNC60, NIH45-46, VRC-PG04, VRC-PG20, CH103, b12, PGT121, PGT128, PGT135 and PG9 were synthesized according to the published sequences (Invitrogen). All these HIV-1 mAbs were produced in our laboratory by transfection of respective expression plasmids into either 293T or 293F cell lines (Invitrogen). Recombinant and purified mAb 3BNC117 was a gift from Dr. Michel C. Nussenzweig at The Rockefeller University in New York, USA. Recombinant protein gp120 derived from HXB2 and CM235 were purchased from Immune Technology company (Suzhou, China) and produced in 293T cell line. Recombinant protein gp120 derived from CNE54 and CNE55 were produced in our laboratory by Bac-to-Bac Baculovirus Expression System. Recombinant trimeric gp140 of NL4-3 was constructed based on the original BG505 SOSIP.664 gp140 trimer framework, and kindly provided by Dr. Yi Shi (CAS, China). RSC3 and RSC3Δ371I proteins were obtained from Drs. Peter Kwong and Tongqing Zhou at Vaccine Research Center of NIH, USA.

Production of Fab and the complex of EAD-VRC01 and VRC01 Fab

Antigen-binding fragments (Fab) of mAbs were produced by protease digestion of full-length antibodies using human rhinovirus (HRV) 3C or Lys-C (Sigma). Specifically, digestion was conducted by mixing 2 mg of each antibody with either HRV 3C protease (2 U/μl) in 1 ml phosphate buffer saline (PBS, pH 7.4) at room temperature (RT) for 2 hrs or with Lys-C protease at a ratio mAb:Lys-C=4000:1 (w/w) in 1 ml Tris-EDTA buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.5) at 37°C for 12 hrs. The digestion mixtures were purified by a Protein A agarose (Pierce) followed by a Superdex 200 column (GE Healthcare).

For generation of the trimeric EAD-VRC01 and VRC01 Fab complex, two milligrams of VRC01 were bound to 500 μl Protein A agarose in 2 ml PBS at 37°C for 1 hr. After extensive washing with PBS, the resin was incubated with 10 mg purified EAD-VRC01 V51tri in 3-fold molar excess over VRC01 in 2 ml PBS at 37°C for 1 hr. The unbound proteins were removed by PBS and the complex of V51tri and VRC01 was digested by HRV 3C protease in 2 ml PBS at RT for 1 hr. The complex of V51tri and VRC01 Fab in the eluate was further incubated with 10 mg VRC01 Fab at 37°C for 1 hr to maximize formation of V51tri-VRC01 Fab complex. The mixture was then purified by a Superdex 200 column and fractions corresponding to the V51tri-VRC01 Fab complex were analyzed and pooled.

Electron microscopy, image reconstruction and model fitting

V51tri and V51tri-VRC01 Fab complex particles were boxed using e2boxer.py of the EMAN2 software package. The particles were extracted with RELION (1) using the box coordinates from e2boxer.py. Several rounds of 2D and 3D classifications were performed using RELION. Aberrant particles were removed and

the remaining good particles were then used for 3D auto-refinements with 3-fold symmetry imposed. The structure model of the V51tri was generated using the crystal structures of the BG505 SOSIP.664 gp140 trimer (PDB: 4NCO). Since the V3 loop has been truncated in the gp120-VRC01 Fab crystal structure (PDB: 3NGB), the V51-VRC01 Fab complex model was generated as a chimera with the V51 fragment from the crystal structure of the BG505 SOSIP.664 gp140 trimer (PDB: 4NCO) and the VRC01 Fab from the crystal structure of the gp120-VRC01 Fab complex (PDB: 3NGB). The chimeric model of the V51-VRC01 Fab complex was fitted separately into each subunit of reconstructed map (cross correlation: 0.931). The V51tri-VRC01 Fab complex map has distinct features that allow unambiguous fitting of the model into the map. Three monomeric V51 models were then fitted into the V51tri map using the fitted V51tri-VRC01 Fab model as a reference (cross correlation: 0.937). The C-terminus of the fitted V51 model has a reasonable distance to the foldon tag that was added at the C-terminus of the recombinant V51 protein for trimerization, indicating the correctness of the fitting. The V51 equivalent trimer was truncated from BG505 SOSIP.664 gp140 trimer (PDB: 4NCO) and used to generate a 20Å map using EMAN2 e2pdb2mrc.py package. The EM maps of V51 equivalent trimer and V51 trimer were merged by 'fit in map' function in UCSF Chimera software.

Triggering VRC01-BCR signalings by EAD-VRC01

The Ramos B cell line was constructed to express VRC01-BCR on the cell membrane by lentiviral infection. The construction steps were initiated by the selection of cells without endogenous expression of any BCR through several rounds of FACS-based sorting (2). Once BCR-negative cells were obtained, the stable Ramos cells expressing VRC01-BCR heavy and light chains (Ramos-VRC01) were generated by the lentiviral infection (pHAGE-VRC01-BCR, psPAX2 and pMD2.G) followed by FACS-based sorting. The stable Ramos-VRC01 cells expressing calcium indicator Gcamp5 (Ramos-VRC01-Gcamp5) were subsequently generated by the additional round of lentiviral infection (pHAGE-Gcamp5, psPAX2 and pMD2.G) followed by several rounds of FACS-based sorting.

The EAD-VRC01 triggering VRC01-BCR signalings was conducted following our well-established protocols. Specifically, planar fluid lipid bilayers (PLBs) with the EAD-VRC01 were prepared with relevant controls such as P01 (a EAD-PG9 from the same library as a negative control), the resurfaced stabilized core 3 (RSC3), RSC3Δ371I (lacked a single amino acid in RCS3 at position 371 that disrupted VRC01 binding), HXB2 gp120 (clade B), CNE54 gp120 (clade CRF08_BC) and CNE55 gp120 (clade CRF01_AE). The EAD-VRC01 recombinant glycoprotein with the 6×histidine tag was attached to the Ni-NTA-containing PLBs for 30 min at 37°C. Ramos-VRC01 B cells were stained with the Fab anti-human Fc fragment conjugated with Alexa Fluor 647 (Jackson ImmunoResearch Laboratory) and then loaded to the prepared chamber with appropriate antigens on the PLBs. The cells were allowed to react for 10 min at 37°C, fixed by 4% paraformaldehyde (PFA), and imaged by an Olympus IX-81 total internal reflection fluorescence microscopy (TIRFM). TIRFM was supported by a TIRF port, Andor iXon+ DU-897D electron-multiplying CCD camera and Olympus 100×1.49 N.A. objective lens. The exposure time for 512×512 pixels TIRFM image was 100 ms. The contact area with antigen containing surface and total fluorescence intensity (TFI) of accumulated BCRs recruited to the immunological synapse were analyzed by Image J (NIH, USA) as described previously (3). Intracellular immunofluorescence staining of the pSyk and total pTyr of downstream signaling molecules was performed in activated the Ramos-VRC01 B cells according to our published protocols. Specifically, after fixation with 4% PFA for 30 min at RT, the Ramos-VRC01 B cells were permeabilized with 0.2% Triton X-100 for 20 min. Subsequently, the cells were blocked with 100 μg/ml the goat non-specific IgG (Jackson Immuno Research Laboratory) for 1 hr at RT. The Ramos-VRC01 cells were then incubated with the anti-phospho-Syk (pY525/526) mAb (Cell Signaling) or the FITC conjugated anti-phosphotyrosine antibody (Millipore) at 37 °C for 1 hr. After extensive washing, the secondary antibody Alexa Fluor 568 conjugated goat $F(ab)_2$ anti-rabbit IgG (Invitrogen) was used for pSyk staining as previously reported. TIRFM Images were analyzed with Image J (NIH, USA) following our previous protocols.

ELISA and competitive ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to define the binding activities of mAbs or sera against various antigens, including the autologous EAD-VRC01 trimer immunogens (V61tri, V51tri, V17tri, clade B), HXB2 gp120 (clade B), NL4-3 SOSIP.664 gp140 trimer (clade B), RSC3 (clade B), CM235 gp120 (clade AE), CNE55 gp120 (clade CRF01_AE) and CNE54 gp120 (clade CRF08_BC). 96 well plates were coated with 2 μg/ml of each antigen in PBS at 4°C overnight. After blocking with 2% BSA in PBS, guinea pig sera were serially diluted in blocking buffer and incubated at 37°C for 1 hr. After thoroughly washing three times with PBST (PBS with 0.05% Tween 20), the secondary goat anti-guinea pig antibody conjugated with horseradish peroxidase (HRP) (Invitrogen) was added before applying the 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Fisher Scientific) substrate for detection. Optical density at 450 nm (OD450) and corresponding plasma dilutions were recorded (Microplate Reader, Bio-Rad). ELISA endpoint titers of the sera were determined at the dilution where OD450 was three-fold higher than the negative control animal sera.

To study antibody specificity of the immunized animals, competitive ELISA was conducted against various biotinylated mAbs with known epitopes. Specifically, all sera samples were 1:100 diluted and mixed with a serial dilution of biotinylated mAbs before applying to ELISA plate coated with EAD-VRC01 V61tri. After three thorough washes with PBST, the HRP-conjugated streptavidin (Thermo Fisher Scientific) was added before applying TMB substrate for detection. The binding titers of biotinylated mAbs were compared in the presence of competitor or the negative control sera. The competitive ability of sera was defined as the residual binding percentage= [(the titer in the presence of the negative control sample)/ (the titer in the presence of competitor) x 100]. The negative control was derived from the sera samples from the NC group. Biotin-XX human IgG labeling kit (Invitrogen) was used to biotinylate the mAbs.

Supplemental References

1. Scheres, S. H. (2012) RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J Struct Biol* **180**, 519-530
2. Lingwood, D., McTamney, P. M., Yassine, H. M., Whittle, J. R., Guo, X., Boyington, J. C., Wei, C. J., and Nabel, G. J. (2012) Structural and genetic basis for development of broadly neutralizing influenza antibodies. *Nature* **489**, 566-570
3. Lakadamyali, M., Rust, M. J., and Zhuang, X. (2004) Endocytosis of influenza viruses. *Microbes Infect* **6**, 929-936

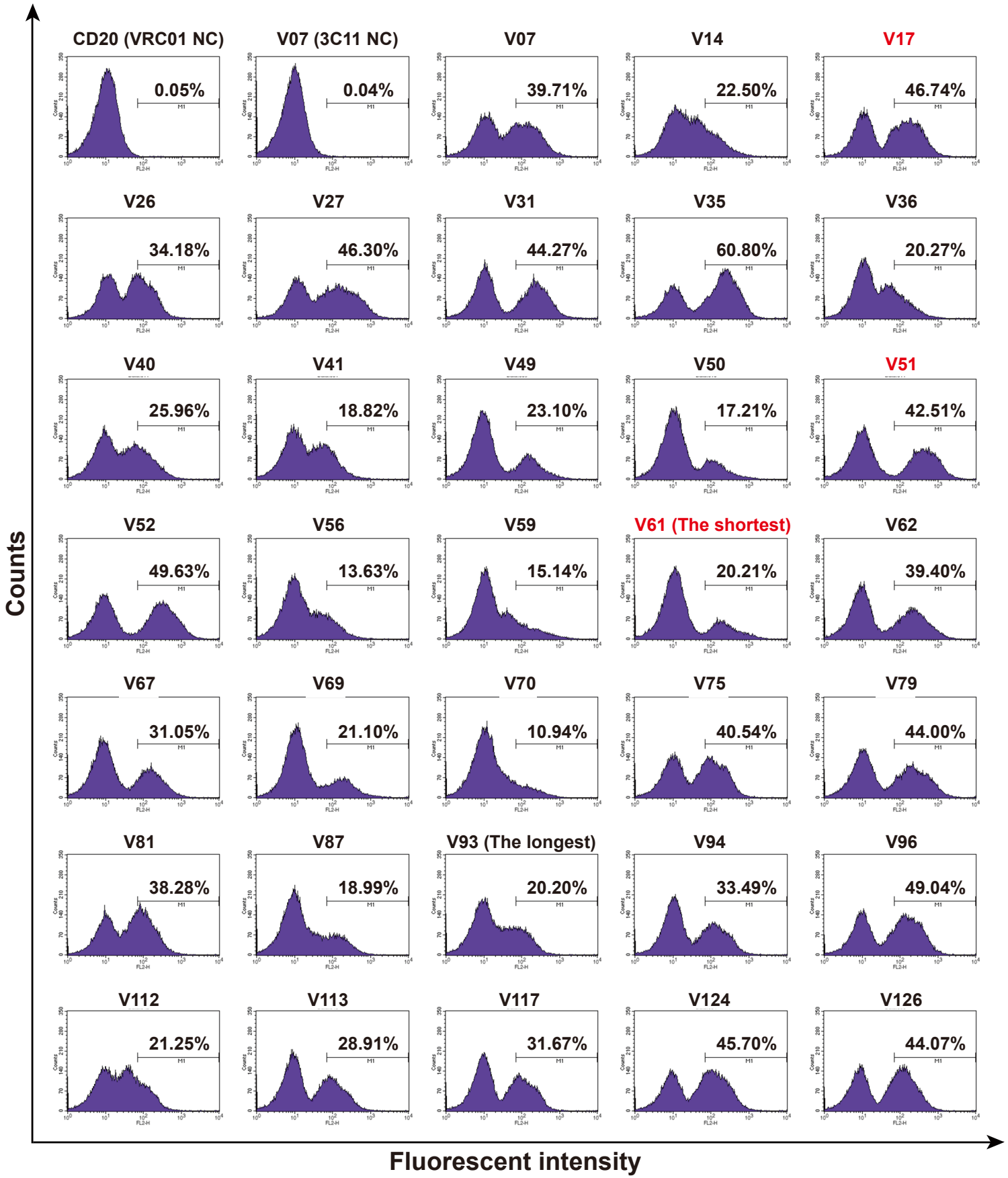


Figure S1. Detection and confirmation of the yeast clones expressing EAD-VRC01.

The yeast clones expressing each different EAD-VRC01 were further verified by staining with VRC01 and FACS analysis. The percentage in each panel indicates the proportion of positive clones among the total yeast clones analyzed. The yeast clones expressing an irrelevant antigen CD20 or expressing EAD-VRC01 but stained with an irrelevant mAb 3C11 were used as negative controls. The V17, V51 and V61 clones were highlighted in red and further selected to express the corresponding EAD-VRC01 in Sf9 cells using the Bac-to-Bac baculovirus expression system.

Figure S2. Sequence alignment of 33 EAD-VRC01 with reference sequences.

The sequence alignment of 33 EAD-VRC01 together with their consensus (con.) and corresponding sequences from CNE11 and HXB2. The contact residues with VRC01 previously identified through structural analysis were indicated with the symbol (○) for main-chain only contacts, (¤) for side-chain only contacts, and (●) for both main- and side-chain contacts. Those colored in yellow were located outside the selected EAD-VRC01 fragments, in purple among the consensus EAD-VRC01 fragment and in dark green in the shortest EAD-VRC01 V61 fragment. Potential glycosylation sites with the signature sequence NXT/S were highlighted in cyan. Dashes represented the gaps introduced to the preserve alignment. The hypervariable regions V1-V5, and Loop D, CD4-binding loop and β20/β21 in gp120 were indicated.

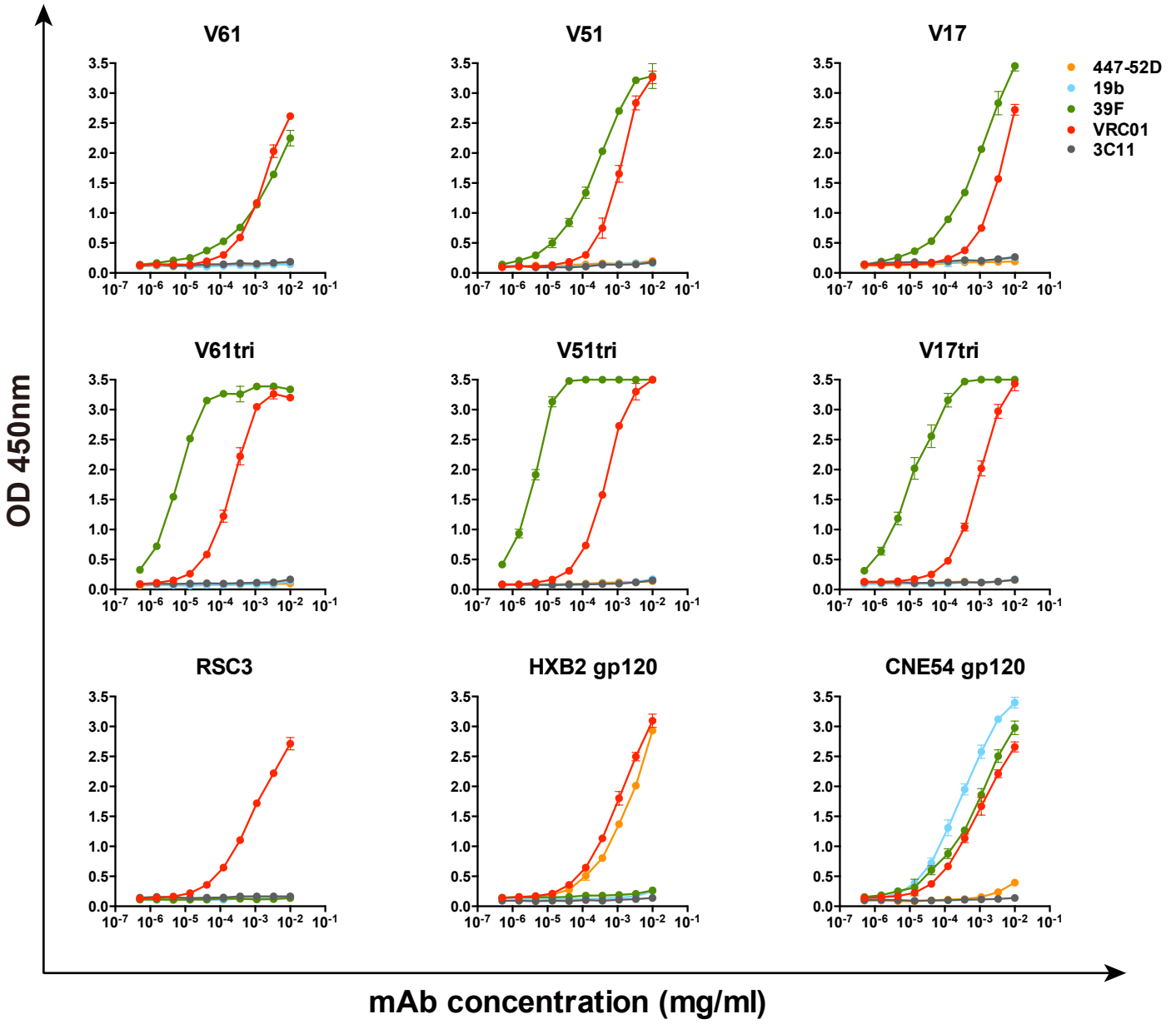


Figure S3. ELISA binding analysis of monomeric and trimeric EAD-VRC01 to anti-V3 narrowly neutralizing mAbs. Binding activities of monomeric and trimeric EAD-VRC01 (V61, V51 and V17) to anti-V3 narrowly neutralizing mAbs (447-52D, 19b and 39F), and control mAbs (VRC01 and 3C11). Recombinant glycoprotein RSC3 (clade B), HXB2 gp120 (clade B) and CNE54 gp120 (clade CRF08_BC) were included as controls. The data represented the average of three repeats and bars indicated the standard deviations.

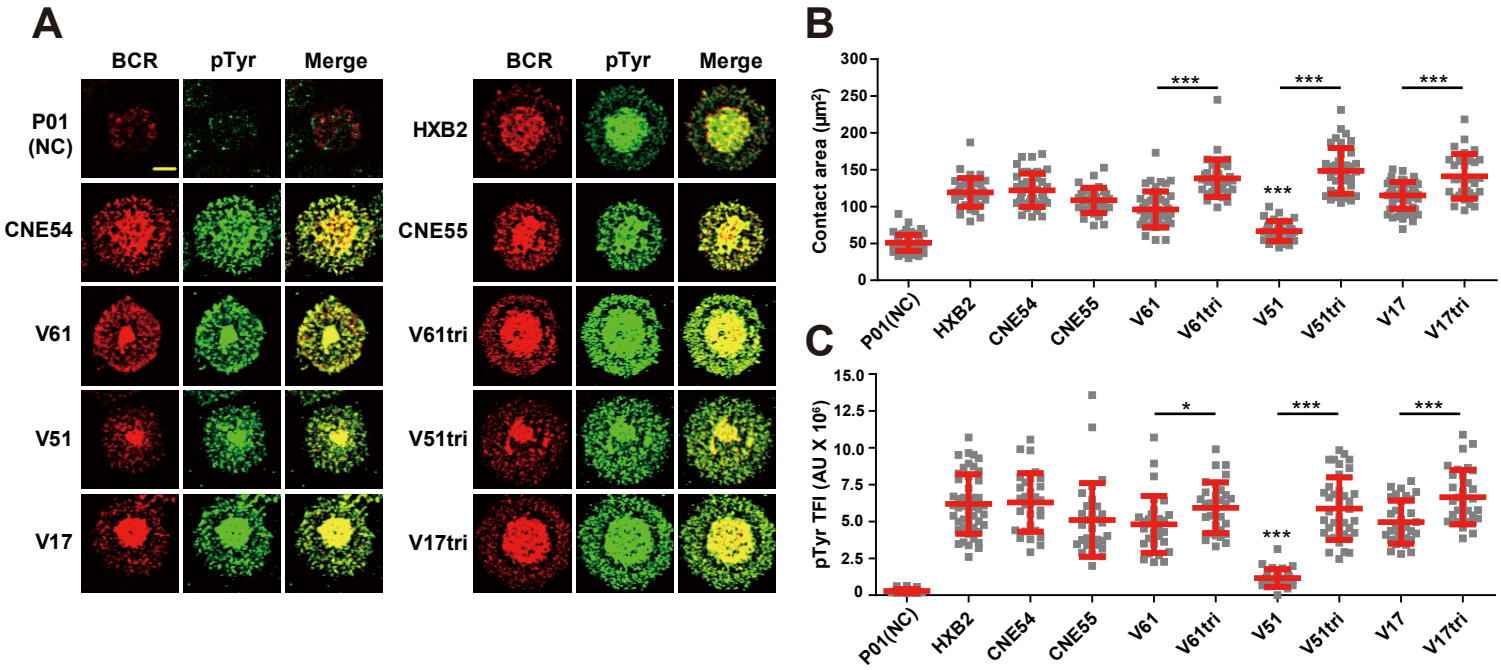


Figure S4. EAD-VRC01 stimulates the total tyrosine phosphorylation (pTyr) of BCR downstream signaling molecules in the Ramos-VRC01 B cells.

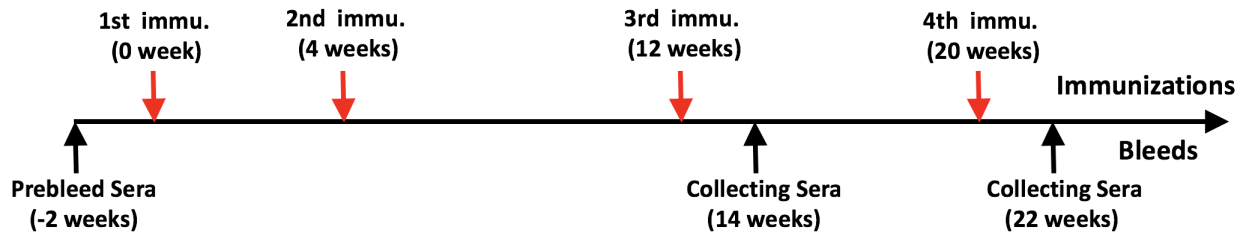
(**A**) The synaptic accumulation of BCR and pTyr of BCR downstream signaling molecules in the Ramos B cells triggered by EAD-VRC01 in TIRFM images. The BCRs and pTyr molecules were labeled in red and green individually. The scale bar represented 1.5 μm.

(**B**) (**C**) Statistical analysis for the contact area (**B**) and total fluorescence intensity (TFI) (**C**) of pTyr in the immunological synapse. Two-tailed t tests were used for the statistical analysis between the monomer and trimer of the same EAD-VRC01. * means P<0.05, *** means p<0.001. Bars indicated the mean values and standard deviations.

A

Group	Group 1 NC	Group 2 Sequencial immunization	Group 3 Mix immunization
ID	NC 01-03 (n=3)	Seq 01-06 (n=6)	Mix 01-06 (n=6)
Species	Guinea pig	Guinea pig	Guinea pig
Adjuvant (Prime)	Freund's complete adjuvant (Sigma)	Freund's complete adjuvant (Sigma)	Freund's complete adjuvant (Sigma)
Adjuvant (Boost)	Freund's incomplete adjuvant (Sigma)	Freund's incomplete adjuvant (Sigma)	Freund's incomplete adjuvant (Sigma)
Route	SC	SC	SC
The volume injected	300μl PBS+ 300μl adjuvant	300μl immunogen in PBS+ 300μl adjuvant	300μl immunogen in PBS+ 300μl adjuvant
1st immunogen	PBS	V17tri 300μg in PBS	The Mix trimer 300μg in PBS (each 100μg)
2nd immunogen	PBS	V51tri 300μg in PBS	The Mix trimer 300μg in PBS (each 100μg)
3rd immunogen	PBS	V61tri 300μg in PBS	The Mix trimer 300μg in PBS (each 100μg)
4th immunogen	PBS	V17tri 300μg in PBS	The Mix trimer 300μg in PBS (each 100μg)

B



C

Virus isolate	Clade	Tier	Prebleed sera neutralization ID_{50}														
			NC 01	NC 02	NC 03	Seq 01	Seq 02	Seq 03	Seq 04	Seq 05	Seq 06	Mix 01	Mix 02	Mix 03	Mix 04	Mix 05	Mix 06
CNE14	B	1B	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CNE4	B	2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CNE10	B	2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CNE11	B	2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CNE57	B	2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CNE1	B	NA	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
JRCSF	B	2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
MMLV			<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

Figure S5. Immunization strategy and schedule of EAD-VRC01 trimers and neutralization activity of the prebleed sera from the guinea pigs.

(**A**) Immunization strategies with indicated details on groups, adjuvant, route and immunogens. The sequential immunization group (Seq. n=6) was immunized sequentially with 300 μg each of EAD-VRC01 V17tri, V51tri, and then V61tri while the mixed immunization group (Mix. n=6) with 300 μg mixture of the three EAD-VRC01 trimers and each contributed 100 μg. The negative control (NC) group was mocked immunized with PBS and the adjuvant.

(**B**) The immunization (red arrows) and blood collection (black arrows) schedule.

(**C**) Neutralization activity of the prebleed sera of all the guinea pigs against a panel of pseudoviruses in the TZM-bl assay. Moloney murine leukemia virus (MMLV) was used as a negative control. NA: Not available. The neutralization assays were conducted at least two independent occasions and each was in duplicate.

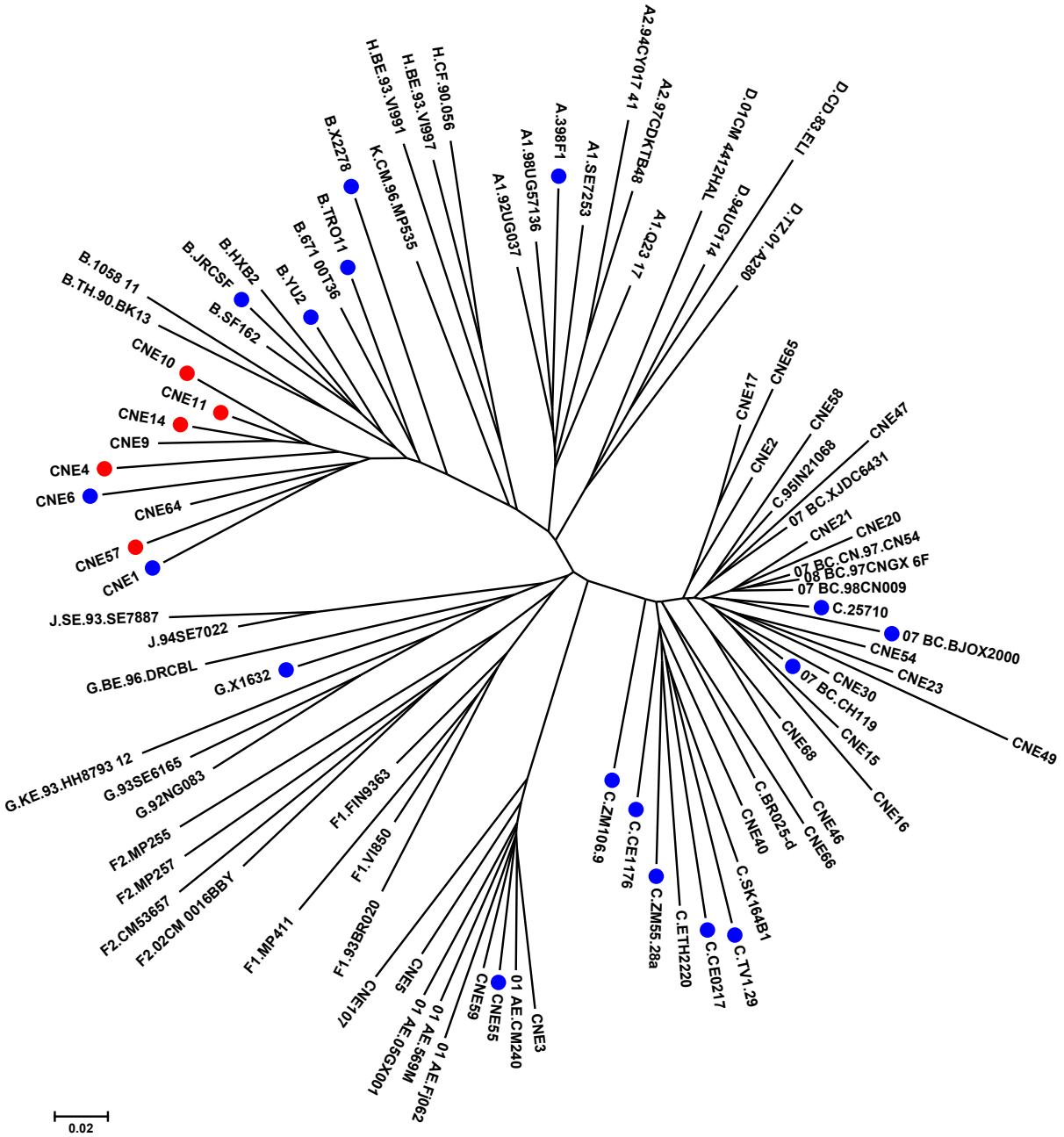


Figure S6. Unrooted neighbor-joining tree depicting the relationship of EAD-VRC01 amino acid sequences among the neutralizing sensitive, insensitive and global panel of control HIV-1 strains.
Neutralizing sensitive strains were indicated by red closed circles, whereas those insensitive ones were by blue closed circles. The branch length was drawn to scale in order to readily assess the relationship among various HIV-1 strains.

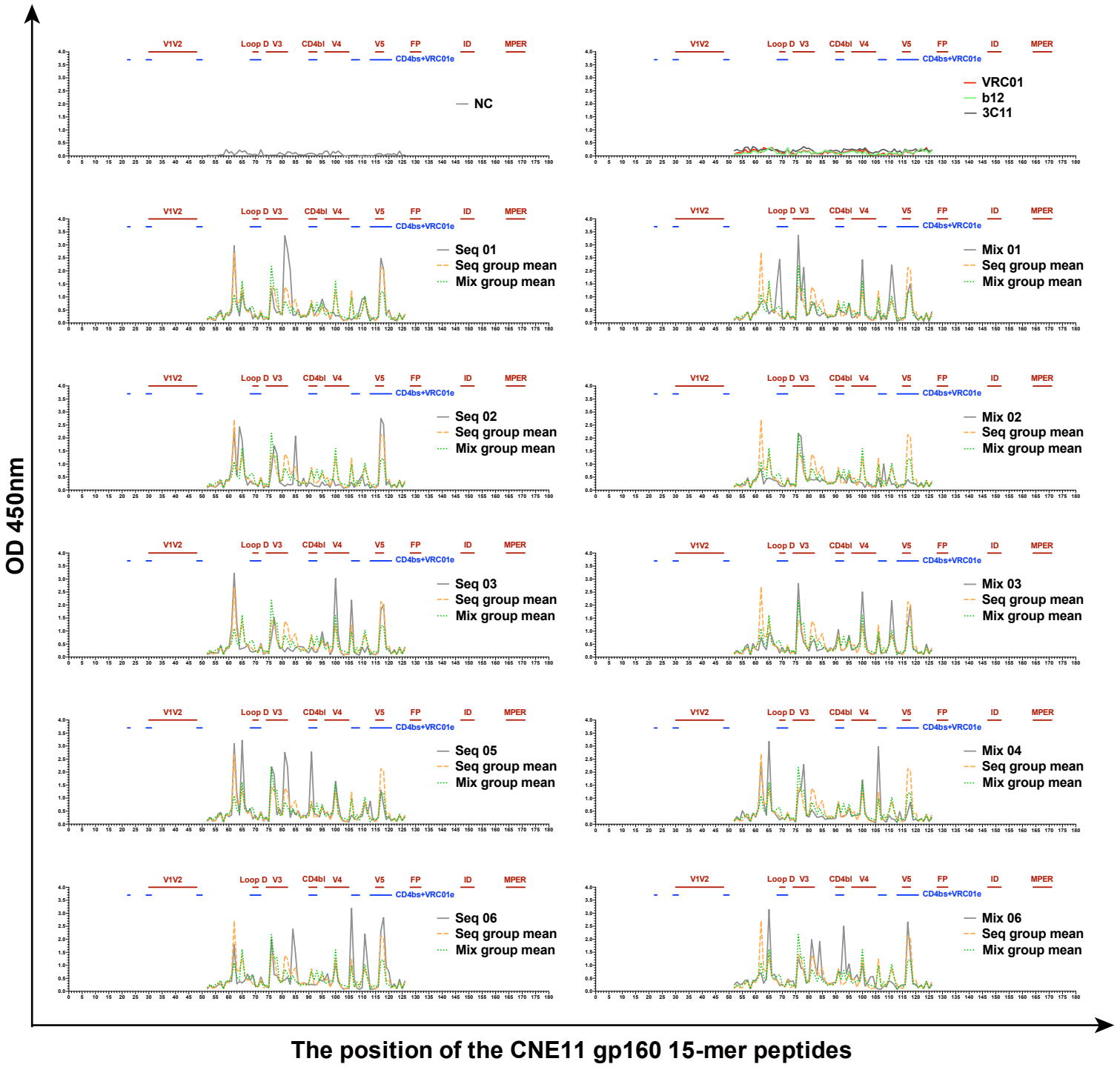


Figure S7. Profiling of the immune guinea pig sera with the CNE11 gp160 15-mer peptides library.
The binding profiling of each immune serum was shown on one plot as a gray solid line. The overall average for the sequential immunized group was indicated in orange dashed line whereas the mixed immunized group in green dotted line. Due to a limited amount of serum, Seq 04 and Mix 05 were not performed in this assay. VRC01, b12 and 3C11 antibodies were performed as controls. The hypervariable regions (V1-V5), Loop D, CD4-binding loop (CD4bl), fusion peptide (FP), immunodominant region (ID), as well as MPER were indicated by the crimson horizontal lines. The CD4bs and VRC01 epitope (VRC01e) were highlighted by the blue horizontal lines.