# Supporting Information

## Alam et al. 10.1073/pnas.0908713106

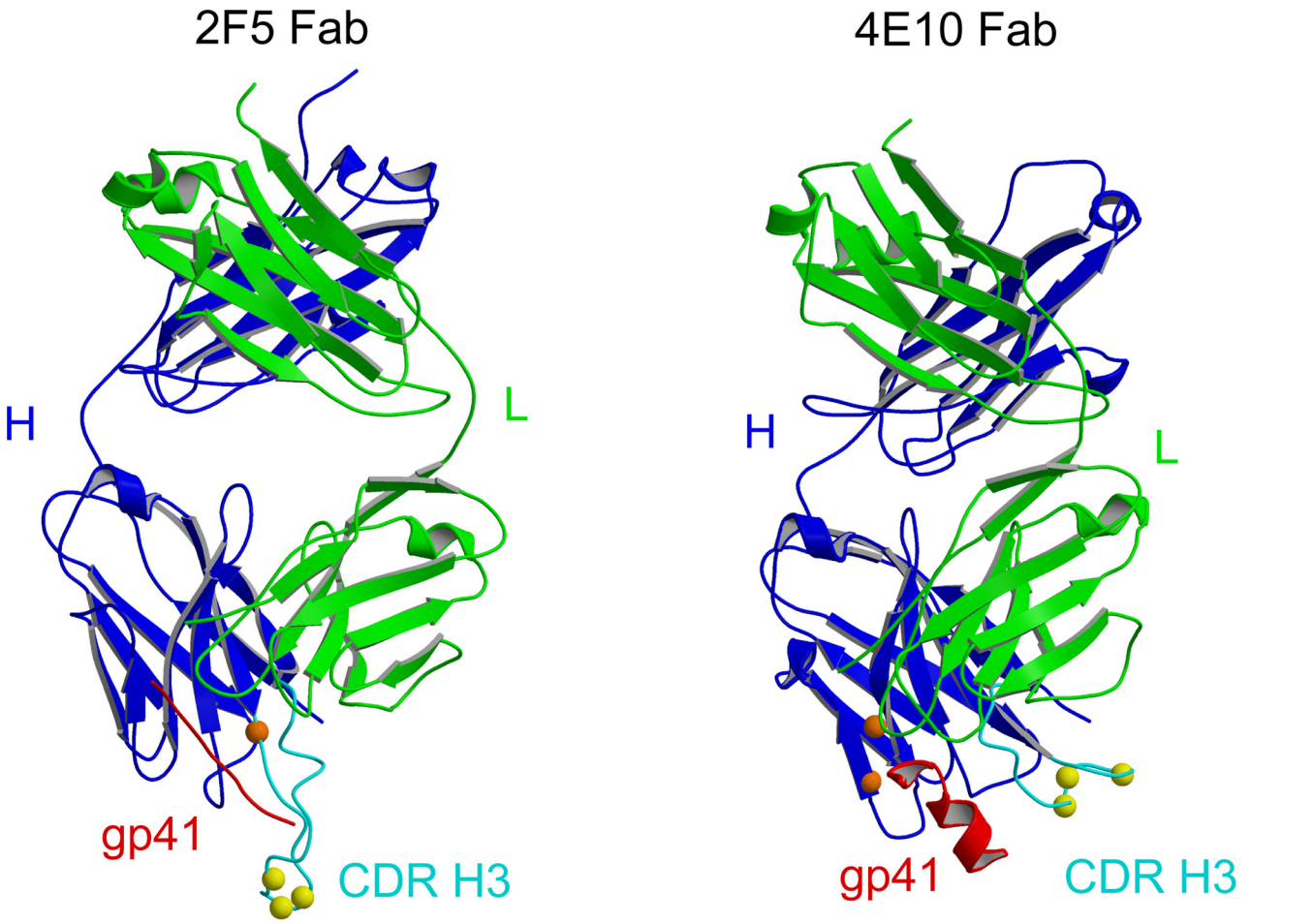
### SI Materials and Methods

**Expression Constructs.** We produced synthetic genes for the Fv fragments of mAb 4E10 (Amoytop) and used pET-21a(+) (Novagen) to express wild-type and mutant 4E10 scFv in *Escherichia coli*. The expression construct, p4E10-scFv, begins with 4E10 light-chain residues L1-L108 (Kabat numbering) (1), followed by a flexible linker (GGGGSGGGGSGGGGS); 4E10 heavy-chain residues H1-H113; a Factor Xa site and a polyhistidine-tag (IEGRHHHHHH). To express mutant 4E10 scFv proteins, we used standard PCR techniques to introduce the following mutations into p4E10-scFv: p4E10-mut1 scFv, H100 (W to A); p4E10-mut2 scFv, H100 (W to A) and H100B (W to A); p4E10-mut3 scFv, H100 (W to A), H100B (W to A), and H100C (L to A); and p4E10-mut4 scFv, L94 (S to R) and H56 (I to A). 2F5 rIgG construct was constructed using a synthetic gene and the mutations were introduced by standard PCR techniques as described (2). Plasmids HV13221 and HV13501 express the variable region of 2F5 heavy-chain (2F5H) in an IgG1 backbone and 2F5 light-chain (2F5L), respectively. To introduce alanine substitutions in the CDR H3 region of 2F5, the plasmid HV13221 was used as a template to generate mutant constructs, including 2F5-mut1 rIgG (L100aA); 2F5-mut2 rIgG (F100bA); 2F5-mut3 rIgG (L100aA/F100bA); and 2F5-mut4 rIgG (R95A). The constructs were verified by restriction digestion and DNA sequencing.

**Protein Expression and Refolding.** Protein refolding in vitro was carried out described previously (3). Briefly, 4E10 scFv constructs were expressed in *E. coli* using BL21-CodonPlus(DE3)-RIPL cells (Stratagene). Bacterial cultures were induced at an $OD_{600}$ of 1.0 by the addition of 1 mM IPTG (Isopropyl $\beta$-D-1-thiogalactopyranoside). Cells were harvested 3 h after induction by centrifugation. Cells were lysed by three cycles of freezing-thawing in PBS with 0.4 mg/mL DNase I and 2 mg/mL lysozyme, followed by brief sonication. scFv constructs were insoluble when expressed in *E. coli*. Inclusion bodies were isolated by centrifugation and solubilized in 6 M guanidine hydrochloride (GdnHCl) with 10 mM imidazole (pH 8.0) and 20 mM $\beta$-mercaptoethanol. After removing insoluble materials by centrifugation, the supernatant was loaded onto Ni-NTA resin (Qiagen). The resin was washed with 10 and 20 mM imidazole (pH 8.0) in 6 M GdnHCl, and eluted with 250 mM imidazole (pH 8.0) in 6 M GdnHCl. The fractions containing His-tagged protein were pooled, and protein refolding was initiated by rapid dilution into ice-cold refolding buffer [1 M arginine, 100 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.2 mM reduced glutathione, 2 mM oxidized glutathione, and one protease inhibitor mixture tablet (Roche)] at a final protein concentration of 50 $\mu$g/mL. The refolding mix was stirred gently at 4 °C for 24–36 h, and then dialyzed against PBS four times and purified on Ni-NTA under native conditions. The imidazole-eluted fractions were pooled, concentrated, and further separated from aggregated species by gel-filtration chromatography on Superdex 200 (GE Healthcare) in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl. Purified protein was concentrated and stored at 4 °C.

To produce recombinant 2F5 IgG, the plasmids HV13221 and HV13501 were cotransfected in 293T cells to generate a stable cell line and the 2F5 rIgG was purified as described (2). 2F5 mutants were also produced in 293T cells by co-transfection of each of the 2F5H mutant plasmids together with the plasmid HV13501, following the protocols, as described (2).


1. Wu TT, Johnson G, Kabat EA (1993) Length distribution of CDRH3 in antibodies. *Proteins* 16:1–7.
2. Liao HX, et al. (2009) High-throughput isolation of immunoglobulin genes from single human B cells and expression as monoclonal antibodies. *J Virol Methods* 158:171–179.
3. Frey G, Peng H, Rits-Volloch S, Morelli M, Cheng Y, Chen B (2008) A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. *Proc Natl Acad Sci USA* 105:3739–3744.
4. Ofek G, et al. (2004) Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with Its gp41 epitope. *J Virol* 78:10724–10737.
5. Cardoso RM, et al. (2005) Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* 22:163–173.




| Mutants: | | |
|---|---|---|
| | 2F5-mut1 rIgG | L100aA |
| | 2F5-mut2 rIgG | F100bA |
| | 2F5-mut3 rIgG | L100aA/F100bA |
| | 2F5-mut4 rIgG | R95A |

| Mutants: | | |
|---|---|---|
| | 4E10-mut1 scFv | W100A |
| | 4E10-mut2 scFv | W100A/W100bA |
| | 4E10-mut3 scFv | W100A/W100bA/L100cA |
| | 4E10-mut4 scFv | I56A/S94R |

**Fig. S1.** Crystal structures of 2F5 and 4E10 and design of mutations in their CDR H3. The ribbon diagrams of the crystal structures of 2F5 and 4E10 in complex with their epitope peptides were derived from the PDB coordinates 1TJH (4) and 1TZG (5), respectively. The heavy chain is shown in blue and the light chain in green; gp41 epitope peptides in red; and the CDR H3 in cyan. The hydrophobic residues in the CDR H3 loops are highlighted by solid spheres in yellow and the residues mutated in the gp41 binding site are represented by spheres in orange. Mutations introduced in CDR H3 loops and gp41 binding site are summarized at the bottom.

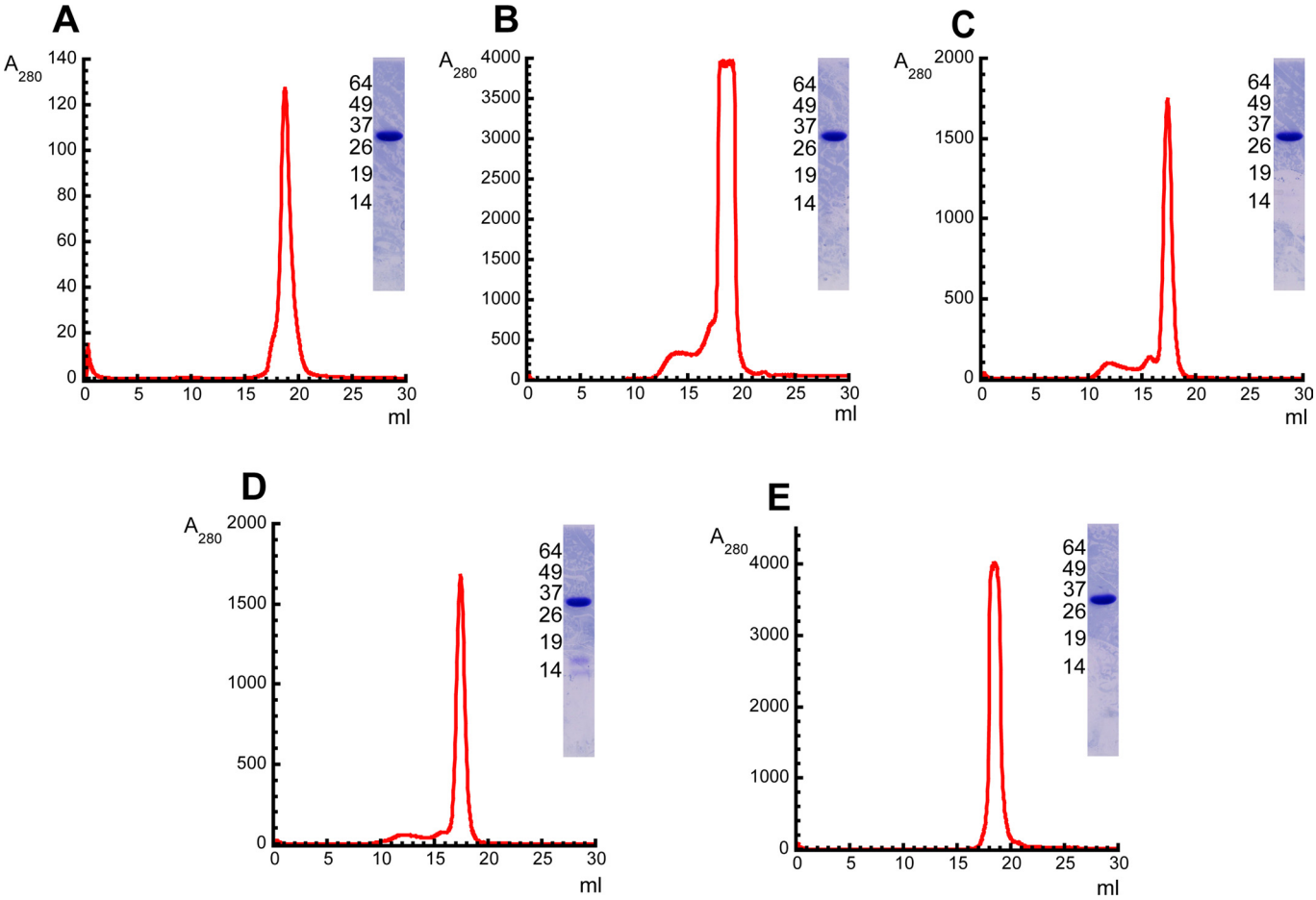



**Fig. S2.** Production of 4E10 scFv and its mutants. The 4E10 scFv and its mutant constructs were expressed in *E. coli* and purified by Ni-NTA under denaturing conditions. The proteins were refolded by a rapid-dilution protocol, further purified by Ni-NTA under native conditions, and then resolved by gel-filtration chromatography using a Superdex 200 column. The apparent molecular masses were calculated based on a standard curve using the following known standards: thyoglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.4 kDa). Peak fractions were pooled and analyzed by Coomassie stained SDS/PAGE (inset). *A*, 4E10 scFv; *B*, 4E10-mut1 scFv; *C*, 4E10-mut2 scFv; *D*, 4E10-mut3 scFv; *E*, 4E10-mut4 scFv.

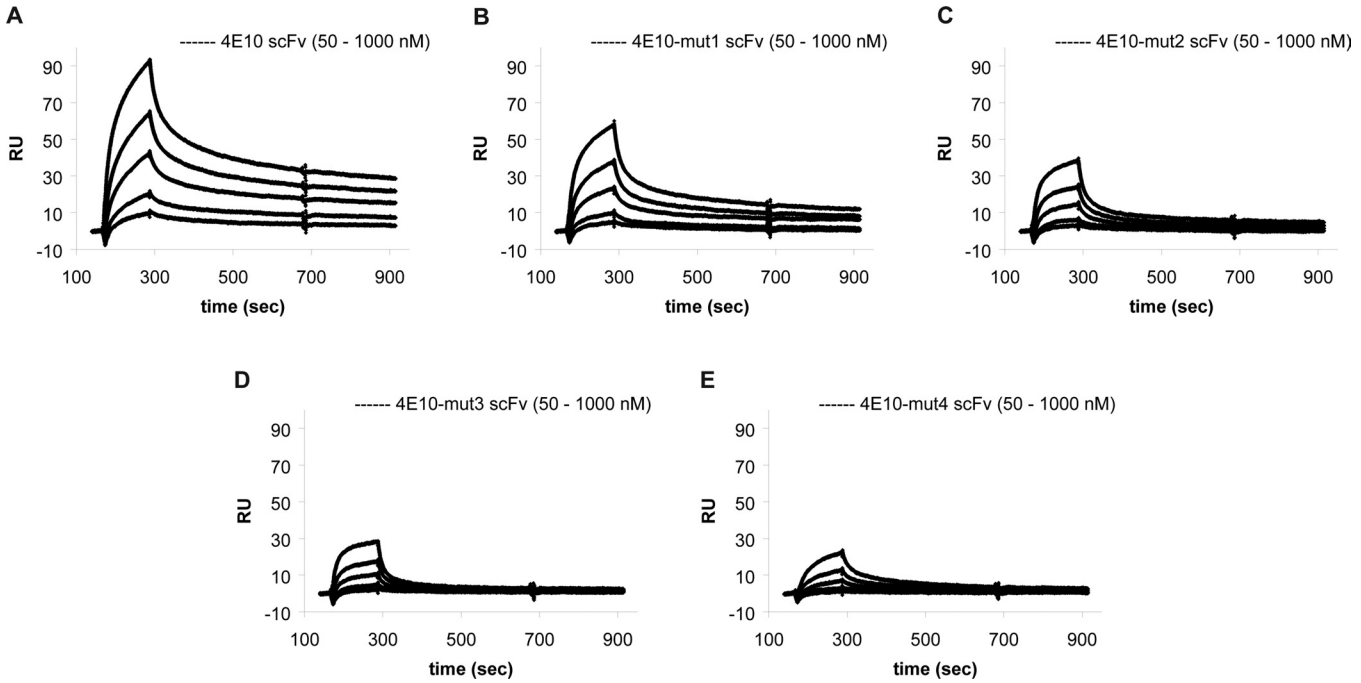



**Fig. S3.** Interaction of 4E10 scFv and its mutants with the gp41 epitope peptide. The biotinylated 4E10 epitope peptide (biotin-SLWNWFNITNWLWYIK) was immobilized on an SA chip, and solutions of 4E10 scFv or its mutants at various concentrations (50, 100, 250, 500, and 1,000 nM) were passed over the chip surface as described in *Materials and Methods*. All injections were carried out in duplicate, which gave essentially identical results. The sensorgrams for one of the duplicates are shown in black. *A*, 4E10 scFv; *B*, 4E10-mut1 scFv; *C*, 4E10-mut2 scFv; *D*, 4E10-mut3 scFv; *E*, 4E10-mut4 scFv.

| Antibody | gp41 epitope binding | Lipid binding | Neutralization |
|---|---|---|---|
| 2F5-wt rIgG | +++ | ++ | +++ |
| 2F5-mut1 rIgG L100aA | +++ | +/- | - |
| 2F5-mut2 rIgG F100bA | +++ | +/- | - |
| 2F5-mut3 rIgG L100aA/F100bA | ++ | - | - |
| 2F5-mut4 rIgG Rh95 | - | +/- | - |

**Fig. S4.** Summary of the properties of r2F5-IgG and its mutants. Recombinant 2F5 IgG and its mutants were produced in 293T cells and purified by elution from an affinity-column using an anti-human H-chain specific antibody, as described previously (*2*). Gp41 epitope binding, lipid binding and neutralization assays were carried out as described in *Materials and Methods*. The gp41 epitope peptide for 2F5 is QQEKNEQELLELDKWASLWN. The liposomes used were phosphatidylserine (PC:PS, 70:30)-liposomes. The pseudotyped viruses used in the neutralization assay contained Envs derived from HIV-1 isolates BG1168 and SF162. These experiments were carried out using whole antibodies, rather than Fabs (to avoid additional loss of material); because of uncertain avidity effects, we have chosen to present the results qualitatively. Symbols: for gp41 epitope binding, $+++$, $K_d$ = or $<$50 nM, $++$, 50 nM $< K_d <$ 100 nM, $-$, no binding was detectable; for lipid binding, $++$, binding was detected, $+/-$, very weak binding was detected, $-$, no binding was detectable; for HIV-1 neutralization, $+++$, $IC_{50} < 3$ $\mu$g/mL, $-$, $IC_{50} > 50$ $\mu$g/mL.

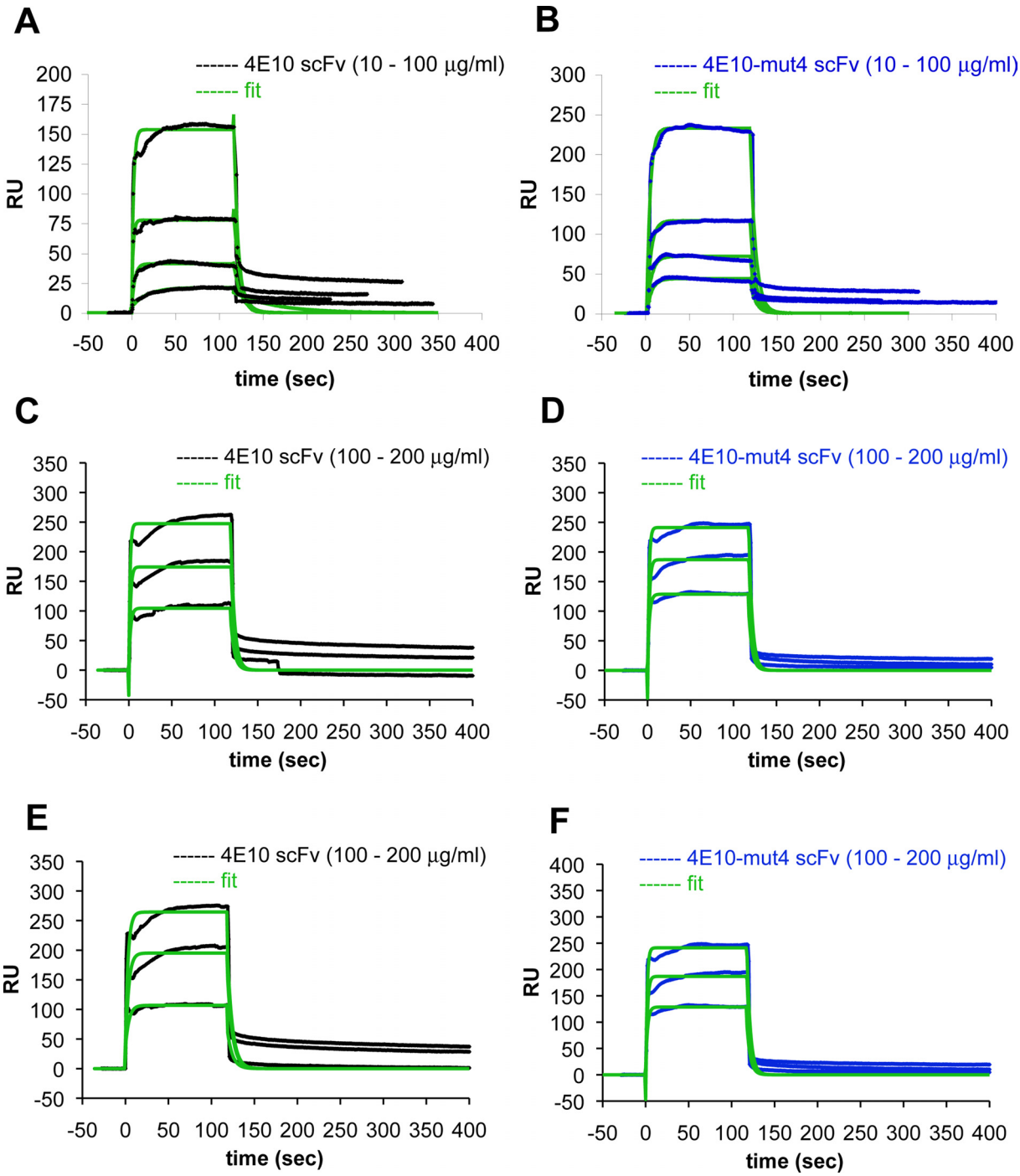



**Fig. S5.** Kinetic studies of interactions of 4E10 scFv wt and mut4 with membranes. In (*A, C* and *E*), viral liposomes in (*A*), AT-2 inactivated HIV-1 ADA in (*C*) or SIV mac239 in (*E*) virion preparations were immobilized on a L1 chip via an alkyl linker. 4E10 scFv at various concentrations (100, 150, and 200 $\mu$g/mL) were passed over the surfaces. In (*B, D* and *F*), viral liposomes in (*B*), HIV-1 in (*D*), or SIV in (*F*) virions were captured on a L1 chip with solutions of 4E10-mut4 scFv (100, 150, and 200 $\mu$g/mL) passing over the surface. A32 mAb and 13H11 Fab were negative controls as in Fig. 1. In (*A* and *B*), the sensorgrams were subtracted from that obtained with 13H11; in (*C–F*), the sensorgrams were subtracted from that obtained with A32. Binding kinetics was evaluated using BIAevaluation software (Biacore). The recorded sensorgrams are shown in black and the fits in green. The dissociation constants derived were summarized in Fig. 1*C*. The experiments were repeated at least twice with similar results.

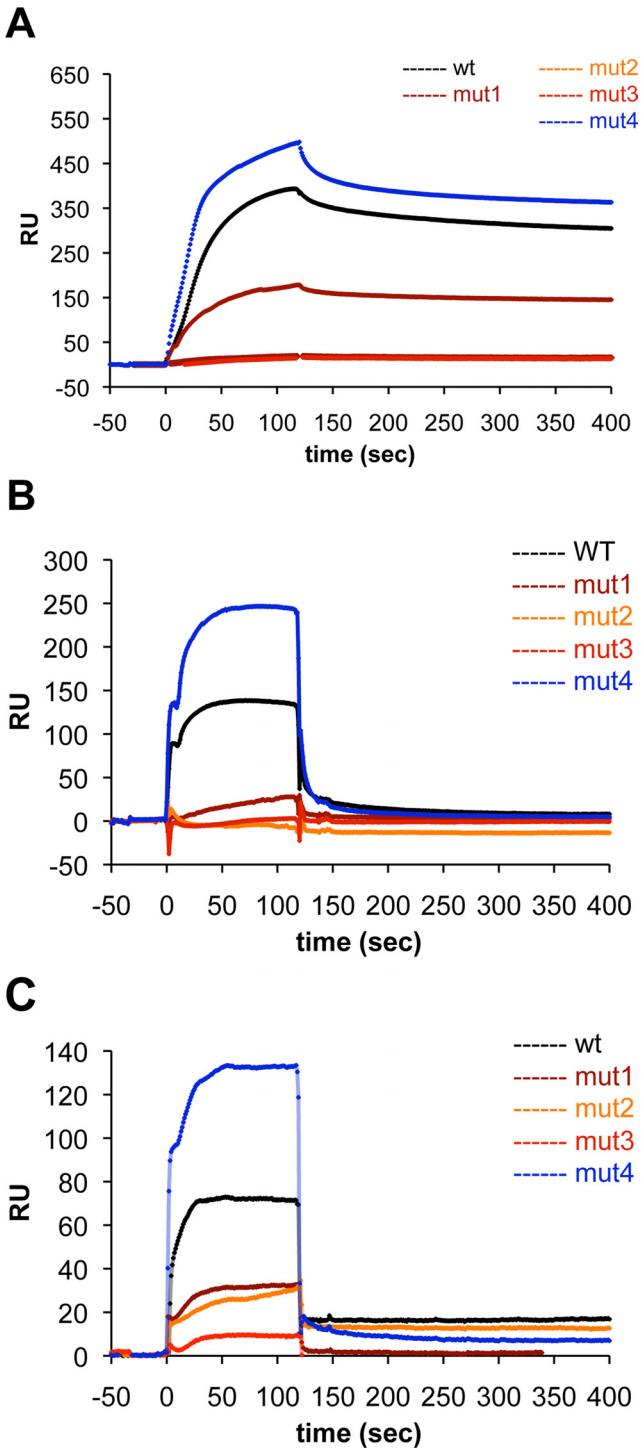



**Fig. S6.** Interactions of 4E10 scFv and its mutants with lipids. In (*A* and *B*), cardiolipin (POPC:CL, 70:30)- and phosphatidylserine (POPC:POPS, 70:30)-liposomes were immobilized on a L1 chip, respectively. 4E10 scFv and its mutants were passed over the surfaces at the concentration of 8 $\mu$M (200 $\mu$g/mL). In (*C*), the AT-2 inactivated SIVmac239 virion preparation was immobilized on a L1 chip. 4E10 scFv and its variants were passed over the surface at a concentration of 8 $\mu$M. mAb A32 was also passed over the surface as a negative control and the sensorgrams in (*C*) were subtracted from the one obtained with A32. The recorded sensorgrams are shown in black for 4E10 scFv; dark red for 4E10-mut1 scFv; orange for 4E10-mut2 scFv; red for 4E10-mut3 scFv; and blue for 4E10-mut4 scFv. The experiments were repeated at least twice with similar results.