Mechanism of HIV-1 Neutralization by Antibodies Targeting a Membrane-Proximal Region of gp41

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Induction of broadly neutralizing antibodies (bNAbs) is an important goal for HIV-1 vaccine development. Two autoreactive bNAbs, 2F5 and 4E10, recognize a conserved region on the HIV-1 envelope glycoprotein gp41 adjacent to the viral membrane known as the membrane-proximal external region (MPER). They block viral infection by targeting a fusion-intermediate conformation of gp41, assisted by an additional interaction with the viral membrane. Another MPER-specific antibody, 10E8, has recently been reported to neutralize HIV-1 with potency and breadth much greater than those of 2F5 or 4E10, but it appeared not to bind phospholipids and might target the untriggered envelope spikes, raising the hope that the MPER could be harnessed for vaccine design without major immunological concerns. Here, we show by three independent approaches that 10E8 indeed binds lipid bilayers through two hydrophobic residues in its CDR H3 (third heavy-chain complementarity-determining region). Its weak affinity for membranes in general and preference for cholesterol-rich membranes may account for its great neutralization potency, as it is less likely than other MPER-specific antibodies to bind cellular membranes nonspecifically. 10E8 binds with high affinity to a construct mimicking the fusion intermediate of gp41 but fails to recognize the envelope trimers representing the untriggered conformation. Moreover, we can improve the potency of 4E10 without affecting its binding to gp41 by a modification of its lipid-interacting CDR H3. These results reveal a general mechanism of HIV-1 neutralization by MPER-specific antibodies that involves interactions with viral lipids.

Fusion of viral and target cell membranes mediated by viral envelope glycoprotein allows HIV-1 to enter host cells (1). The mature envelope spikes [trimeric (gp160)₃ cleaved to (gp120/gp41)₃] are the sole viral antigens on the virion surface. Conformational changes in gp120, when triggered by binding to a receptor (CD4) and coreceptor (e.g., CCR5 or CXCR4), lead to a cascade of refolding events in gp41 in which its N-terminal fusion peptide translocates and inserts into the host cell membrane. A proposed transient, extended conformation of gp41, with the fusion peptide in the host cell membrane and the transmembrane anchor still in the viral membrane, has been called the “pre-hairpin intermediate” (2). It is the target of HIV-1 entry inhibitors, such as T-20/enfuvirtide, the first approved fusion-inhibiting antiviral drug (3). Further transition from the fusion intermediate to the postfusion state of gp41 is believed to bring together the two membranes and ultimately to induce membrane fusion.

Strong antibody responses to the envelope glycoprotein are often detected in HIV-1-infected patients, even during acute infection, but antibodies that can effectively block HIV-1 transmission or disease progression are rare, as most detected antibodies are nonneutralizing or strain specific (4–6). Recent studies indicate that 10 to 25% of patients indeed produce broadly neutralizing antibodies (bNAbs) at low levels during the course of HIV-1 infection (7, 8), raising the hope that a search for immunogens that can induce stronger responses of this kind may lead to an effective vaccine. Major challenges to generating bNAbs by vaccination include unprecedented sequence diversity, conformational flexibility, and a protective “glycan shield” of the envelope glycoprotein, as well as potential host immunoregulatory constraints and lack of reactivity by envelope immunogens to germ line antibodies of known bNAbs (5–7, 9–11). Thus, detailed studies of isolated broadly reactive neutralizing antibodies that recognize

conserved regions of the envelope glycoprotein are critical for guiding strategies to mimic their corresponding immunogens.

Human bNAbs 2F5 and 4E10 interact with the membrane-proximal external region (MPER) of gp41, the segment of the protein adjacent to the viral membrane (12, 13). Both block viral infection by attacking the pre-hairpin intermediate conformation of gp41 with the help of their ability to bind the viral membrane (14, 15), although additional mechanisms of HIV-1 neutralization by these antibodies have also been suggested (16, 17). A useful MPER-based immunogen would therefore likely include a protein component to present the relevant gp41 conformation and a lipid component to induce membrane binding. The autoreactivity of both 2F5 and 4E10, however, is a potential barrier to effective induction of similar antibodies by a vaccine immunogen (9). Another MPER-specific neutralizing antibody, 10E8, has been isolated recently (18); it was reported not to bind either lipid bilayers or any other human autoantigens. It also showed better recognition of the envelope glycoprotein expressed on a cell surface than do 2F5 and 4E10, igniting excitement that the MPER might be harnessed to induce protective antibody responses without major concerns regarding the unwanted immunological limitations associated with 2F5 and 4E10 (9). The reported accessibility of the

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10E8 epitope on the native envelope spikes is much lower, however, than that of the epitopes for antibodies targeting the untriggered envelope with similar neutralization potencies (18). Moreover, the affinity of 10E8 variants for gp41 does not fully correlate with their neutralizing strengths (18), suggesting that a better understanding of the action of the antibody is needed to guide vaccine design efforts to induce 10E8-like antibodies.

In this study, we show that 10E8 does bind lipid bilayers through two hydrophobic residues in its CDR-H3 (third heavy-chain complementarity-determining region). Its affinity for membranes is weak in general, but it has a preference for cholesterol-rich membranes that may account for its great potency in neutralization, as it is less likely than other MPER-specific antibodies to bind cellular membranes nonspecifically. Moreover, it binds with high affinity to gp41-inter, a construct designed to mimic the fusion intermediate of gp41 (15), but fails to recognize the epitope on envelope trimers representing the native, untriggered conformation. Furthermore, we can improve the potency of 4E10 by a modification of its lipid-interacting CDR H3 without affecting its binding to gp41. Thus, MPER bNAbs are likely to neutralize by a common mechanism. These results have important implications for MPER-based vaccine design.

MATERIALS AND METHODS

Production of antibodies and recombinant proteins. The expression constructs of 10E8 and a small amount of purified 10E8 antibody were obtained from the NIH AIDS Reagent Program (heavy chain, catalog no. 12290, and light chain, catalog no. 12291; purified immunoglobulin G [IgG], catalog no. 12294). The 10E8 mutants (F100aA, W100bA, F100aA-W100bA, and P100fA) were generated by PCR-based mutagenesis, and the mutations were confirmed by DNA sequencing. The antibodies were expressed in 293T cells by transient transfection and purified by affinity chromatography using GammaBind Plus resin (GE Healthcare, Little Chalfont, United Kingdom) following protocols described previously (19). The 10E8 concentration was measured by the absorbance at 280 nm, assuming that 1 optical-density (OD) unit equals 1 mg/ml. The wild-type 10E8 and mutant P100fA have low solubility at 4°C and were stored at a low concentration (~0.7 mg/ml). All antibodies were buffer exchanged into phosphate-buffered saline (PBS) and filtered through Utrafree centrifugal filters (0.22 µm; EMD Millipore, Billerica, MA) before use. The VRC01 expression construct was kindly provided by John Mascola (Vaccine Research Center [VRC], NIH), the 17b hybridoma by James Robinson (Tulane University), and the 1281 hybridoma by Susan Zolla-Pazner (New York University). We generated expression constructs of 2G12 IgG and 4E10 Fab using synthetic genes made by GeneArt (Life Technologies Corporation, Grand Island, NY). 4E10 Fab mutants were created by Genewiz, Inc. (South Plainfield, NJ). 4E10 IgG was purchased from Polymun (Klosterneuburg, Austria). Other intact antibodies or Fab fragments were produced in 293T cells or hybridoma following protocols described previously (20). The production of soluble CD4, 92UG037.8 gp140 trimer, and gp41-inter was described previously (15, 19, 21).

The 92UG037.8 gp41-post construct containing the sequence AVVEL GAVFIGFLGTAGSTMGAASITLTVQVRKLLSGIVQQQSNLLRAIEAQ QHLLKLTWVGKQLQARVLAVERYLRDQQLGIWGCSSGKLICTTN PWNSSWSNKSEREIWENMTWLQWDKEISNYTHIYELIESQKQQ EKNEQELLELDKWANLWNWFDISNWLWYIKIFIMIVGGILGLRIVF AVLSVI with an N-terminal honeybee melittin signal sequence and a C-terminal His tag was generated by PCR and cloned into the expression vector pFastBac1 (Life Technologies Corporation). The gp41-post protein was expressed in insect cells using the Bac-to-Bac system (Life Technologies Corporation) as described previously (15). Briefly, recombinant baculovirus was generated according to the manufacturer's protocol and amplified in Sf9 insect cells. For protein production, *Trichoplusia ni*

(Hi-5) cells (2×10^6 cells/ml) were infected at the optimal multiplicity of infection (MOI) and harvested 72 h postinfection by centrifugation. The cell pellets were resuspended in PBS containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and then subjected to three cycles of freeze-thaw and brief sonication to disrupt the membranes. Soluble proteins were removed by centrifugation, and membrane-containing pellets were extracted with a buffer containing 20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10% glycerol, and 1% Fos-Choline-12 (Affymetrix, Santa Clara, CA) at 4°C for 24 h. The extract was clarified by high-speed centrifugation ($2 \times 10^5 \times g$) and filtration through a 0.22-µm filter; diluted 4-fold with a solution containing 20 mM Tris-HCl, pH 8.0 and 150 mM NaCl; and then loaded onto a 5-ml HisTrap column (GE Healthcare) that was equilibrated in 20 mM Tris-HCl, 150 mM NaCl, and 0.2% Fos-Choline-12. The His-tagged gp41-post protein was eluted from the HisTrap column with a buffer containing 300 mM imidazole, concentrated, and further purified by gel filtration chromatography on a Superdex 200 column in a running buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Fos-Choline-12. Fractions containing the gp41-post trimers were pooled and incubated with 1281 Fab at room temperature for 1 hour. To replace Fos-Choline-12 with dodecyl maltoside and remove unbound 1281 Fab, the gp41-post-1281 complex was further purified on a Superdex 200 column in a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% dodecyl maltoside.

Liposome preparation. All lipids, including chicken egg phosphatidylcholine (PC), chicken egg phosphatidylethanolamine (PE), porcine brain phosphatidylserine (PS), porcine brain sphingomyelin (SM), bovine heart cardiolipin (CL), and ovine wool cholesterol, were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were prepared following a modified sonication-extrusion protocol as described previously (<http://www.avantilipids.com/images/PDF/MorrisseyLabProtocolForPrepSuvBySonication.pdf>). Briefly, the lipids were dissolved in chloroform and mixed in a glass vial at various molar ratios, including PC-CL at 3:1 for PC-CL liposomes, PC-PS at 3:1 for PC-PS liposomes, PC-PE-PS-SM-cholesterol at 1.1:2.3:1.0:2.2:5.5 for low-cholesterol HIV-1-like membrane, and PC-PE-PS-SM-cholesterol at 1.2:2.6:1.0:2.4:11.6 for high-cholesterol HIV-1-like membrane. The lipid ratio for the high-cholesterol HIV-1-like membrane was chosen empirically based on liposome stability and signals for 10E8 binding. Chloroform was evaporated under a stream of N₂ for ~10 min, and the lipids were further vacuum dried overnight. The dry lipid films were then hydrated in PBS either on ice or at room temperature, followed by brief sonication for 20 s, and subjected to 21 to 25 cycles of extrusion through 0.2-µm polycarbonate filter membranes (GE Healthcare) using an Avanti extruder (Avanti Polar Lipids). Liposomes were usually used immediately but were stable for at least 5 days, as judged by dynamic light scattering (DLS) (see below). For liposomes used for surface plasmon resonance (SPR) experiments, 0.5% (molar ratio) biotin-labeled PE was included to allow capture by streptavidin-coated (SA) sensor chips. In addition, the biotinylated liposomes were extruded through 0.05-µm polycarbonate filter membranes (GE Healthcare) for analysis by a Biacore instrument. To rule out the possibility that the observed binding of liposomes by antibodies was due to minor contaminants in purified natural phospholipids, we produced liposomes by the same protocol using synthetic phospholipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and N-palmitoyl-D-erythro-sphingosylphosphorylcholine (SM), purchased from Avanti Polar Lipids, and obtained the same results.

Liposome flotation assay. Each antibody (0.5 mg/ml) and 1 mg/ml liposomes prepared with 0.2-µm filters were mixed and incubated either at 37°C or on ice for 1 hour. In a centrifuge tube, 0.15 ml of the protein-liposome mixture was gently mixed with 0.3 ml of 70% (wt/vol) sucrose in PBS to give 0.45 ml of 46.7% (wt/vol) sucrose solution that formed the bottom layer; 1 ml of 25% (wt/vol) sucrose in PBS was carefully overlaid on top of the 46.7% (wt/vol) sucrose solution as the middle layer, and 0.15

ml of 5% (wt/vol) sucrose in PBS was then overlaid as the top layer. After centrifugation at 52,000 rpm in a Beckman Optima TL Ultracentrifuge using a TLS55 rotor for 3 h at either 37°C or 4°C, 6 fractions in 0.2 ml and one last fraction in 0.3 ml were collected from the top to the bottom of the sucrose gradient and analyzed by Western blotting using an anti-human Fab antibody conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, MO).

DLS analysis. Liposomes (0.5 mg/ml) prepared with 0.2- μ m filters and 0.5 mg/ml of each antibody were preincubated at either 37°C or 20°C and then mixed in a cuvette, from which light scattering was immediately recorded in a DynoProtein Solution instrument (Wyatt Technology, Santa Barbara, CA). Fifty consecutive acquisitions were averaged for each measurement. The program DYNAMICS v5 was used for data analysis.

SPR binding assays. All experiments were performed in duplicate or repeated at least twice with a Biacore 3000 (GE Healthcare). Interactions of antibodies with envelope constructs were analyzed using a single-cycle kinetic (SCK) method, following the manufacturer's protocol (GE Healthcare), with a 1-min association phase and a 5-min dissociation phase without regeneration. Experiments to compare gp140 trimer, gp41-inter, and gp41-post were carried out in HBS buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA) containing 0.1% dodecyl maltoside. Each antibody IgG was captured on a sensor chip coated with protein A to an immobilization level of 1,800 response units (RU), and envelope proteins at various concentrations (2.5 to 40 nM) were passed over at a flow rate of 30 μ l/min without regeneration. All other protein-binding experiments were performed in a standard HBS-EP running buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant polysorbate 20). Each antibody IgG was captured on a sensor chip coated with protein A to an immobilization level of 2,000 RU, and envelope proteins at various concentrations (2.5 to 40 nM for binding to 10E8, 2G12 and VRC01; 10 to 160 nM for binding to 10E8 mutants) were passed over at a flow rate of 30 μ l/min without regeneration. The binding kinetics was evaluated using BiaEvaluation software (GE Healthcare), and all sensorgrams were fitted to a 1:1 binding model. A script for analyzing SCK data obtained with Biacore 3000 was kindly provided by GE Healthcare.

Lipid binding experiments were carried out in PBS without detergent. PC-CL and HIV-1-like liposomes incorporated with 0.5% (molar ratio) biotin-PE were prepared using 0.05- μ m filter membranes as described above. All liposome preparations were monitored for homogeneity by DLS analysis and were stable for at least 5 days. Biotinylated liposomes were captured on a streptavidin (SA) chip (GE Healthcare) to an immobilization level of ~6,000 RU for HIV-1-like liposomes or ~3,000 RU for PC-CL liposomes. Each antibody IgG at a concentration of 1.0 μ M was passed over the lipid surface at a flow rate of 50 μ l/min. Sensorgrams were recorded with a 2-min association phase and a 5-min dissociation phase.

Flow cytometry. Transfection of 293T cells with HIV-1 92UG037.8 gp160 was carried out as described previously (21). Forty hours posttransfection, transfected and control cells were detached from plates using PBS containing 1% bovine serum albumin (BSA) and washed with ice-cold PBS containing 1% BSA. Cells (10^6 /ml) were incubated for 1 hour at 4°C with various anti-HIV-1 envelope monoclonal antibodies at 50 μ g/ml in PBS containing 1% BSA, washed in PBS with BSA three times, and then stained with 5 μ g/ml of R-Phycoerythrin AffiniPure F(ab')₂ fragment goat anti-human IgG and F(ab')₂ fragment-specific secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 1% BSA. The labeled cells were then washed in cold PBS with 1% BSA three times and analyzed immediately using a BD FACScan instrument and CellQuest software (BD Biosciences, San Jose, CA).

Antibody neutralization assay in TZM.bl cells. Antibody neutralization was measured using a protocol of the luciferase-based HIV-1 neutralization assay in TZM.bl cells as described previously (22, 23). Briefly, 5-fold serial dilutions of 10E8 or 4E10 were performed in duplicate (in a 96-well flat-bottom plate) in 10% Dulbecco's modified Eagle's medium (DMEM) growth medium (100 μ l/well). TZM.bl cells were added (1×10^4 /well in a 50- μ l volume), and the plates were incubated for 1 h at 37°C.

Ten selected HIV-1 Env pseudoviruses were then added (100 μ l/well) in 10% DMEM growth medium containing DEAE-dextran (Sigma-Aldrich) at a final concentration of 11 μ g/ml. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48-h incubation at 37°C, 150 μ l of assay medium was removed from each well, 100 μ l of Bright-Glo luciferase reagent (Promega, Madison, WI) was added, and luminescence was measured using a Victor 3 luminometer (PerkinElmer, Waltham, MA). The 50% inhibitory concentration (IC₅₀) and 80% inhibitory concentration (IC₈₀) were calculated based on the antibody dilution that caused a 50% or 80% reduction in relative luminescence units (RLU) compared to the virus control wells after subtraction of cell control RLU.

RESULTS

Production of 10E8 and its mutants. We set out to determine the conformational state of the envelope glycoprotein recognized by 10E8 and to understand why 10E8 is much more potent than 4E10 despite its lower affinity for a similar epitope on gp41 (18). We obtained the 10E8 expression constructs from the NIH AIDS Reagent Program, contributed by J. Huang and colleagues; expressed the 10E8 antibody in 293T cells; and purified it by protein G affinity chromatography. The IC₅₀ and IC₈₀ neutralization titers of our preparation using 10 selected HIV-1 isolates with a wide range of sensitivities to the antibody are largely concordant with values reported previously (see Table S1 in the supplemental material), confirming that our antibody is indistinguishable from the reported preparation (18). The freshly purified 10E8 had unusually low solubility (<1 mg/ml) for an IgG in PBS and precipitated over time at 4°C, suggesting that there might be exposed hydrophobic surfaces causing aggregation. Close examination of the crystal structure of 10E8 in complex with the epitope peptide suggested that two hydrophobic residues, F100a and W100b, in the CDR H3 may be exposed in the unliganded form (18) and could interact with phospholipids (see Fig. S1 in the supplemental material). We therefore produced three 10E8 mutants—F100aA (Phe to Ala), W100bA (Trp to Ala), and F100aA-W100bA (double mutant)—to test the functions of these residues. An additional mutant, P100fA, in the gp41-binding site was also generated as a control. The mutant antibodies were produced by the same protocol, but the first three mutants showed much higher solubility (up to 10 mg/ml) in PBS after purification at 4°C, confirming our hypothesis that the low solubility of 10E8 correlates with its surface hydrophobicity.

Interactions of 10E8 with lipid bilayers. We used three methods to test whether 10E8 interacts with membrane lipids. The first was a liposome flotation assay with liposomes of three different compositions: PC and CL (at 3:1), PC and PS (at 3:1), and a mixture mimicking the lipid composition of the HIV-1 membrane (PC-PE-PS-sphingomyelin-cholesterol at 1.2:2.6:1.0:2.4:11.6 [24]). The high cholesterol level in the HIV-1-like liposomes enhanced signals in lipid binding assays, although the liposomes with the composition used in published studies (PC-PE-PS-sphingomyelin-cholesterol at 1.1:2.3:1.0:2.2:5.5 [24]) also gave positive results (see below). The sensitivity of the lipid composition may be significant, because HIV-1 membranes have been reported to have a cholesterol level 2- to 3-fold higher than that of plasma membranes (24–26), and their high cholesterol content is critical for viral infectivity and budding (27, 28). The experiments were carried out at both 37°C (Fig. 1; see Fig. S2 in the supplemental material) and 4°C (see Fig. S3 in the supplemental material). As expected, the known membrane-binding antibody, 4E10, associ-

FIG 1 Interactions of 10E8 with lipid bilayers. A liposome flotation assay was used to detect binding to HIV-1-like membranes at 37°C by various antibodies, i.e., 17b, 4E10, 10E8, 10E8-F100aA, and 10E8-W100bA. Seven fractions throughout the sucrose gradient (from top [t] to bottom) after centrifugation were collected and analyzed by Western blotting. The heavy and light chains of antibodies are indicated by H and L, respectively.

ated with liposomes of all three types after centrifugation in a sucrose gradient, while a gp120-specific antibody, 17b, that does not interact with membranes sedimented to the bottom of the gradient as free protein (Fig. 1; see Fig. S2 and S3 in the supplemental material). Like 4E10, wild-type 10E8 also floated with both HIV-1-like and PC-CL liposomes, but not with PC-PS liposomes (Fig. 1; see Fig. S2 and S3 in the supplemental material), indicating that the antibody binds phospholipids with certain compositional preferences. Mutation of either of the two hydrophobic residues in the CDR H3 (F100aA or W100bA) led to much weaker binding to PC-CL liposomes and no detectable binding to HIV-1-like liposomes, while the double mutation (F100aA-W100bA) completely abolished binding to both types of lipid bilayers. The P100fA mutant still bound lipid bilayers as expected (see Fig. S2 in the supplemental material). We conclude that the two hydrophobic residues, F100a and W100b, in the CDR H3 of 10E8 mediate its interaction with the membrane.

As a second assay for membrane binding, we used dynamic

light scattering to monitor cross-linking of liposomes in solution by intact, bivalent IgGs at both 37°C (Fig. 2; see Fig. S4 in the supplemental material) and 20°C (see Fig. S5 in the supplemental material). Our freshly prepared liposomes had an average hydrodynamic radius of 100 nm, stable for at least 5 days and unperturbed by addition of PBS (Fig. 2; see Fig. S4 and S5 in the supplemental material). 10E8 and 10E8-P100fA, which tended to aggregate at high concentrations and low temperature, did not form large aggregates under the experimental conditions we used (see Fig. S4A and S5A in the supplemental material). As expected, addition of 17b IgG did not have any effect on the size of the liposomes under all conditions tested. In contrast, addition of lipid binding 4E10 IgG led to a dramatic increase in the apparent size of the liposomes (up to 1×10^6 nm) for all three types of liposomes at both temperatures (Fig. 2; see Fig. S4 and S5 in the supplemental material), consistent with previous observations of strong membrane interaction by 4E10 (9, 14, 29). Addition of 10E8 IgG produced a moderate but significant increase in the sizes

FIG 2 Interactions of 10E8 with lipid bilayers. Dynamic light scattering analysis was used to monitor cross-linking of liposomes with an HIV-1-like lipid composition at 37°C by various antibodies, i.e., 17b, 4E10, 10E8, 10E8-F100aA, and 10E8-W100bA. PBS was used as a negative control. Regularization histograms plotting percentages of scattering intensity (%intensity) against the hydrodynamic radii of particles (Rh) in nanometers are shown.

FIG 3 Interactions of 10E8 with lipid bilayers. Interactions of lipid bilayers with an HIV-1-like lipid composition (A) and with a PC/CL ratio of 3:1 (B) with various antibodies were measured by SPR at room temperature. Liposomes incorporated with 0.5% biotin-labeled PE were immobilized on the surface of a sensor chip coated with streptavidin, and various antibodies, i.e., 17b, VRC01, 4E10, 10E8, 10E8-F100aA, 10E8-W100bA, and 10E8-FW-AA (10E8-F100aA-W100bA), at 1 μ M were passed over the lipid bilayer surfaces. The recorded sensorgrams are shown.

of HIV-1-like liposomes at both 37°C and 20°C (Fig. 2; see Fig. S5B in the supplemental material) and of PC-CL liposomes at 20°C (see Fig. S5C in the supplemental material) but no changes in the size of PC-PS liposomes at either temperature (see Fig. S4C and S5D in the supplemental material). A 10E8 preparation obtained from the NIH AIDS Reagent Program also gave the same results (see Fig. S4B and C and S5B and C in the supplemental material). Liposomes lack lipid asymmetry in biological membranes. Removing PE in the HIV-1-like liposomes with a lower cholesterol level to mimic the lipid composition of the outer leaflet of biological membranes significantly enhanced their binding to 10E8, while their strong binding to 4E10 was not sensitive to the change in composition (see Fig. S6 in the supplemental material). These observations agreed with the notion that 10E8 interacts weakly with lipid bilayers, with a preference for the cholesterol-rich, HIV-1-like membrane. Mutation of the hydrophobic residues at positions 100a and 100b in the 10E8 IgG (F100aA IgG, W100bA IgG, and F100aA-W100bA IgG) did not affect the size of any liposomes under any of the conditions tested, nor did the monovalent 10E8 Fab. The mutant P100fA behaved similarly to wild-type 10E8 in this assay (see Fig. S4 and S5 in the supplemental material). These results indicate that the large particles observed by dynamic light scattering are indeed the result of cross-linking of liposomes, not of antibody-mediated liposome fusion, and further confirm that the two hydrophobic residues in the CDR H3 mediate 10E8 interaction with lipid bilayers. These findings provide direct evidence that both 10E8 and 4E10 interact with lipid bilayers in solution.

As a third assay for lipid interaction, we used SPR at both 37°C and room temperature (Fig. 3; see Fig. S7 in the supplemental material). Biotin-labeled PE was incorporated into liposomes that were then immobilized on the surface of a sensor chip by streptavidin, and various antibodies were passed over the liposome surfaces. As controls, 4E10 showed high levels of binding to HIV-1-like and PC-CL liposomes, and gp120-specific antibodies, 17b and VRC01, did not bind detectably to any liposomes. 10E8 bound both types of liposomes, with a high off-rate for the HIV-1-like membrane and a low off-rate for the cardiolipin-containing membrane. Mutating the two hydrophobic residues reduced binding

for both types of liposomes to an almost undetectable level. We observed a similar effect of these mutations, but with a weaker signal for HIV-1-like liposomes with a lower cholesterol level (see Fig. S7 in the supplemental material). These results are consistent with the data obtained from the liposome flotation and DLS analyses.

10E8 neutralizes by targeting the fusion intermediate of gp41. To determine which conformational state of the envelope spikes 10E8 recognizes, we first analyzed direct binding of 10E8 to the envelope protein expressed on the surfaces of 293T cells using a fluorescence-activated cell sorting (FACS) assay. We chose to use a 10E8-sensitive primary HIV-1 A clade isolate, 92UG037.8, from which we have produced stable, homogeneous preparations of the envelope glycoprotein, to represent its distinct conformational states (15, 19, 21). We verified that the expressed protein was active by showing that it mediated cell-cell fusion. As shown in Fig. 4, one of the most potent CD4 binding site bNAbs, VRC01, which targets the untriggered, native conformation of the envelope trimer with a potency similar to that of 10E8, bound strongly to the cell surface-expressed 92UG037.8 envelope spikes, but not to the surfaces of control cells. In contrast, 4E10, which targets the pre-hairpin intermediate state of gp41, bound weakly, but detectably, to the cell surfaces whether or not the envelope protein was present, consistent with the previous results showing that 4E10 binds cell membranes, but not the untriggered envelope spikes (14). Addition of soluble CD4, which induced formation of the CD4i epitope (see Fig. S8 in the supplemental material), did not lead to significant exposure or formation of the 4E10 epitope, suggesting that coreceptor binding is probably needed to induce the pre-hairpin intermediate conformation of gp41. In short, like 4E10, but unlike VRC01, 10E8 did not interact with the functional, cell surface-expressed envelope trimers, with or without addition of soluble CD4, indicating that the prefusion conformational state of the envelope is not the antibody target. The most striking difference between 10E8 and 4E10 is that the former shows much weaker binding to the 293T cell membrane, which has a lipid composition distinct from that of the HIV-1 membrane (24). Tighter association with cellular membranes by 4E10 might hinder its ability to locate the transient, fusion-intermediate state

FIG 4 10E8 targets the pre-hairpin intermediate conformation of HIV-1 gp41. Antibody binding to the HIV-1 92UG037.8 envelope trimer was expressed on 293T cell surfaces by FACS analysis. 293T cells were transfected with either 92UG037.8 gp160 or no DNA as a negative control. Binding of antibodies VRC01, 4E10, 10E8, 4E10 plus 2D CD4, 10E8 plus 2D CD4, 10E8-F100aA, 10E8-W100bA, and 10E8-F100aA-W100bA to the cell surfaces was detected with a phycoerythrin-conjugated goat anti-human secondary antibody. Histograms plotting cell counts against fluorescence intensity are shown. The vertical dashed red lines indicate the peak centers of the untreated 293T cells as a negative control. The experiments were repeated three times with similar results.

of gp41; the preference of 10E8 for cholesterol-rich HIV-1-like membranes and weaker association with cellular membranes might account for its greater potency.

If 10E8 does not bind native envelope spikes, what, then, is its neutralization target? We tested binding of 10E8 with our stringently characterized recombinant proteins in three distinct conformational states: for the prefusion conformation, a stable, homogeneous 92UG037.8 gp140 trimer (15, 19); for the pre-hairpin intermediate state, our construct called gp41-inter (15, 21); and for the postfusion conformation, a construct termed gp41-post, which contains the entire MPER, the transmembrane segment, and the fusion peptide in complex with a six-helix bundle-specific monoclonal antibody, 1281 (see Fig. S9 in the supplemental material) (21). As shown in Fig. 5A and Table S2 in the supplemental material, 10E8 bound with high affinity (K_d [dissociation constant] = 1.85 nM) to gp41-inter and with lower affinity (K_d = 11.7 nM) to the gp41-post–1281 complex in the presence of detergent, which was required to solubilize the gp41-post construct (because of the hydrophobic fusion peptide and transmembrane segment). Under the same conditions, 10E8 had almost undetectable binding to the gp140 trimer, consistent with our FACS data. Since the SPR response is proportional to the molecular mass of the binding analyte, the differences between gp41-inter and gp140 are much greater than those shown in the figure. Thus, the binding profile of these preparations is almost the same as that of 4E10: high affinity (K_d = 2.34 nM) for gp41-inter and somewhat lower affinity (K_d = 7.69 nM) for the gp41-post–1281 complex, but barely detectable binding to gp140 (Fig. 5B). The similarity suggests that these two antibodies neutralize by a common mechanism. Under conditions without detergent, 10E8 again bound strongly to gp41-inter (K_d = 0.05 nM), but not to the gp140 trimer (Fig. 5C). Mutant W100bA still had relatively high affinity for gp41-inter (K_d = 0.23 nM), but mutants F100aA, F100aA-W100bA, and P100fA bound much more weakly. In particular, binding of the P100fA mutant

(K_d = 1.23 μ M; see Fig. S10 and Table S2 in the supplemental material) was over 3 orders of magnitude weaker than that of W100aA. As controls, we showed that our gp140 trimer had high affinity for the two anti-gp120 bNAbs, 2G12 (K_d = 10.2 nM) and VRC01 (K_d = 0.75 nM) (Fig. 5D; see Table S2 in the supplemental material). We therefore conclude that 10E8, like 4E10, neutralizes by targeting the fusion-intermediate conformational state of gp41.

In a luciferase-based HIV-1 neutralization assay (see Table S1 in the supplemental material), mutants F100aA and W100bA both had much lower potency than wild-type 10E8. P100fA was only slightly less potent than wild-type 10E8 but much more potent than W100bA, despite a much lower affinity for its gp41 target, in agreement with data reported previously (18). Thus, gp41 binding alone cannot account for neutralization potency, further suggesting that loss of membrane binding has a greater impact on neutralization by 10E8 than loss of affinity for gp41.

Improving the neutralization potency of 4E10. Antibodies 4E10 and 10E8 both neutralize by targeting the same region of gp41. Although 4E10 binds the gp41 intermediate at least as tightly as does 10E8, it is a much less effective neutralizer. The comparison suggests that altering the lipid binding properties of 4E10 might improve its neutralization potency. We therefore tested whether we could increase 4E10 potency by manipulating its lipid binding CDR H3 loop. We made several 4E10 Fab variants with single mutations in the CDR H3 to alter its hydrophobicity or structural flexibility (see Fig. S11 in the supplemental material). Although many of these mutations led to reduced or unchanged neutralization, we found one variant, with the mutation G100dA, designed to rigidify the CDR H3, that was consistently more potent at neutralizing all selected viruses (Table 1). Its binding to gp41-inter was even slightly reduced (see Fig. S11 in the supplemental material). Thus, optimization of the membrane-interacting CDR H3 of 4E10 may

FIG 5 10E8 targets the pre-hairpin intermediate conformation of HIV-1 gp41. (A) 10E8 was analyzed by SPR assay for binding to HIV-1 gp140, gp41-inter, and gp41-post in complex with an antibody, 1281, in the presence of 0.1% dodecyl maltoside. 10E8 IgG was captured on the surface of a sensor chip coated with protein A to avoid potential artifacts introduced by protein immobilization. gp140, gp41-inter, and gp41-post-1281 at various concentrations were passed over the antibody surface individually without regeneration for single-cycle kinetic analysis. The recorded sensograms are shown. The molecular masses of the three envelope constructs are also indicated. Since the SPR response is proportional to the molecular mass of the binding analyte, the differences between gp41-inter and gp140 are much greater than those shown. (B) Similar to panel A, 4E10 was analyzed for binding to HIV-1 gp140, gp41-inter, and gp41-post in complex with anti-gp41 antibody 1281. (C) Binding of 10E8 and its variants 10E8-F100aA, 10E8-W100bA, and 10E8-F100aA-W100bA to gp41-inter was compared by single-cycle kinetic analysis in the standard HBS buffer. Binding of 10E8 to gp140 was also performed to evaluate the interaction without dodecyl maltoside. (D) Control experiments by single-cycle kinetic analysis to demonstrate binding of gp140 used in panels A, B, and C to the gp120-specific antibodies 2G12 and VRC01. All of the binding constructs are summarized in Table S2 in the supplemental material.

lead to generation of a 10E8-like neutralizing antibody. The result suggests that we could consider 10E8 simply a more potent version of a 4E10-like antibody.

DISCUSSION

Our results indicate that 10E8 is not an “outlier” among MPER-specific bNAb, but rather, a 4E10-like antibody with greater neutralization potency. We suggest that most MPER bNAb, if not all,

are likely to neutralize by a common mechanism: targeting the fusion-intermediate state of gp41 with the help of their lipid binding activity. The greater neutralization potency of 10E8 than of 4E10 highlights the importance of optimal binding to the HIV-1 membrane and low levels of binding to host cellular membranes. 4E10 is a weaker neutralizer, despite its higher affinity for gp41, probably because of its relatively strong, nonselective binding to lipid bilayers and, in particular, to the surfaces of cellular mem-

TABLE 1 Improved neutralization potency of 4E10 by modifying its lipid-interacting CDR H3 loop

Virus	Clade	4E10 IgG		4E10 WT ^a Fab		4E10 mutant Fab	
		IC ₅₀ (μg/ml)	IC ₈₀ (μg/ml)	IC ₅₀ (μg/ml)	IC ₈₀ (μg/ml)	IC ₅₀ (μg/ml)	IC ₈₀ (μg/ml)
Du156.12	C	0.245	1.010	0.667	2.324	0.358	1.103
92UG037.8	A	1.104	7.113	2.373	9.658	1.349	5.319
TRO.11	B	1.204	7.167	2.417	9.563	1.127	5.546
ZM197M.PB7	C	1.044	8.427	4.969	20.458	3.488	12.452
T266.60	AG	3.573	21.570	39.182	>50	23.701	>50
928-28	AG	5.871	32.092	14.060	46.442	10.669	38.748
Q842.d12	A	12.300	49.613	9.955	30.329	5.942	16.391
C2101.c1	AE	15.529	>50	17.308	49.851	8.158	26.802
PVO.4	B	18.952	>50	40.485	>50	19.063	>50
RHPA4259.7	B	12.811	>50	>50	>50	>50	>50

^a WT, wild-type.

branes (Fig. 4). Tight binding to the cell membrane could diminish its capacity to capture a transient gp41 target, even though some association with the viral membrane is needed to create proximity.

There has been considerable debate about how the MPER-specific antibodies neutralize. Ruprecht and colleagues reported that irreversible HIV-1 neutralization by 2F5 and 4E10 might involve gp120 shedding when the virus was exposed to the antibodies in the absence of target cells (17). In this case, virion neutralization was found to be a very slow process that required several hours. Neutralization in the presence of target cells by these antibodies occurs much more rapidly (well within an hour) (18, 30), however, suggesting gp120 shedding is unlikely to be their primary mode of neutralization. Without receptor engagement, it is conceivable that a small population of gp41 can sample the prehairpin intermediate conformation, which could be captured by 2F5 or 4E10 over a long incubation time. Subsequently, stabilization of the intermediate state by the antibodies could lead to gp120 dissociation. It would be interesting to test whether 10E8 is more or less potent than 2F5 or 4E10 under these conditions. Kim et al. and Sun et al. have proposed yet another mechanism of neutralization based on studies using short monomeric MPER peptides embedded in lipid bilayers. They concluded that 2F5 and 4E10 might need to extract their core epitopes from the viral membrane in order to form a high-affinity complex with gp41 to inhibit viral entry (16, 31). It is unclear what conformational state of gp41 these short peptides represent and which step during the viral fusion process these antibodies would block. Moreover, 10E8 has a lower affinity for gp41 and shows weaker binding to membranes than 4E10. As it would presumably be less effective in extracting its epitope from the membrane, why would it be much more potent than 4E10 in neutralization? Finally, several gp140 preparations have been reported to bind 4E10 or 2F5 (32, 33). Many preparations of envelope trimers or oligomers used in previous studies have “monomeric gp120-like” characteristics, for example, binding to CD4-induced antibodies in the absence of CD4 and exhibiting high affinity with nonneutralizing CD4 binding site antibodies. They also either aggregate or dissociate into dimers and monomers during expression or purification and probably fail to mimic the native envelope spikes. In contrast, our stable, homogeneous gp140 trimers, despite lacking the cleavage between gp120 and gp41, show a number of properties expected for a native, functional envelope trimer but bind 4E10 or 2F5 only when denatured in a Western blot (15, 19). We demonstrate here that our gp140 trimer also fails to bind native 10E8, fully consistent with the characteristics of the functional envelope trimers expressed on cell surfaces (Fig. 4).

Biochemical studies of protein-membrane interaction are technically challenging. Huang and colleagues did not detect phospholipid binding by 10E8 using SPR with an L1 sensor chip (18). We found that relatively high levels of background binding of hydrophobic proteins to the lipophilic alkyl chains, which capture liposomes on the L1 chip, as detected in the control channel in the absence of detergent, could mask weak signals. Additionally, compositional preference for lipids may also account for lack of binding to certain liposomes by 10E8. We chose for that reason to use three independent approaches to demonstrate lipid binding by 10E8. In particular, for our SPR experiments, we used a streptavidin-coated sensor chip (SA chip) to capture biotinylated liposomes; this sensor chip showed almost no background binding of

these antibodies in the absence of detergent. The liposome flotation assay detects the amount of protein-liposome complexes that survive 3 h of centrifugation. It is therefore not surprising that the signals for both 4E10 and 10E8 binding to membranes are low, because of their relatively high dissociation rate. Dense precipitates due to cross-linking were clearly visible when IgG of 10E8 or 4E10 was first mixed with liposomes, thereby ruling out the possibility that only a small population of the antibody can bind the liposomes.

Our findings that 10E8 and other MPER antibodies neutralize by a common mechanism may guide MPER-based vaccine development and help avoid potentially wasteful efforts to chase elusive immunogens that may not be meaningful. These results emphasize the need for an immunogen with two components: a protein to present the relevant gp41 structure, such as gp41-inter, and lipids to select for lipid binding. In our study, the postfusion gp41 construct containing the intact MPER showed relatively high-affinity binding to both 10E8 and 4E10. Because of its stability, it might be a better vaccine antigen than those studied so far (34–36). Buzon and colleagues reported that 4E10 did not interact with gp41 including the MPER in the postfusion conformation, in agreement with clashes revealed by molecular docking (37). It is possible that the presence of the fusion peptide and transmembrane segment in our gp41 construct has some impact on the local conformation of the 4E10 epitope, as we found that both 4E10 and 10E8 also show binding to our gp41 protein expressed on the insect cell surface (data not shown). Likewise, liposomes with a cholesterol-rich, HIV-1-like lipid composition might be more effective for inducing potent MPER-specific bNAbs than any previously tested lipid-based immunogens (35, 38, 39). We note that a single-chain antibody (VHH), 2H10, recently isolated from llamas immunized with a liposome-based gp41-MPER immunogen, requires a hydrophobic CDR3 for neutralization, but not for antigen recognition (35). The mode of neutralization used by this vaccination-induced antibody appears to be consistent with the general mechanism of HIV-1 neutralization by MPER-specific antibodies we propose here.

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REFERENCES

- Harrison SC. 2008. Viral membrane fusion. *Nat. Struct. Mol. Biol.* 15: 690–698. <http://dx.doi.org/10.1038/nsmb.1456>.
- Chan DC, Kim PS. 1998. HIV entry and its inhibition. *Cell* 93:681–684. [http://dx.doi.org/10.1016/S0092-8674\(00\)81430-0](http://dx.doi.org/10.1016/S0092-8674(00)81430-0).
- Kilby JM, Eron JJ. 2003. Novel therapies based on mechanisms of HIV-1 cell entry. *N. Engl. J. Med.* 348:2228–2238. <http://dx.doi.org/10.1056/NEJMr022812>.
- Cohen MS, Shaw GM, McMichael AJ, Haynes BF. 2011. Acute HIV-1 infection. *N. Engl. J. Med.* 364:1943–1954. <http://dx.doi.org/10.1056/NEJMr1011874>.
- Richman DD, Wrin T, Little SJ, Petropoulos CJ. 2003. Rapid evolution

- of the neutralizing antibody response to HIV type 1 infection. *Proc. Natl. Acad. Sci. U. S. A.* 100:4144–4149. <http://dx.doi.org/10.1073/pnas.0630530100>.
6. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. 2003. Antibody neutralization and escape by HIV-1. *Nature* 422:307–312. <http://dx.doi.org/10.1038/nature01470>.
 7. Mascola JR, Haynes BF. 2013. HIV-1 neutralizing antibodies: understanding nature's pathways. *Immunol. Rev.* 254:225–244. <http://dx.doi.org/10.1111/imr.12075>.
 8. Stamatatos L, Morris L, Burton DR, Mascola JR. 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nat. Med.* 15:866–870. <http://dx.doi.org/10.1038/nm.1949>.
 9. Haynes BF, Fleming J, St Clair EW, Katinger H, Stiegler G, Kunert R, Robinson J, Searce RM, Plonk K, Staats HF, Ortel TL, Liao HX, Alam SM. 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* 308:1906–1908. <http://dx.doi.org/10.1126/science.1111781>.
 10. Haynes BF, Kelsoe G, Harrison SC, Kepler TB. 2012. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nat. Biotechnol.* 30:423–433. <http://dx.doi.org/10.1038/nbt.2197>.
 11. Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* 420:678–682. <http://dx.doi.org/10.1038/nature01188>.
 12. Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, Ruker F, Katinger H. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J. Virol.* 67:6642–6647.
 13. Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F, Katinger H. 2001. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* 17:1757–1765. <http://dx.doi.org/10.1089/08892220152741450>.
 14. Alam SM, Morelli M, Dennison SM, Liao HX, Zhang R, Xia SM, Rits-Volloch S, Sun L, Harrison SC, Haynes BF, Chen B. 2009. Role of HIV membrane in neutralization by two broadly neutralizing antibodies. *Proc. Natl. Acad. Sci. U. S. A.* 106:20234–20239. <http://dx.doi.org/10.1073/pnas.0908713106>.
 15. 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. U. S. A.* 105:3739–3744. <http://dx.doi.org/10.1073/pnas.0800255105>.
 16. Kim M, Sun ZY, Rand KD, Shi X, Song L, Cheng Y, Fahmy AF, Majumdar S, Ofek G, Yang Y, Kwong PD, Wang JH, Engen JR, Wagner G, Reinherz EL. 2011. Antibody mechanics on a membrane-bound HIV segment essential for GP41-targeted viral neutralization. *Nat. Struct. Mol. Biol.* 18:1235–1243. <http://dx.doi.org/10.1038/nsmb.2154>.
 17. Ruprecht CR, Krarup A, Reynell L, Mann AM, Brandenburg OF, Berlinger L, Abela IA, Regoes RR, Gunthard HF, Rusert P, Trkola A. 2011. MPER-specific antibodies induce gp120 shedding and irreversibly neutralize HIV-1. *J. Exp. Med.* 208:439–454. <http://dx.doi.org/10.1084/jem.20101907>.
 18. Huang J, Ofek G, Laub L, Louder MK, Doria-Rose NA, Longo NS, Imamichi H, Bailer RT, Chakrabarti B, Sharma SK, Alam SM, Wang T, Yang Y, Zhang B, Migueles SA, Wyatt R, Haynes BF, Kwong PD, Mascola JR, Connors M. 2012. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. *Nature* 491:406–412. <http://dx.doi.org/10.1038/nature11544>.
 19. Kovacs JM, Nkolola JP, Peng H, Cheung A, Perry J, Miller CA, Seaman MS, Barouch DH, Chen B. 2012. HIV-1 envelope trimer elicits more potent neutralizing antibody responses than monomeric gp120. *Proc. Natl. Acad. Sci. U. S. A.* 109:12111–12116. <http://dx.doi.org/10.1073/pnas.1204533109>.
 20. Freeman MM, Seaman MS, Rits-Volloch S, Hong X, Kao CY, Ho DD, Chen B. 2010. Crystal structure of HIV-1 primary receptor CD4 in complex with a potent antiviral antibody. *Structure* 18:1632–1641. <http://dx.doi.org/10.1016/j.str.2010.09.017>.
 21. Frey G, Chen J, Rits-Volloch S, Freeman MM, Zolla-Pazner S, Chen B. 2010. Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. *Nat. Struct. Mol. Biol.* 17:1486–1491. <http://dx.doi.org/10.1038/nsmb.1950>.
 22. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, Voss G, Goepfert P, Gilbert P, Greene KM, Bilska M, Kothe DL, Salazar-Gonzalez JF, Wei X, Decker JM, Hahn BH, Montefiori DC. 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J. Virol.* 79:10108–10125. <http://dx.doi.org/10.1128/JVI.79.16.10108-10125.2005>.
 23. Mascola JR, D'Souza P, Gilbert P, Hahn BH, Haigwood NL, Morris L, Petropoulos CJ, Polonis VR, Sarzotti M, Montefiori DC. 2005. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. *J. Virol.* 79:10103–10107. <http://dx.doi.org/10.1128/JVI.79.16.10103-10107.2005>.
 24. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG. 2006. The HIV lipidome: a raft with an unusual composition. *Proc. Natl. Acad. Sci. U. S. A.* 103:2641–2646. <http://dx.doi.org/10.1073/pnas.0511136103>.
 25. Aloia RC, Tian H, Jensen FC. 1993. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc. Natl. Acad. Sci. U. S. A.* 90:5181–5185. <http://dx.doi.org/10.1073/pnas.90.11.5181>.
 26. Lorizate M, Sachsenheimer T, Glass B, Habermann A, Gerl MJ, Krausslich HG, Brugger B. 2013. Comparative lipidomics analysis of HIV-1 particles and their producer cell membrane in different cell lines. *Cell. Microbiol.* 15:292–304. <http://dx.doi.org/10.1111/cmi.12101>.
 27. Guyader M, Kiyokawa E, Abrami L, Turelli P, Trono D. 2002. Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. *J. Virol.* 76:10356–10364. <http://dx.doi.org/10.1128/JVI.76.20.10356-10364.2002>.
 28. Ono A, Freed EO. 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc. Natl. Acad. Sci. U. S. A.* 98:13925–13930. <http://dx.doi.org/10.1073/pnas.241320298>.
 29. Scherer EM, Leaman DP, Zwick MB, McMichael AJ, Burton DR. 2010. Aromatic residues at the edge of the antibody combining site facilitate viral glycoprotein recognition through membrane interactions. *Proc. Natl. Acad. Sci. U. S. A.* 107:1529–1534. <http://dx.doi.org/10.1073/pnas.0909680107>.
 30. Dimitrov AS, Jacobs A, Finnegan CM, Stiegler G, Katinger H, Blumenthal R. 2007. Exposure of the membrane-proximal external region of HIV-1 gp41 in the course of HIV-1 envelope glycoprotein-mediated fusion. *Biochemistry* 46:1398–1401. <http://dx.doi.org/10.1021/bi062245f>.
 31. Sun ZY, Oh KJ, Kim M, Yu J, Brusica V, Song L, Qiao Z, Wang JH, Wagner G, Reinherz EL. 2008. HIV-1 broadly neutralizing antibody extracts its epitope from a kinked gp41 ectodomain region on the viral membrane. *Immunity* 28:52–63. <http://dx.doi.org/10.1016/j.immuni.2007.11.018>.
 32. Alam SM, McAdams M, Boren D, Rak M, Searce RM, Gao F, Camacho ZT, Gewirth D, Kelsoe G, Chen P, Haynes BF. 2007. The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J. Immunol.* 178:4424–4435. <http://www.jimmunol.org/content/178/7/4424.full>.
 33. Xu H, Song L, Kim M, Holmes MA, Kraft Z, Sellhorn G, Reinherz EL, Stamatatos L, Strong RK. 2010. Interactions between lipids and human anti-HIV antibody 4E10 can be reduced without ablating neutralizing activity. *J. Virol.* 84:1076–1088. <http://dx.doi.org/10.1128/JVI.02113-09>.
 34. Dawood R, Benjelloun F, Pin JJ, Kone A, Chanut B, Jospin F, Lucht F, Verrier B, Moog C, Genin C, Paul S. 2013. Generation of HIV-1 potent and broad neutralizing antibodies by immunization with postfusion HR1/HR2 complex. *AIDS* 27:717–730. <http://dx.doi.org/10.1097/QAD.0b013e32835cfa5>.
 35. Lutje Hulsik D, Liu YY, Strokappe NM, Battella S, El Khattabi M, McCoy LE, Sabin C, Hinz A, Hock M, Macheboeuf P, Bonvin AM, Langedijk JP, Davis D, Forsman Quigley A, Aasa-Chapman MM, Seaman MS, Ramos A, Pognard P, Favier A, Simorre JP, Weiss RA, Verrips CT, Weissenhorn W, Rutten L. 2013. A gp41 MPER-specific llama VHH requires a hydrophobic CDR3 for neutralization but not for antigen recognition. *PLoS Pathog.* 9:e1003202. <http://dx.doi.org/10.1371/journal.ppat.1003202>.
 36. Montero M, van Houten NE, Wang X, Scott JK. 2008. The membrane-proximal external region of the human immunodeficiency virus type 1 envelope: dominant site of antibody neutralization and target for vaccine

- design. *Microbiol. Mol. Biol. Rev.* 72:54–84. <http://dx.doi.org/10.1128/MMBR.00020-07>.
37. Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W. 2010. Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions. *PLoS Pathog.* 6:e1000880. <http://dx.doi.org/10.1371/journal.ppat.1000880>.
38. Dennison SM, Sutherland LL, Jaeger FH, Anasti KM, Parks R, Stewart S, Bowman C, Xia SM, Zhang R, Shen X, Searce RM, Ofek G, Yang Y, Kwong PD, Santra S, Liao HX, Tomaras G, Letvin NL, Chen B, Alam SM, Haynes BF. 2011. Induction of antibodies in rhesus macaques that recognize a fusion-intermediate conformation of HIV-1 gp41. *PLoS One* 6:e27824. <http://dx.doi.org/10.1371/journal.pone.0027824>.
39. Grundner C, Li Y, Louder M, Mascola J, Yang X, Sodroski J, Wyatt R. 2005. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* 331:33–46. <http://dx.doi.org/10.1016/j.virol.2004.09.022>.