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Immunogenicity of recombinant human immunodeficiency virus type 1-like particles expressing gp41 derivatives in a pre-fusion state

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Abstract

The conserved membrane proximal external region (MPER) of the ectodomain of human immunodeficiency virus type 1 (HIV-1) gp41 is the target of two broadly neutralizing antibodies, 2F5 and 4E10. However, no neutralizing antibodies have been elicited against immunogens bearing these epitopes. Given that structural and biochemical studies suggest that the lipid membrane of the virion is involved in their proper configuration, HIV-1 gp41 derivatives in a pre-fusion state were expressed on the surface of immature virus like particles (VLP) derived from Sf9 cells. Guinea pigs were immunized with three doses of VLPs or Sf9 cells presenting gp41 derivatives with or without *E. coli* heat-labile enterotoxin (LT) as an adjuvant. While immune sera contained high titer anti-VLP antibodies, the specific anti-gp41 antibody responses were low with no neutralizing antibodies detected. An explanation for this absence may be the low level of gp41 expression relative to the many other proteins derived from host cells which are incorporated onto the VLP surface. In addition, the anti-gp41 immune response was preferentially directed to the C-helical domain, away from the MPER. Future vaccine design needs to contend with the complexity of epitope display as well as immunodominance.

Keywords

HIV; gp41; MPER; immunogen; immunodominance; neutralizing antibody; 2F5; 4E10; virus-like particle; vaccine

1. Introduction

Neutralizing antibodies (NAbs) are among the first agents which block viral entry. Direct antiviral activity against HIV is attributed to antibodies directed against specific epitopes on the envelope glycoproteins gp120 and gp41. These NAbs inhibit viral entry by blocking virion

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attachment to its receptors or membrane fusion [1]. During natural infection the effect of the autologous neutralization response appears to be limited, since the virus rapidly escapes the immune pressure in most individuals [2–6]. Therefore, eliciting robust neutralizing antibody responses against diverse HIV strains remains a major obstacle for vaccine development. Nevertheless, rare potent monoclonal antibodies (mAbs) with broad neutralizing activity have been isolated from infected individuals. The two NAb b12 and 2G12 are directed against exterior gp120 protein [7–11], and the three NAb 2F5, 4E10 and Z13 are directed against the transmembrane gp41 protein [12–14]. Passive immunotherapy studies using combinations of some of these mAbs have resulted in protection against intravenous and/or mucosal simian–human immunodeficiency virus (SHIV) challenge in monkeys [15–18]. In addition, 2F5 and/or 4E10 plus 2G12 immunotherapy have resulted in reduced viremia in established HIV-1 infection or delay of HIV-1 rebound in individuals undergoing interrupted antiretroviral treatment (ART) [19,20].

The existence of these broadly neutralizing mAbs provides some hope that a vaccine inducing broadly NAb can be exploited by targeting conserved epitopes on HIV. Among those, 2F5, 4E10 and Z13 recognize adjacent, but distinct linear epitopes within the C-terminal membrane proximal external region (MPER) of the HIV-1 gp41 protein [13,14]. The MPER is a highly conserved region among various HIV-1 isolates and is critical for HIV-1 mediated membrane fusion and infection [21,22]. In an *in vitro* study of cross-clade neutralization using a pseudotyped virus assay, 4E10 appeared to be the broadest neutralizing antibody described to date with activity against isolates from different clades [23]. 2F5 and 4E10 neutralized 80 and 100%, respectively, of 91 viruses that were cloned directly from newly infected patients that had a negative or indeterminate serology [24]. Recently, it was shown that 2F5 and 4E10 mAbs were more potent in inhibiting viruses from acutely infected individuals than viruses from chronically infected patients [25]. However, experiments revealed that Env - pseudotyped viruses were more sensitive to neutralization than were their classical peripheral blood mononuclear cell (PBMC)-grown viruses, against which these antibodies manifest less potent activity with reduced breadth [23,26]. The molecular bases of these observed differences between pseudovirus and PBMC-derived virus remains to be fully explained [27].

Many efforts have focused on immunogens that can induce 2F5-like NAb. In addition to simple linear or structurally constrained peptide immunogens, recombinant protein immunogens including displays of constrained 2F5 epitope in the contexts of various protein scaffolds have been also pursued [28–37]. While inducing high antibody titers against the primary amino acid sequence of the epitope, these immunogens have failed to elicit NAb against primary HIV-1. More recently, some very weak NAb against TCLA strains have been detected with immunizations using bovine papilloma-HIV-1 gp41 chimeric virus-like particles [38] and with the porcine endogenous retrovirus p15E fragment fused with the MPER [39]. However, these approaches will require rigorous evaluation. Collectively, the findings suggest that the conformation of the native 2F5 epitope on the virion requires more than just a single constrained secondary structure and/or linear core epitope sequence to adequately mimic native epitope conformation against which a NAb can be elicited through immunization.

Difficulty in eliciting neutralizing antibodies like 2F5 has prompted studies to elucidate the structure of the MPER segment to which it binds. Nuclear magnetic resonance (NMR) studies of the membrane-proximal region and a crystal structure of the 2F5 Fab in complex with a core 7-mer peptide have shown the core epitope to adopt either a ³10-helix or a β -turn conformation, respectively [40–42]. Later, the crystal structure of 2F5 Fab in complex with a 17-mer peptide, EKNEQELLELDKWASLW revealed that this portion of the MPER is rather in an extended conformation with a distinct Type I β -turn at the ‘DKW’ in the core of the peptide epitope, and further biochemical analyses confirmed the importance of the lipid membrane and hydrophobic context for the binding of 2F5 and 4E10 [43,44]. To date, few immunogenicity studies focused

on the 4E10 epitope have been reported. The extended helical structure of an MPER peptide, KWASLWNWFNITNWLWYIK in DPC micelles was also revealed by an NMR study offering a model of possible interaction of this region with the membrane [45]. More recently, a crystal structure of a 4E10 Fab in complex with a peptide bearing the sequence, WNWFDITNW revealed its major contact residue segment, WFDIT to be in a helical conformation [46]. A further study showed improved 4E10 binding affinity to a helix-stabilized peptide relative to the starting untethered peptide [47].

Although the structures of gp41 on the virion in CD4-unligated or fusion intermediate states are currently unavailable, the above observations provide a clue as to a more optimized immunogen design; the conformation of 2F5 and 4E10 neutralizing epitopes may be induced better in a membrane environment than in solution. Given that mAb 2F5 favorably binds to its epitope in native gp160 and/or transient fusion intermediate state of gp41 [48–50], we have constructed three different gp41 variants in a prefusion state and presented these target epitopes in the MPER on the surface of HIV VLP. We report here an immunogenicity study of the VLP/gp41 variants and the attendant complexities of epitope display and immunodominance observed.

2. Materials and Methods

2.1. Production and purification of HIV–1 VLPs

Recombinant baculovirus (rBV) expressing membrane bound gp140 4cSSL24 was constructed as previously described in detail [51], but with further additional modification by overlapping PCR to append the MPER region and transmembrane domain (TMD) of gp41 to the C-terminus of 4cSSL24. The rationale for individual constructs is as detailed in the Results and Discussion section to follow. The cytoplasmic domain of gp41 was deleted to enhance the production of protein [52]. The rBV expressing membrane bound gp41d4mt consisted of gp41 in which the fusion peptide and cytoplasmic domain of gp41 were removed, and 4 residues (V549, L556, Q563, and V570) within the N-helix (residue 546–581) were mutated to aspartic acid. The loop region between N- and C-helices including upstream and downstream flanking residues (residue 582–627) was also replaced by a 5 residue linker (GSSGG) in gp41d4mt. The plasmid harboring the 5helix (D4) variant (kindly provided by Peter Kim and Michael J. Root) was used as template for overlapping PCR. The rBV expressing BAFF-C56 was constructed by overlapping PCR. The plasmid harboring human BAFF (residues 136–285) was a kind gift from Biogen and was used as a template to amplify BAFF. The C56 segment (residues 628–683) including the TMD of gp41 from ADA was amplified and fused to the C-terminus of BAFF by overlapping PCR. The full length Pr55^{gag} was amplified by PCR using a plasmid pTM1 harboring the gag-GFP fusion gene, kindly provided from the AIDS Research and Reference Reagent Program. The final sequence of all constructs was verified by DNA sequencing.

The recombinant baculovirus DNA was used to transfect Sf9 insect cells, and supernatants were collected at 48 to 72 h post-transfection, followed by virus amplification according to manufacturers' protocol (Bac-to-Bac baculovirus expression system, Invitrogen). For large scale production of VLPs, Sf9 insect cells were co-infected with rBV expressing HIV-1HXB2^{gag} at a multiplicity of infection (m.o.i.) of 5 and rBV expressing gp140 4cSSL24, BAFF-C56 or gp41d4mt at a m.o.i. of 10, respectively. At 3 days post-infection, the culture medium was collected and centrifuged at 2500 rpm for 20 min. The supernatant was then filtered through a 0.45 µm filter and VLPs were pelleted at 37,000 rpm (120,000 × g) for 2 h at 4 °C. The sample was then layered on top of a 20% sucrose cushion and spun for 2 h at 28,000 rpm in an SW40 rotor. The pellet was resuspended in 200 µl of either PBS or 10mM Tris pH 7.4, layered on top of either 12–30% modified OptiPrep (60% [wt/vol] iodixanol; Life Technologies) velocity gradient containing NaCl in 10mM HEPES pH 7.4 or 14–30% modified

OptiPrep gradient containing sucrose in 10mM Tris pH 7.4/1mM EDTA and centrifuged for 3 h at 37,000 rpm in an SW41 Ti rotor. 0.9 ml of each fractions were collected from the top of the gradient. The efficient incorporation of Env protein was determined by Western blot. Protein concentrations of purified VLPs were determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

2.2. Immunizations in guinea pig

Out-bred guinea pigs were purchased from Charles River Laboratory and housed at Biacom Inc. (Rockville, MD). Animals were studied under an animal use protocol approved by the Animal Use and Care Committee in Maryland. Female guinea pigs, each group consisting of five animals were administered either Sf9 cells (5×10^6 cells/guinea pig) or purified VLPs at various doses ranging from 2 μ g to 10 μ g, by the i.m. or i.d. route in the presence or absence of adjuvant LTG33D (IOMAI). The immunization schedule was based on a three-dose regimen with two boosters every two week apart. Serum samples were collected 10 days after each immunization and stored at -20°C until use in ELISA and neutralization assays.

2.3. Neutralization assays

Neutralization was measured as a function of reductions in luciferase reporter gene expression after a single round of infection in TZM-bl cells as described [26,53]. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Briefly, 200 TCID₅₀ of virus was incubated with serial 3-fold dilutions of serum sample in triplicate in a total volume of 150 μ l for 1 hr at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 μ l of growth medium containing 75 μ g/ml DEAE dextran) were added to each well. Indinavir was added at a final concentration of 1 μ M to prevent virus replication in the case of HIV-1 MN and ADA. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After a 48 hour incubation, 100 μ l of cells was transferred to a 96-well black solid plates (Costar) for measurements of luminescence using Bright Glo substrate solution as described by the supplier (Promega). An assay stock of HIV-1 MN was prepared in H9 cells. Assay stocks of the Env-pseudotyped virus, SF162.LS, and the full-genome clone, ADA, were prepared by transfection in 293T cells [26]. All virus stocks were made cell-free by 0.45-micron filtration and were stored at -70°C until use.

2.4. ELISA

Antibody titers specific for VLPs used in immunization study were measured by ELISA. Ninety-six well plates (Dynex, Alexandria, V a.) were coated at 100 μ l/well containing 20 μ g of purified VLPs in PBS and incubated overnight at 4°C . The following day, the plates were blocked with PBS containing 4% bovine serum albumin. Serum samples were incubated for 2 h at 37°C , washed four times with PBS containing 0.05% Tween 20, and then incubated with a 1:2000 dilution of peroxidase conjugated goat anti-guinea pig IgG for 1 h at 37°C (Sigma Chemical Co., St. Louis, Mo.). The specific antibody titers for HIV-1ADA gp41 was measured in standard ELISA assay. The gp140 or 4cSSL24 proteins were coated overnight at 100 μ l/well each at a concentration of 2 μ g/ml. Additional ELISAs were performed with 6-helix protein (300 ng/well) and peptide corresponding to the MPER of HIV-1 MN gp41 (ELDKWASLWNW FNITNWLWYIK) or of ADA gp41 (ALDKWASLWNWFDISNWLWYIK). Peptides were absorbed to 96 well plates at 300 ng/well overnight at 4°C . Bound antibodies were detected using horseradish peroxidase-conjugated anti-guinea pig or anti-human IgG at a wavelength of 490 nm.

2.5. Western blotting

To test the incorporation of gp41 variant protein into VLP, particulate antigens were purified by either of two Optiprep gradients as described above. Ten gradient fractions were collected and subjected to SDS-PAGE followed by western blotting. The incorporation of gp41 variant protein was determined by incubation of membrane with 2F5 antibody and mouse anti-p24 antibody (Advanced Biotechnologies, Columbia, MD) for 2h followed by detection using horseradish peroxidase-conjugated anti-mouse or anti-human IgG antibodies. To investigate the antigenic map of gp41 with a panel of immune sera from guinea pigs immunized with VLP/gp41 variants, GB1-GCN4-C56 fusion protein or a peptide corresponding to the MPER sequence of the ADA were subjected to SDS-PAGE and western blotting. Peptides were run on 10% Tris-N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl] glycine (Tricine) gel. Each immune sera was incubated either with GB1-GCN4-C56 at a 1:100 dilution or with peptide at a 1:20 dilution for 2h at RT. The fusion protein GB1-GCN4-C56 was expressed in *E. coli* and purified by human IgG affinity column chromatography. N-terminally acetylated and C-terminally amidated peptides were synthesized using standard synthesis protocols and purified to homogeneity by reverse phase high-performance liquid chromatography using a C5 column. The predicted peptide sequence was confirmed by mass spectrometry.

2.6. Analysis of antibody binding by flow cytometry

Sf9 cells were infected with each rBV expressing gp140 4cSSL24, BAFF-C56 or gp41d4mt at an m.o.i. of 10 for 2 days. After being washed thoroughly, infected cells (1×10^6) were incubated with 100–200 ng of each antibody 2F5, 4E10, 50–69 or 98-6 at 4°C for 1hr. After washing, FITC-conjugated mouse anti-human IgG (Sigma) diluted at 1:100 was added and incubated for 30 min at 4°C. The cells were then washed twice and analyzed by FACS Caliber flow cytometer using CellQuest software (Becton Dickinson, San Jose, Calif.).

2.7. Electron microscopy

Sf9 insect cells were co-infected with rBV expressing HIV-1HXB2^{gag} at a multiplicity of infection (m.o.i.) of 5 and rBV expressing gp41d4mt at a m.o.i. of 10 for 3 days and supernatants were purified by Optiprep density gradient, as described above. The fractions were diluted in PBS buffer, centrifuged, fixed in 2.5% buffered glutaraldehyde, and stored in 0.01M phosphate buffer. Specimens were enmeshed in 2% agar, processed for embedding in epoxy resins according to published protocols [54], sectioned, and examined in a Philips transmission electron microscope EM410LS.

2.8. Surface plasmon resonance measurements of peptide binding to large unilamellar vesicles (LUV)

Biosensor experiments were carried out with a Biacore 3000 using the Pioneer L1 sensor chip (Biacore AB, Uppsala, Sweden) at 25 °C. The Pioneer L1 sensor chip is composed of alkyl chains covalently linked to a dextran-coated gold surface. The running buffer was 0.01M HEPES buffer; the washing solution was 40mM CHAPS, and the regeneration solution was 10 mM sodium hydroxide. All solutions were freshly prepared, degassed, and filtered through a 0.22 µm filter. The LUV (100 nm) of 1, 2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) were prepared in 0.01 M HEPES buffer by extrusion. Briefly, dry DMPC was dissolved in ethanol-free chloroform. The solvent was evaporated under a stream of nitrogen, and the lipids were held under vacuum overnight. The lipids were then resuspended in 0.01 M HEPES buffer via vortex mixing. The resultant lipid dispersion was then extruded 20 times through polycarbonate filters to obtain LUV at 100 nm size.

The Biacore instrument was cleaned extensively and left running overnight using Milli-Q water to remove trace amounts of detergent. The surface of the L1 sensor chip was cleaned by

an injection of the nonionic detergent 40 mM CHAPS (25 μ l) at a flow rate of 5 μ l/min. LUV (80 μ l, 0.5 mM) was applied to the sensor chip surface at a low flow rate, and the liposomes were captured on the surface of the sensor chip by the lipophilic constituents and provided a supported lipid bilayer. To remove any multilamellar structures from the lipid surface, sodium hydroxide (30 μ l, 10 mM) was injected at a flow rate of 50 μ l/min, which resulted in a stable baseline corresponding to the immobilized liposome bilayer membrane.

Peptide solutions (4 μ M) were prepared by dissolving each peptide in 0.01 M HEPES buffer, pH 7.4. The solution (80 μ l) was injected over the lipid surface at a flow rate of 5 μ l/min, and antibody solution was passed over peptide-liposome complex for 3 min at a flow rate of 5 μ l/min. Since the peptide-lipid interactions are very hydrophobic, the regeneration of the liposome surface was not possible. The immobilized liposomes were therefore completely removed with an injection of 40 mM CHAPS, and each peptide injection was performed on a freshly prepared liposome surface. All binding experiments were carried out at 25 °C.

3. Results

3.1. Constructs, expression and production of VLP/gp41 variants

To investigate whether NABs like 2F5 and 4E10 can be raised against immunogens incorporating the MPER on a biomembrane surface, three different gp41 variant constructs were made as shown in Fig. 1. Design considerations include the need for a lipid environment for proper configuration of epitopes in the MPER, the exclusion of dominant immune responses to cluster I (residue 579–613) and II (residue 644–663) regions during natural HIV-1 infection and the requirement to present epitopes expressed on gp41 in a pre-fusion state. First, the gp140 4cSSL24 protein consists of gp41 covalently associated with the C1 and C5 region of gp120 and virtually all other segments of gp120 was replaced by SH3 domain from CD2BP1, a cdc15-like adaptor protein, which binds to the cytoplasmic tail of CD2 and regulates CD2-triggered adhesion as previously described for 4cSSL24 [51]. Unlike 4cSSL24, gp140 4cSSL24 incorporates the MPER and TMD of gp41. The native SH3 domain in gp140 4cSSL24 was verified by the conformation- dependent mAb 8c9 (Qiao et al., 2005 and data not shown). Second, gp41d4mt was constructed to mutate four residues in the N-domain (V549, L556, Q563, and V570) to aspartic acids thereby preventing gp41d4mt from forming a fusogenic 6-helix bundle comprised of antiparallel packing of N – and C-domains [55]. In addition, the fusion peptide and immunodominant cluster I region including the loop (582–622) and the cytoplasmic domain were removed. Third, the chimeric BAFF-C56 comprises a homotrimeric, extracellular domain fragment of human B cell activating factor (BAFF, residue 136–285) fused to the N-terminus of the C- domain of gp41, linking BAFF to HR-2, MPER and the TMD of gp41 (residues 628–683). In essence, gp120 and the HR-1 of gp41 have been replaced by the trimeric BAFF domain in this construct. BAFF plays an important role in peripheral B-cell survival, a crucial function that has a considerable impact on B-cell maturation, immune responses and the pathogenesis of autoimmune diseases [56]. BAFF can potentiate antibody responses and increase the number of plasma cells [57,58]. In fact, excessive BAFF production seems to be able to subvert B-cell tolerance [59,60]. In view of the dramatic induction of IgA responses to Pneumovax in BAFF- treated mice [58], we speculated that BAFF may be a beneficial factor that enhances immune responses with an adjuvant-like effect in the modulation of BAFF levels.

Each of the three VLP-gp41 constructs was produced in a baculovirus expression system by co-infection with rBV HIV HXB2 Pr55^{gag}. VLP/gp41d4mt and VLP/BAFF-C56 were subsequently purified by Optiprep-sucrose (14–30% iodixanol) velocity gradient and VLP/gp140 4cSSL24 by Optiprep-NaCl velocity gradient ranging from 12–30%. As shown in Fig. 2A, most of the gp140 4cSSL24 proteins were found in density gradient fractions ranging from 1.08 to 1.14 g/ml, while the viral Gag protein distribution was present at the density ranging

from 1.08 to 1.13 g/ml. Analysis of collected fractions by immunoblotting with 2F5 antibody and anti-p24 antibody also revealed coincidence of Pr55^{gag} precursor and fragments and the gp41d4mt or BAFF-C56 proteins in Optiprep-sucrose fractions 2–7 at a density ranging from 1.1–1.15 g/ml (Fig. 2B for gp41d4mt and data not shown with BAFF-C56). The concentration of the gp41 variant proteins incorporated into VLP was calculated by western blot analysis with 2F5 and comparison with a calibration curve obtained by 2-fold dilution of purified soluble BAFF-C56 proteins. Approximately, 0.1 µg to 1 µg of particle-entrapped gp41 variant proteins per 100 µg of purified VLP preparations were expressed (data not shown). Further analysis of purified VLP/gp41dmt4 by EM (Fig. 2C) indicated that the morphology and the size of purified VLP/gp41dmt4 was similar to those of HIV-1 VLPs previously reported [61]. Some baculovirus particles were observed by EM in the VLP preparations. However, given that gp41 variants were expressed on the surface of baculovirus derived from the same host Sf9 cells, the purified VLPs preparations were suitable to test the immunogenicity of gp41 variants intended to elicit 2F5- and 4E10- like neutralizing antibodies.

3.2. Antigenic properties of gp41 variants on the Sf9 cell surfaces

Next, to test antigenicity of the three molecularly engineered gp41 variants on the cell surface, their reactivity with several mAbs was assessed by flow cytometric analysis. For that purpose, Sf9 cells were infected with rBV expressing each gp41 variant at a m.o.i of 5 and infected cells were harvested at day 2 post infection for immunofluorescence analysis. This early time point avoids rBV mediated cytopathic effects. All gp41 variants were expressed on the cell surface as judged by a panel of anti-gp41 specific antibodies and mean fluorescence intensity (MFI). In contrast, wild type baculovirus infected Sf9 cells were not recognized by any of the anti-gp41 specific antibodies (Fig. 3 and data not shown). The surface level of gp140 4cSSL24 stained by 2F5 was comparable to gp160 with MFI of 485 and 531, respectively, whereas somewhat lower expression levels of gp41d4mt and BAFF-C56 were observed with MFI of 395 and 389. We further observed that the targeting of gp41 variants to the surface of Sf9 cells was not affected by the replacement of transmembrane domain of gp41 with EBV gp220/350-derived transmembrane domain [52] in the absence and presence of co-infection with rBV Pr55^{gag} (data not shown). In addition, the epitope exposure and accessibility to 2F5 and 4E10 antibody of gp41 variants expressed on the Sf9 cell surface is evident in Fig. 3A. On the other hand, gp41d4mt and BAFF-C56 were not recognized by antibody 50–69 as evidenced by MFI of 13 and 15, whereas the recognition of gp140 4cSSL24 by 50–69 was quite comparable to gp160 with MFI of 531 and 441, respectively, (Fig. 3B). The 50–69 antibody recognize immunodominant cluster I (residue 579–613) spanning sequences containing two cysteine residues located in the loop connecting the N- and C- domain of gp41. Note that this epitope is not present in the gp41d4mt and BAFF-C56 proteins employed here (Fig. 1). We further observed that gp41d4mt was not recognized by antibody NC-1, whereas a 6-helix protein thought to be representative of gp41 in the post fusion configuration was recognized well by this antibody, indicating that the gp41d4mt is different from 6-helix bundle (data not shown). The gp41 variants tested by antibody 98-6 mapping to immunodominant II region (residue 644–663) spanning sequences N-terminal to the 2F5 epitope were detected well except for the BAFF-C56. The reason for this lack of reactivity may be due to structural constraints resulting from covalent linkage between BAFF and HR-2 in BAFF-C56. In fact, this apparent constraint reduced BAFF's ability to bind to its receptor, soluble BCMA, to a level too low to be detected (data not shown)

3.3. Immunogenicity of the VLP/gp41 variants

To assess the humoral immune response elicited by the three VLP/gp41 variants, groups of guinea pigs were primed with either 2 µg or 10 µg of each VLP/gp41 variant via either intradermal or intramuscular routes, in the absence or presence of the adjuvant LT (G33D), followed by two successive boosts two weeks apart (Table 1). A group of guinea pigs was

injected with PBS as a negative control. High titer antibody responses were observed with maximal endpoint titers ranging from 1×10^4 to 2×10^4 in all groups of animals immunized with VLP or Sf9 transfectant. The results of an ELISA that employed VLP/gp41d4mt as the coating antigen is shown in Fig. 4. No significant differences in the antibody titers were observed between groups immunized with 2 μ g of antigens and groups immunized with 10 μ g of antigens. The adjuvant LT(G33D) showed no effect on the ensuing anti-VLP/gp41 variants antibody titers, suggesting that the VLP stimulates innate and adaptive immune responses without the requirement for additional adjuvants [62]. PBS alone as negative control induced negligible anti-VLP/gp41 d4mt antibody titer (Fig. 4). Although the immunogenicity data of the VLP/gp41 derivatives were shown against only VLP/d4mt and gp41 specific antibody titer against 4cSSL24 in Fig. 4 in an attempt at brevity, antibody titers were measured against VLP/4cSSL24 as well. The antibody titers directed to VLP/4cSSL24 immunogen were of similar magnitude to those directed to VLP/d4mt. The specific 2F5 antibody reactivity was observed with VLP/4cSSL24 at O.D of 0.986, whereas background nonspecific reactivity was observed with VLP/gag at O.D of 0.30 at 490 nm (data not shown). To measure anti-gp41 antibody titer, gp140, 4cSSL24 or peptide corresponding to the MPER of ADA were coated on the 96 well plates. Note that both gp140 and 4cSSL24 lack the MPER segment. Therefore, the 98.6 antibody was used as a control for ELISA and was reactive with 4cSSL24 antigens (data not shown). The specific antibody titers directed to gp41 was low with maximal endpoint titers at about 1 to 8×10^2 in all animal groups, regardless of antigens used in ELISA assay. No further increment of anti-gp41 antibody titers were observed with immunization of guinea pigs with 100 μ g of VLPs (data not shown). The higher titers of gp41 binding antibodies were elicited by immunization with infected Sf9 cells expressing gp140 4cSSL24 rather than with VLP/gp140 4cSSL24. The basis of this differences remains to be elucidated. However, no neutralizing activity was detected against MN, SF162.LS, ADA or NL-ADAr in any of the sera (Table 2). The MN and the SF162.LS are known to be highly sensitive to neutralization by 4E10 and, as expected, yielded herein an ID50 of 0.01 and 0.3 μ g/ml, respectively in a luciferase reporter gene assay in TZM-b1 cells. The ADA was less sensitive to neutralization by 4E10 (ID50 of 6.6 μ g/ml) than CD4 independent, NL-ADAr (ID50 of 0.9 μ g/ml) (unpublished data).

3.4. The MPER is weakly immunogenic

Since no NAb were elicited by immunization of VLP/gp41 variants, we further addressed whether the lack of observed neutralization was a result of insufficient antibody titer directed to the MPER or the result of an irrelevant conformation of the MPER in the context of immunogens tested in these experiments. For that purpose, we have used a panel of immune sera from all animal groups to perform antigenic mapping of gp41. Given that 2F5 and 4E10 react well with linear epitope sequences both in native and denatured condition, pooled immune sera (at 1:100 dilution) from 5 animals per each group were tested against the fusion protein GB1-GCN4-C56. GB1-GCN4-C56 consists of the protein G B1 domain fused at its C-terminus with GCN4 followed by HR-2 and MPER (C56) of ADA. In addition, peptide corresponding to the MPER sequence of ADA was interrogated at 1:20 in denatured SDS-PAGE followed by western blot. As shown in Fig. 5A, all animal groups elicited antibodies directed to the HR2 region, whereas no antibody was generated against the ADA MPER. Of note, both 2F5 and 4E10 antibodies were reactive against GB1-GCN4-C56 (data not shown) and the MPER, respectively. The same result was observed with immune sera from guinea pigs immunized with VLP/ADA gp160 in pilot experiments (data not shown), indicating that lack of antibody reactivity to MPER peptide with anti-VLP/gp41 variants sera may not be due to the result of an irrelevant conformation of the MPER in the context of VLP/gp41 variants immunogens. Neither control animal group immunized with PBS buffer nor animal group immunized with mock Sf9 cells elicited antibodies directed to HR2 or MPER (data not shown). In a subsequent experiment, two immune sera (#307 and #309) from group 1 were tested against either 6-helix protein or peptide corresponding to the MPER sequence of MN. These immune

sera were generated by immunization of Sf9 cells/gp140 4cSSL24 and elicited the highest specific anti-gp41 antibody titers among all animals tested. Both ELISA and BIAcore assays were used for detection taking into consideration of possible difference in secondary structure of peptides under experimental conditions. Immune sera (#314) raised from mock Sf9 cells was compared as a negative control. No immune sera were reactive with the MPER peptide by ELISA, while antibodies reactive with 6-helix proteins were detected both in 307 and 309 sera at the antibody titers of 1:50–1:100 (Fig. 5B). By BIAcore, purified polyclonal antibodies reactive with fusion inhibitor DP-178 (residue 638–673, also known as T20) were detected in 307 sera at titers ranging from 1:20 to 1:100, but not with the MPER peptide (Fig. 5C). The 314 control immune sera was tested against DP 178 and were unreactive as expected (data not shown).

The 2F5 and the 4E10 antibody were passed over either liposome/DP-178 or liposome/MPER peptide complexes as positive controls. Similar BIAcore results were observed using immune sera 309 and immune sera raised against VLP/gp41d4mt or VLP/BAFF-C56 (data not shown). The non-specific binding to uncomplexed liposomes of polyclonal antibodies purified from 307 and 314 sera was subtracted from the specific antibody binding to liposome/D P 178 or liposome/MPER peptide complexes, respectively. These results indicate that the MPER is weakly immunogenic and that our modification of gp41 in each of the three constructs was not sufficient to redirect the immune response toward this segment, despite partial removal of the immunodominant region.

4. Discussion

During natural infection, non-neutralizing antibodies appear to dominate the antibody response against HIV-1 gp41. On the other hand, NABs that target the MPER of gp41 are rare in infected individuals, suggesting that such epitopes are weakly immunogenic [28,63–66]. Many gp41 antibodies are raised against the membrane-distal immunodominant epitope I (residue 597–613) and immunodominant epitope II (residue 644–663) regions and are incapable of mediating viral neutralization [67–69]. This may be explained by the fact that the non-covalent interaction of gp120 with gp41 is labile and CD4 binding to gp120 stimulates further shedding of gp120 from the functional envelope spike. Upon dissociation from gp120, gp41 assumes a post-fusion state [70] with concomitant exposure to the immune system of gp41 surfaces that are occluded in the context of the functional trimer, favoring antibody response to irrelevant epitopes [50, 71,72].

Many vaccine studies have pursued immunization with gp160 or gp140 in different contexts. The antibodies elicited by gp140 react relatively more to the non-neutralization sites within the gp120 than gp41 [73,74]. Furthermore, important neutralizing epitopes in gp41 are absent from soluble gp140 protein immunogens because of protein aggregation attributed to the hydrophobic properties of the MPER [51,75]. No NABs directed to gp41 were elicited with these immunogens lacking the MPER segment. In addition, when gp41 and its truncated fragments containing the MPER were used as immunogens, we found that such fragments formed either insoluble or soluble aggregates, consistent with other reports [51,70,76,77]. Not surprisingly, it is difficult to obtain conformationally relevant antibodies against MPER in the context of such aggregated proteins. Furthermore, the outcome of vaccine studies with immunogens targeted to the 2F5 epitope have suggested that there is greater conformational complexity of the epitope on the virion than revealed by current structural studies [44]. Hence, in addition to the practical difficulties mentioned above in immunogen design, the possible contribution of lipid membrane requirements for native configuration of the MPER provides impetus both for further modification of gp41 and expression of immunogens on a membrane surface.

In this study, we have reported on the immunogenicity of modified gp41 proteins using three different constructs. We examined whether the native conformation of the MPER could be displayed when incorporated into a membrane in the context of a modified pre-fusion or intermediate state gp41, distinct from a post-fusion configuration. To this end, we evaluated immune sera using these immunogens for the existence of NAbs. We addressed whether modification of gp41 via protein engineering could enhance the immune response to a poorly immunogenic target by elimination of certain immunodominant or irrelevant epitopes.

We previously constructed 4cSSL24 protein in which the majority of gp120 had been removed while retaining C1 and C5 regions to preserve gp41 in a non-fusogenic state [51]. No neutralizing epitopes in N- and C-domains of gp41 were identified through immunogenicity studies of 4cSSL24, although high titer antibodies were elicited against gp41. Since the MPER of gp41 is absent, and hypervariable, redundant, immunodominant epitopes of gp120 that decoy the ability of immune response to focus on more protective targets are removed from 4cSSL24, for this current study, MPER and the transmembrane domain of gp41 were appended to the 4cSSL24 C-terminus and expressed on the surface of VLP as gp140 4cSSL24. Given the absence of structural information about gp41 in pre-fusion and/or intermediate states and the knowledge that antibodies are elicited to immunodominant epitopes during natural infection without protective value to the host, two additional constructs were made to remove the irrelevant epitopes while keeping gp41 from adopting a 6-helix bundle configuration. Gp41d4mt is comprised of 36 residues (N36) of the N-domain, the entire C-domain, the MPER and the TMD of gp41 and a linker replacing the immunodominant loop between the two helical regions. Mutations at 4 residues in the N36 of gp41d4mt were introduced to prevent 6-helix formation. In BAFF-C56, the entire N-domain of gp41 was eliminated and replaced by the B AFF ectodomain.

All three proteins were surface-expressed as judged by FACS analysis, showing that the 2F5 and the 4E10 mAbs could bind to their respective epitopes when expressed on insect cells using rBV. As the VLP derives from exosomal budding from these insect cells, the recombinant proteins are exposed on the surface membrane of VLP. Although these VLP candidates were strongly immunogenic, eliciting high titer antibody without any adjuvant requirement, the specific antibody titers directed to gp41 were low as is generally observed in DNA or viral vector vaccines. This low titer may be a consequence of the limited expression levels of the target antigen compared to the many diverse number of endogenous surface proteins derived from the host cell exosomal pathway, raising a concern about the poor immunogenicity of the target antigen in general. Note that quantities of the particle-entrapped gp41 variant proteins ranged from 0.05 μ g to 1 μ g per 100 μ g of purified VLP preparations. More importantly, no neutralizing activity was detected from immune sera tested against MN, ADA, SF162.LS and NL-ADAr strains. Furthermore, the dominant anti-gp41 antibody responses in all tested sera were directed to regions of gp41 other than the MPER. No antibodies reactive with peptides corresponding to the highly conserved MPER were detected by western blot, ELISA or BIAcore assays (despite testing of sera at high concentrations, i.e. only a 1:20 dilution). In fact, the amount of 4cSSL24 antigen expressed on the surface of Sf9 cells used for guinea pig immunization was ~4.0 to 50 μ g per 5×10^6 cells. This range is comparable to the amount of soluble GB1-GCN4-C56 fusion protein used to immunize guinea pigs in our previous study. Furthermore, immunogenicity study with immune sera from guinea pigs immunized with GB1-GCN4-C56 (50 μ g/injection, prime and two boosts) showed that antibody directed to the MPER was not induced even at the titer of 1:10 by sensitive western blot assay, whereas antibodies directed to DP178 were induced at titers of 1:5000 (data not shown).

Although the 2F5 and 4E10 mAbs themselves bind to the MPER, perhaps most immunogen-elicited antibodies may avoid this segment. The membrane proximity of the segment could be difficult to access by antibody and even in part be occluded by other membrane structures.

Alternatively, some non-neutralizing antibodies to irrelevant conformations of the MPER may be elicited at a very low level and/or recognize conformations different from that of antigens tested in this assay, but are not favored, given that all immune sera including sera from guinea pigs immunized with VLP/gp160 showed no antibody reactivity to MPER peptide. The poorly immunogenic nature of 2F5 and 4E10 epitopes was recently interpreted to be due to autoantigen mimicry of these conserved epitopes [78]. Haynes et al. have noted that all of HIV-1 NAb have extended CDRH3 loops, typical of autoantibodies, and that these mAbs cross-react with human antigens targeted during autoimmune responses [78]. On the other hand, our result is inconsistent with a recent observation employing a fluorescence-based binding assay to determine antigenic maps to gp41 among several seropositive individuals. In that study, no region of the gp41 ectodomain was immunologically silent [79]. Nevertheless, no correlation between neutralizing titer and total antibody titer against any of the gp41 antigens tested was further shown. In another study, an immunodominant site was identified in which the residues WNWFDI were most critical for mAb recognition from patient sera [80]. The basis for the differences between these data remains to be resolved.

Both antigenicity and immunogenicity of a protein can change due to the context in which an epitope is presented, emphasizing the importance of antigen display, especially in the face of structural complexity. The pattern of immunodominance of one response over another might therefore be altered. Modifications of Env immunogens through deletion of variable loops [81–83] or elimination of carbohydrate attachment sites [84,85], for example, have been made to improve immunogenicity of functionally relevant epitopes. Alternatively, introduction of glycosylation sites or other site-specific mutations have been performed in an attempt to redistribute immune responses away from unwanted dominant epitopes towards neutralizing sites of relevance [86,87]. To date, these strategies as well as our own have not yielded desired immunogenicity to key epitopes. Despite our current inability to elicit neutralizing antibodies employing the gp41 immunogen variants in the current vaccination protocol, it is possible that enhanced antigen expression *per se* may foster a higher titer immune response to the targeted epitopes capable of mediating viral neutralization. More likely, however, further protein engineering needs to be undertaken to focus antibody onto the target epitopes. Our results suggest that for success, future vaccine design needs to consider the complexity of epitope display and attendant immunodominance.

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HIV_{ADA} gp41 immunogen constructs for VLP expression from Sf9 insect cells

Fig. 1. Schematic representation of the gp41 variant constructs

The segments of the ADA HIV-1 gp160 precursor are shown at the top and labeled. Conserved (C) and variable (V) regions are labeled sequentially. The fusion peptide (FP), N- and C-terminal heptad repeat (HR-1, HR-2) and transmembrane (TM) regions are shown. The C-terminal ectodomain segment of e-gp41 (CTE) refers to the MPER of gp41. For clarity, the numbering of ADA gp160 follows that of HXBc2. Potential N-linked glycosylation sites are represented by small tree marks. Fusion peptide replacement by a 24 linker sequence and insertion of the SH3 domain from CD2BP1, an adapter protein binding to the cytoplasmic tail of CD2 are indicated in 4cSSL24. Mutations of each of four residues in the N-domain (V549, L556, Q563, and V570) to aspartic acid are indicated at the top of HR1 and replacement of the loop region by a 5 residue linker sequence is also shown in gp41d4mt. HM leader refers to the honeybee melittin secretion signal sequence. Gag is comprised of matrix protein (MA), capsid protein (CA), two spacer peptides (SP1 and SP2), nucleocapsid (NC) and proline rich protein (p6).

Fig. 2. Production of Pr55^{gag}/gp41 variant virus-like particles (VLP) used for immunization
 (A) OptiPrep velocity gradient analysis of VLP. HIV HXB2 Pr55^{gag} and gp140 4cSSL24 protein were co-expressed in Sf9 cells after coinfection with rBVs expressing gag and gp140 4cSSL24. Supernatants were harvested 3 days postinfection. Concentrated particulate antigens harvested through a 20% sucrose cushion were layered onto Optiprep/NaCl velocity gradient (12–30%). Eleven gradient fractions were collected with fraction 1 referring to the top of the gradient and assayed for gag and gp140 4cSSL24 contents by western blotting using 2F5 and anti-p24 antibody. Bands of gag and gp140 4cSSL24 were quantified by densitometry using Kodak ID software. The amounts of gag and gp140 4cSSL24 in each fraction were represented by the normalized mean intensity values of the region of interest (ROI) of each band. Gradient fraction densities are given. (B) Distribution of total gag and gp41d4mt in a representative Optiprep/sucrose gradient ranging from 14 to 30% as analyzed by western blot with anti-p24 and 2F5 antibodies. (C) Electron micrograph of purified VLP/gp41d4mt. Spherical VLPs (arrows) contain a peripheral electron- dense layer of Gag proteins. Bar, 100nm.

Fig. 3. Expression and epitope exposure of gp41 variants on the surface of Sf9 cells
Sf9 cells infected with rBV expressing gp140 4cSSL24, gp41d4mt, BAFF-C56 or gp160, respectively, for 2 days at a m. o. i. of 5 were stained for FACSscan analysis with mAbs 2F5 and 4E10 (A) or 50-69 and 98-6 (B), and then FITC-conjugated anti-human IgG antibody. Filled histograms represent background staining of wild type baculovirus infected Sf9 cells with 2F5 in A or 50-69 in B. Fluorescence intensities are indicated in log scale.

Fig. 4. Immunogenicity of VLP/gp41 variants

Antibody binding to VLP and the gp41 variants was evaluated 10 days after the third immunization with each VLP/gp41 variant. ELISA plates were coated with either VLP/gp41d4mt or 4cSSL24 proteins. Mean values of the end-point titers of immune sera $\pm$ standard error from five guinea pigs per group are plotted as log₁₀ arithmetic values.

Fig. 5. Analysis of MPER reactivity profiles of sera from guinea pigs immunized with VLP/gp41 variants

A) Western blot analysis of pooled sera from 5 guinea pigs per each group. Antibody reactivity was determined by using the soluble fusion protein GB1-GCN4-C56 at the titer of 1:100 and peptide corresponding to the MPER sequence of ADA at the titer of 1:20 with 4E10 serving as positive control. Soluble GB1-GCN4-C56 was expressed from *E. coli* and purified by human IgG affinity column. Note that the C56 segment consists of HR2 and the MPER of ADA (B) Antibody reactivity determined by ELISA. 6-helix protein and peptide corresponding to the MPER of MN were coated on the plate at the concentration of 3µg/ml each. Sera 307 and 309 derived from guinea pigs immunized with Sf9 cells expressing gp140 4cSSL24 (showing highest specific antibody titers to gp41 among all animals immunized with VLP/gp41 variants) were tested in comparison with sera 314 from guinea pigs immunized with Sf9 cells/wt BV at serial serum dilution. (C) BIAcore analysis of sera using the L1 sensor chip. The LUV (100 nm) of DMPC was captured on the surface of the sensor chip by the lipophilic constituents providing a supported lipid bilayer. The peptide solution was injected next over the liposome

surface and then purified polyclonal antibodies from sera 307 and 314 were passed over peptide-liposome complexes. The representative sensogram of antibody binding to liposome-DP-178 peptide complex from sera 307 at the antibody titer ranging from 1:20 to 1:10 was shown with 2F5 as a positive control. Purified polyclonal antibody binding to the MPER peptide from sera 307 was compared with negative control polyclonal antibodies from sera 314. 4E10 antibody served as positive control over liposome-MPER peptide complex.

Table 1

Scheme for immunization of guinea pigs with VLP/gp41 variants

Antigen (μg)	Adjuvant (10 μg)	Route	Group
Sf9 cells/gp140 4cSSL24 ^I	-----	id	1
	LT(G33D)	id	2
Mock Sf9 cells	LT(G33D)	id	3
VLP/gp140 4cSSL24	(2 μg) -----	id	4
	(2 μg) LT(G33D)	id	5
	(10 μg) -----	id	6
	(10 μg) LT(G33D)	id	7
VLP/gp41 d4mt	(10 μg) -----	id	8
	(10 μg) LT(G33D)	id	9
	(10 μg) -----	im	10
	(10 μg) LT(G33D)	im	11
VLP/BAFF-C56	(10 μg) -----	im	12
	(10 μg) LT(G33D)	im	13
PBS	-----	id	14

^I
(5 × 10⁶ cells)

Table 2

Lack of neutralizing activity of sera from guinea pigs immunized with VLP and Sf9-gp41 variant

Group	ID50 in TZM-bl cells ^I			
	MN	SF162.LS	ADA	NL-ADArS
1	30	<20	<20	----
2	29	<20	<20	----
3	29	<20	<20	----
4	29	<20	<20	----
5	20	<20	<20	----
6	20	<20	<20	----
7	20	<20	<20	----
8	<20	----	<20	<20
9	<20	----	<20	<20
10	<20	----	<20	28
11	<20	----	<20	20
12	<20	----	<20	23
13	<20	----	<20	23
14	<20	<20	<20	----

Assay: Neutralization in TZM-bl cells

Virus Stocks: HIV-1 MN (H9-grown); SF162.LS (293T transfection); ADA(293T transfection); NL-ADArS (PBMC-grown).

^I Values are reciprocal serum dilution at which RLU were reduced 50% relative to no sample, and represent average numbers from five guinea pigs per group.