Title

Design, display and immunogenicity of HIV1 gp120 fragment immunogens on virus-like particles.

Journal

Vaccine, 36(42)

Authors

Purwar, Mansi
Pokorski, Jonathan
Singh, Pranveer
et al.

Publication Date

2018-10-08

DOI

10.1016/j.vaccine.2018.07.032

Peer reviewed

HHS Public Access

Author manuscript

Vaccine. Author manuscript; available in PMC 2019 October 08.

Published in final edited form as:

Vaccine. 2018 October 08; 36(42): 6345–6353. doi:10.1016/j.vaccine.2018.07.032.

Design, display and immunogenicity of HIV1 gp120 fragment immunogens on virus-like particles

Mansi Purwar[#1], **Jonathan K. Pokorski**[#2,5], **Pranveer Singh**[1,6], **Sanchari Bhattacharyya**[1], **Heather Arendt**[3], **Joanne DeStefano**[3], **Celia C. La Branche**[4], **David C. Montefiori**[4], **M.G. Finn**[2,7,*], and **Raghavan Varadarajan**[1,*]

[1]Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India 560012

[2]Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA

[3]International AIDS Vaccine Initiative, Brooklyn, NY 11226, USA

[4]Department of Surgery, Duke University Medical Center, Durham, NC, USA

[#] These authors contributed equally to this work.

Abstract

The broadly neutralizing antibody against HIV-1, bl2, binds to the CD4 binding site (CD4bs) on the outer domain (OD) of the gp120 subunit of HIV-1 Env. We have previously reported the design of an *E.coli* expressed fragment of HIV-1 gp120, b122a, containing about 70% of the b12 epitope with the idea of focusing the immune response to this structure. Since the b122a structure was found to be only partially folded, as assessed by circular dichroism and protease resistance, we attempted to stabilize it by the introduction of additional disulfide bonds. One such mutant, b122al-b showed increased stability and bound bl2 with 30-fold greater affinity as compared to b122a. Various b122a and OD fragment proteins were displayed on the surface of Qβ virus-like particles. Sera raised against these particles in six-month long rabbit immunization studies could neutralize Tierl viruses across different subtypes with the best results observed with b122a1-b displayed particles. Significantly higher amounts of antibodies directed towards the CD4bs were

[*]**Corresponding Authors:** Raghavan Varadarajan (Email: varadar@mbu.iisc.ernet.in). M.G. Finn (mgfinn@gatech.edu).

[5]Current address: Department of Macromolecular Science and Engineering, Case Western Reserve University, Case School of Engineering, Cleveland, Ohio 44106, USA

[6]Current address: Department of Zoology, Mahatma Gandhi Central University Bihar, Motihari-845401, Bihar, India

[7]Current address: School of Chemistry & Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, Georgia 30332, USA

Author Contributions

SB and RV designed the immunogens; SB and PS carried out the biophysical characterization and SPR studies of the immunogens; JKP and MGF were responsible for production and characterization of Qβ VLP's displaying fragment immunogens; HA and JDS planned the immunization studies; DCM, CC and MP were responsible for neutralization assays; MP also determined ELISA titers against priming immunogens, competition binding experiments and *in vivo* stability experiments; MP, RV JKP and MGF wrote the manuscript.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article

also elicited by particles displaying b122a1-b. This study highlights the ability of fragment immunogens to focus the antibody response to the conserved CD4bs of HIV-1.

Keywords

protein stability; immune focusing; neutralizing antibodies; vaccine; nanoparticles

INTRODUCTION

HIV-1 is the causative agent for AIDS. About 37 million people are living with HIV worldwide. There are many circulating HIV-1 strains, which are further divided into subtypes [1] [2]. When more than one strain infects an individual, circulating recombinant forms are produced. Partly because of this high diversity, a successful vaccine remains elusive. The virus infects cells of the immune system when the envelope glycoprotein of HIV-1 binds to CD4 receptors on T helper cells [3]. The envelope glycoprotein (Env) is the primary target of the humoral immune response elicited against HIV-1. Env is synthesized as a single polypeptide precursor (gp160) which is processed in the Golgi to produce two chains, gp120 and gp41. These associate in a non-covalent manner to form a trimer of heterodimers of surface-exposed gp120 and membrane-anchored gp41 chains. gp120 contains the conserved CD4 receptor binding site and gp41 drives the fusion of viral and host cell membranes [4, 5].

In natural infection, most of the antibodies produced at any given time are unable to efficiently neutralize the virus present [6–8]. Viral sequence diversity and the presence of misfolded/unfolded forms of Env shift the focus of the antibody response away from conserved sites [9]. A large amount of glycosylation on the surface of gp120 also occludes the exposure of conserved sites [10]. In an effort to focus the immune response on conserved epitopes of the CD4 binding site, we previously reported the design of a non-glycosylated, bacterially expressed outer domain fragment of HIV-1 Env which was folded and could bind its cognate ligands b12, CD4 and VRC01 [11, 12]. In rabbit immunization studies, it elicited neutralization of primarily Tier 1 viruses. We also designed a small fragment of gp120, called b122a, comprised of a compact beta barrel located on the lower part of the outer domain (Figure S1A) [13]. When bacterially expressed, b122a was found to be only partially folded but able to bind the broadly neutralizing antibody b12 with micromolar affinity. Upon immunization in rabbits and subsequent boosting with gp120, the anti-sera showed broad, albeit moderate neutralization of a 16 virus panel. Competition experiments and serum depletion studies showed that the neutralization was mediated by CD4 binding site antibodies.

Since b122a was only partially structured as assessed by CD and protease resistance [13], we attempted to enhance its ability to elicit neutralizing antibodies by stabilizing the structure of the gp120 fragment immunogen with additional disulfides, and by displaying it on the surface of simple virus-like particles (VLPs) to present the epitope in polyvalent fashion [14–17]. The design, production, and immunogenic properties of these constructs are described here.

Foreign polypeptide antigens can be incorporated into VLPs to create “chimeric” structures, either as fusions to either end of the VLP capsid (coat) protein (CP) or as additions to well-presented loops [18]. This kind of direct fusion to the coat is usually good for small peptides, however, larger proteins may not be properly folded. In such cases, chemical conjugation to the VLP surface, usually through reactive amino or thiol groups, can be a better option [19, 20] as the folding of the coupled antigen is not dependent on how the VLP monomer folds and the site of attachment can be suitably engineered so that it does not cover important epitopes. The platform used here, derived from bacteriophage QP is a 133 amino acid CP (14.3 kDa), 180 copies of which self-assemble into icosahedral VLPs when expressed in *E. coli.* There are four externally-exposed amino groups on each subunit, which are available for conjugation to foreign antigens. Engineered versions of the Qβ CP have been used as vaccine candidates, with variable efficacy but consistently high safety [21, 22].

In this work, the gp120 fragment proteins were displayed either by conjugation of the purified protein on the surface of the VLPs using click chemistry, or by direct display as a fusion to the coat protein of the phage. When used for rabbit immunization studies (with and without adjuvant), these protein-particle structures elicited high priming immunogen specific titers. Significant anti-gpl20 titers were observed only after boosting animals with gpl20. However, the resulting sera primarily induced a Tier 1 neutralization response. Weak, sporadic Tier 2 neutralization was seen in antisera raised by VLP’s displaying b122a1-b.

MATERIALS AND METHODS

Brief descriptions are below. More information is provided in supplementary materials.

Protein Purification and Characterization

The K383F mutation was introduced in the background of b122a, referred to here as b122a1. Cysteine mutations were introduced in the background of b122a1. b122a1 with 293–448 disulfide bond is referred to as b122a1-b. For conjugation to Qβ virus like particles, the free cysteine in b122a was introduced as an insertion in the pET15b plasmid encoded sequence after residue 19 (b122a-19iC). Proteins were expressed and purified from *E. coli* BL21(DE3) cells essentially as described previously [13]. The OD_{Ec}protein was expressed and purified as described previously [11]. Biophysical characterization was done by Far-UV CD, Fluorescence Spectroscopy, Gel Filtration, Iodoacetamide labeling and Reverse phase HPLC.

Genetic Screen to assess *in vivo* stability of b122a and b122a1-b

b122a and b122a1-b were PCR amplified from the pET 15b vector and cloned in pBR322*bla*-link* vector in between residues 196 and 197 of β-lactamase gene (*bla*) using BamHl and Xhol restriction sites and henceforth referred to as pBR.322*bla*-link*bl 22a and pBR322*bla*-Iink*b122al-b respectively. This plasmid is a derivative of pBR322 plasmid where a 33 residue Gly-Ser-Ala linker was introduced between residues 196 and 197 of the *bla* gene. The fusion proteins were monitored for antibiotic resistance as described previously [23]. Briefly, 1 ml of mid log phase cells were taken and the A_{6}oo was adjusted to 1.0. The cells were serially diluted 10 fold in 170 mM NaCl under sterile conditions. 2 µl

of each dilution was spotted onto LB Agar supplemented with different concentrations of PenG. Plates were incubated at 37°C for 18–20 hours.

Binding affinity studies using surface plasmon resonance

All binding studies were performed with a Biacore 2000 (Biacore, Uppsala, Sweden) optical biosensor at 25°C as described previously [11]. Four different concentrations of gpl20, b122a, b122a1, OD_{Ec} or the disulfide and cysteine insertion mutants were passed across each sensor surface in a running buffer of PBS, pH 7.4, containing 0.005% P20 surfactant. The kinetic parameters were obtained by fitting the data to the simple 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software.

Qβ particles displaying fragment immunogens as coat protein extensions.

b122a, OD_{Ec}, and b122a1-b were individually cloned immediately downstream of the Qβ coat protein gene with an 8 amino acid hydrophilic spacer separating the two domains. Particles were purified and characterized as previously described [16], the latter was carried out by FPLC size exclusion chromatography on Superose 6, dynamic light scattering (DLS) (Wyatt Dynapro), and microfluidic gel electrophoresis (Agilent Bioanalyzer 2100, Series II Protein 80 chips). The number of extended coat proteins per particle was determined by integration of the electrophoretic bands in the Bioanalyzer software (Table S2).

Synthetic Manipulations of Virus-Like Particles-Synthesis of Qβ-b122a

The alkyne-bearing Qβ particle was prepared as described previously (Figure 2A) [24]. Azide-functionalized b122a-19iC was generated by reacting the engineered free Cys with an azido-modified succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) linker. A solution of b122a-19iC-azide was reacted with Qβ-alkyne particles under ligand-accelerated copper catalysed azide-alkyne cycloaddition conditions [25]. The reaction was allowed to proceed for 2 hours at room temperature and the product was purified using a 10–40% sucrose density gradient. The purified protein was resuspended in phosphate-buffered saline (PBS) and characterized using DLS, FPLC, and microfluidic protein quantification.

Immunization studies in rabbits

This study C0913–11 was performed at Covance, Inc., Denver, PA. The facility is iAAALAC accredited and all work was approved by the Covance Institutional Animal Care and Use Committee.

Six groups, each having four rabbits (New Zealand White, female, 2 months old) were used for immunization studies. All rabbits were injected intramuscularly with 50μg of the VLP/protein immunogen. Priming was done at weeks 0 and 4 and boosts were given at weeks 12 and 20. Four weeks following the last boost, the rabbits were terminated. Serum samples were collected at weeks 0 and two weeks post each immunization, heat inactivated and stored in aliquots for further analysis.

Determination of Serum Antibody Titers

Antibody titers against test immunogens were determined by ELISA. Antibody titer was defined as the reciprocal of the highest dilution that gave an OD value above the mean plus two standard deviations of control wells.

Neutralization Assay

Neutralizing antibody activity in sera was measured in a standardized *in vitro* assay in TZM-bl cells using Tat-regulated luciferase reporter gene expression to quantify reductions in virus infection in TZM-bl cells. Assays were carried out with Env-pseudotyped viruses as described previously [26]. Neutralization titers (ID_{50}) was calculated as the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells.

Competition experiments

Competition ELISA between the week 22 sera and antibodies bl2, VRC01, VRC-PG04, FI05 and PGT128 was carried out as described elsewhere [27]. Briefly, 96-well plates (Nunc MaxiSorp) were coated with 300 ng of gpl20 in 50 μL PBS and kept overnight at 4 °C. Plates were then washed with PBST and blocked for 1 h with PBSB. 50 μL of week 22 sera was added to each well starting at a 1:50 dilution followed by a threefold serial dilution in PBSB. As a negative control, pre-immune sera from each group was used starting at a 1:50 dilution followed by a threefold serial dilution in PBSB. After 2 h of incubation, the plates were washed with PBST. The competing antibody was then added to each well at a fixed concentration (10 times EC50, as determined from the titration curve of the antibodies with gpl20) of lμg/ml for bl2 and F105, 2μg/ml for both VRC01 and PGT 128, 4μg/ml for VRC-PG04. After 2 h of incubation with competing antibody, the plates were washed with PBST. The wells were then probed with 50 μL of ALP-conjugated goat anti-human antibody (Sigma) at a predetermined dilution (1:10,000) to detect the bound competing antibody. The plates were washed and developed using the chromogenic substrate p-nitrophenyl phosphate (Sigma). The optical density was measured at 405 nm (SPECTRAmax Plus 384L; Molecular Devices). The percent competition was calculated as follows: % competition = [(PI-W22)/PI] x 100, where PI is the signal of competing antibody binding to gpl20 in the presence of pre-immune sera and W22 is the binding signal of competing antibody to gpl20 in the presence of week 22 sera for that group. All SPR competition experiments were carried out on a BioRad ProteOn instrument. Approximately 6000 RUs of monoclonal antibody bl2 were immobilized on the GLC chip surface. The week 22 serum from all groups was normalized for gpl20 titers and was incubated with lOOnM of JRFL gpl20 for 30 min at room temperature. JRFL gpl20 alone or with the pre-incubated serum was passed over the surface.

RESULTS

b122al

The b122a construct had a lysine residue that was buried in the corresponding gpl20 structure. As buried charges are destabilizing, a mutant was made where this residue was converted to phenylalanine. This mutant is referred to as b122al (Table S3). The protein was soluble and the CD spectrum showed slightly improved secondary structure compared to the

parent construct b122a (Figure 1C). The intrinsic fluorescence spectrum showed a red shift upon denaturation (Figure S2A). From gel filtration studies, it appeared to be a monomer (Figure 1A, 1B). In reverse-phase HPLC studies, it eluted primarily as a single major species under both oxidized and reduced conditions. (Figure S2B). The K_D for binding of b122a1 to b12, was 2.4 μM (Figure S3B and Table 1) exhibiting approximately 6 times greater affinity than b122a.

b122a1 disulfide mutants

In an attempt to impart structural rigidity and further increase the stability of b122a1, disulfides were introduced in various regions of the molecule [28, 29]. The possible disulfide positions were chosen based on the high-resolution structure of b12 bound to gp120 (2NY7), which was used as input for the program MODIP (Modeling Disulfide bridges in Proteins) [30, 31]. The list of these positions is given in Supplementary Table S1; some were not suitable because they were located at hydrogen bonding positions of anti-parallel beta strands or were in close proximity to the b12 binding site (Table S1). In addition, several other exposed sites on anti-parallel beta strands were chosen by visual inspection, inspired by a study showing most naturally occurring cross-strand disulfides in anti-parallel beta-strands to be located at non-hydrogen bonded positions [32]. The positions finally chosen were 422–438, 295–446, and 293–448, indicating the amino acids substituted with cysteine to form disulfide bonds between them (Figure S1B) in the background of b122a1. These mutant proteins were produced and purified from the soluble fraction by the above procedure, giving a typical yield of approximately 20 mg per liter. No increase in protein mass was observed upon iodoacetamide treatment in the absence of reducing agent, confirming the spontaneous formation of disulfide linkages for these polypeptides. The same treatment after disulfide reduction showed the incorporation of eight iodoacetamide molecules per protein. However, far UV CD (Figure 1D) measurements showed that all the mutants had structure similar to b122al indicating that all the proteins were only partially folded. All the three proteins showed a small red shift upon denaturation, conforming the presence of a partially folded conformation prior to denaturation (Figure 1E, S2C, S2E). By this measure, the introduction of disulfide bonds did not lead to a substantial gain of protein secondary structure.

The proteins were also analyzed for their oligomeric state using gel filtration on a Superdex-75 column (Figure 1A). Comparison to a calibration curve (Figure 1B) showed all the disulfide mutants to have higher elution volumes than the parent construct, indicating that they are more compact. Furthermore, mixtures of peaks for the oxidized proteins were observed on reversed phase chromatography, indicating that they consist of more than one combination of disulfide bonded isomers (Figure 1F, S2D, S2F). In contrast, the reduced mutant proteins eluted as a single peak and at a higher acetonitrile concentration than the oxidized protein. Disulfide mutants 422–438 and 295–446 in the background of b122al were found to exhibit no improvement in binding to immobilized bl2 IgG by SPR (Figure S3C, S3D and Table 1) compared to b122al. However, the 293–448 disulfide mutant in the background of b122al bound to bl2 about five times better than b122al (Figure S3E and Table 1); this construct is referred to as b122al-b (Table S3).

The b122al-b protein was found to be significantly more stable in *E. coli* cells than the parent b122a protein, as assessed by the split β-lactamase assay [23]. Thus, cells containing pBR322*bla*-link*b122a, pBR322*bla*-link*b122al-b, and wild type β-lactamase (see Experimental section for descriptions) were spotted on LB agar containing different concentrations of penicillin G ranging from 1 mg/ml to 3.5 mg/ml. Figure 1G shows that pBR322*bla*-link*b122al-b confers higher antibiotic resistance than pBR322*bla*-link*b122a, suggesting greater stability of the folded structure owing to introduction of an additional disulfide.

Cysteine Insertion Mutant of b122a

For conjugation to Qβ virus like particles, the free cysteine in b122a was introduced as an insertion in the pET15b plasmid encoded sequence after residue 19. This construct is referred to as b122a-19iC (Table S3). Protein b122a-19iC was purified from the soluble fraction of the *E. coli* BL21(DE3) cell lysate. The protein was about 90% pure, and the identity was confirmed by mass spectrometry. The far-UV CD spectrum of the protein was similar to the b122a construct (Figure S4A) indicating that introduction of the single cysteine close to the N-terminus did not perturb the protein structure. Gel filtration studies confirmed that the protein eluted at the same volume as the wild-type protein (Figure S4B), indicating that it is not aggregated. Iodoacetamide labeling studies under native conditions, confirmed the presence of one free cysteine. This mutant bound to bl2 IgG [33] with K_D in the range of 8–12 μM (Figure S4C and Table 1), similar to b 122a.

Immunization and neutralization studies

Rabbit immunization studies were carried out with fragment immunogens b122a and b122al-b displayed on Qβ virus like particles (Table S4). In addition, the previously described construct OD_{Ec}, consisting of residues 255–474 of the gpl20 outer domain [11] was displayed on the surface of Qβ virus like particles as a fusion to the coat protein (Figure 2A). A prime-boost rabbit immunization study was employed as described previously [13] which involved priming with the b122a/OD_{Ec}/b122al-b protein fragments displayed on the particles at weeks 0,4 and boosting with full-length gp120 at weeks 12, 20. Sera were collected at two weeks after each immunization. Briefly, we hypothesized that the designed fragments may not adopt exactly the same conformation as the corresponding regions in the whole molecule, in the absence of antibody b12/VRC01. Hence boosting with gp120 might elicit gp120 cross-reactive antibodies some of which could be targeted to the broadly neutralizing antibody epitopes that are present in the priming immunogen.

Group 1 was primed with b122a conjugated to the particles. To test the difference between the display strategies, group 2 rabbits were primed with b122a fragment expressed as a fusion to the coat protein on the surface of the particles and compared to group1. Our earlier immunization studies with soluble OD_{EC} showed modest neutralization [11]. In an attempt to improve the immune response, group 3 was primed with outer domain based construct OD_{EC} displayed on particles as a fusion to the coat protein. Group 4 was primed with the b122a1-b displayed on particles as a fusion to coat protein. We expect to see a better immune response generated by this protein as it showed better biophysical properties in terms of stability as well as significantly improved binding to antibody b12 when compared

to b122a. VLPs can generally elicit a high immune response without adjuvant. However, to see if adjuvant can further enhance titers, group 5 rabbits were primed with b122a conjugated to the particles, administered with the adjuvant and compared to group 1. Group 6 animals were primed with empty particles. All groups were boosted with full length gp120 in the presence of adjuvant.

Week 2 and week 4 ELISA titers obtained after particle immunizations, did not have any significant cross-reactivity to gp120. After one boost with full-length gp120, at least one rabbit from all groups showed weak gp120 titers (~400), except group 6 which received empty particles for priming. After two boosts, the week 22 sera showed good gp120 ELISA titers in the range of 10^5 (Table 2). Comparable gp120 and b122a

ELISA titers were seen for group 1 and group 2 showing that the display strategy (genetic fusion vs. covalent coupling) did not contribute to immunogenicity of the particles. The mean gp120 titers for group 1 and group 5 showed a very small difference but b122a titers were significantly increased for group 5 which persisted even after 2 boosts with gp120 showing the presence of antibodies with higher cross reactivity to the b12 epitope. This was also reflected in the neutralization ID_{50} values (Table 3), where group 5 sera showed significantly higher titers when compared to group 1 sera both against subtype B and subtype C tier 1 pseudoviruses.

The mean gp120 titer for the week 22 sera was highest for group 4, which received particles displaying a stabilized disulfide mutant of b122a1 (293–448) as the priming immunogen. Priming immunogen ELISA titers were high for group 4 when compared to other groups except group 5 (Table 2). This could be possibly due to use of b122a instead of b122a1-b to measure priming immunogen titers. Neutralization titers against a panel of four HIV-1 pseudoviruses were also highest for group 4. Two of four animals from Group 4 also showed weak neutralization of Tier 2 subtype B JRFL strain (Table 3). ELISA titers and neutralization titers were lowest for group 6 (primed with empty particles) showing the specificity of the immune response generated against our molecules.

To test for the presence of CD4bs antibodies, competition experiments were carried out using SPR to probe for competition between b12 antibody and the week 22 sera from all groups, for the CD4bs on gp120. gp120 binding to b12 was measured in the presence and absence of sera. Addition of sera containing CD4bs/b12-like antibodies should result in reduction of gp120 binding to b12. However an increase in binding was observed (Figure 2B) presumably because antibodies to other binding sites on gp120 would also have been elicited following the boosts with gp120. Such antibodies can be expected to bind to gp120 and the entire complex will then bind to b12 on the chip surface, thus showing an increase in RU in SPR. Sera eliciting the highest titer of CD4bs antibodies are expected to show the lowest increase. Consistent with ELISA and neutralization data described above, gp120 incubated with group 4 sera show the least increase upon binding to b12 followed by group 5 sera indicating the presence of a higher fraction of CD4bs directed antibodies than other groups. Group 6 sera showed the highest increase in binding, indicating the lack of a CD4bs focused response, consistent with the absence of a CD4bs focused primary immunogen in this group (Figure 2B). To further characterize the antisera, competition ELISAs were

carried out to probe for competition between various CD4bs antibodies and the week 22 sera from all groups, for the CD4bs on gp120. Table 4 lists percent competition at 1:50 dilution of week 22 sera. As a negative control, competition was carried out with the V3 epitope targeting broadly neutralizing antibody PGT128. None of the sera showed any significant competition (<15%). Group 4 sera show the highest competition with antibody b12 compared to other groups consistent with ELISA (Table 2) and neutralization data (Table 3). This was observed for competition experiments performed with SPR also (Figure 2B). We also performed competition with other CD4bs antibodies like VRC01, VRC-PG04 and F105. For both VRC01 and VRC-PG04 all groups show very little competition. It is known that residues in loop D and variable loop five are part of the VRC01 but not the b12 epitope and hence are not present in b122a based immunogens [34]. From Figure S1A it is clear that b122a contains a small part of the VRC01 epitope and so its lack of VRC01 binding is expected. Consistent with this, sera raised against b122a immunogens do not compete with VRC01 and VRC-PG04. OD_{Ec} does contain the VRC01 epitope but the sera did not compete well with VRC01 possibly due to low valency display of this antigen on the VLP. Competition studies were also performed with the CD4bs non-neutralizing antibody F105 whose epitope significantly overlaps with bl2 [35, 36]. The data show that Group 4 sera which show the highest competition with bl2, also compete with FI05.

DISCUSSION

In many cases, protective immunity against various viral and bacterial diseases has been achieved using live attenuated vaccines, because they closely mimic natural infection. However, for certain infections like HIV, live attenuated viruses are not considered safe, both because of viral integration into the chromosome and because of mutations that can reactivate the virus. In such cases, virus like particles or VLPs are an attractive alternative, as they resemble intact virions in terms of size and presentation of antigens, but are non-pathogenic, non-replicative and hence are safe for administration. Chimeric VLPs are an efficient way to display foreign epitopes in multivalent form and are able to elicit a robust immune response without adjuvant [37]. In this study, we displayed various designed gpl20 fragment proteins on the surface of Qβ virus like particles. We improved the stability and bl2 affinity of our previously described fragment immunogen b122a [13] by incorporating the K383F mutation with additional disulfides. Construct b122al-b resulted in the largest improvement in affinity to bl2. No significant improvement was observed in the secondary structure of the protein as assessed by CD spectroscopy, but increased *in vivo* stability was seen in a β-lactamase activity assay [23]. For b122a-19iC conjugated particles, the final gpl20 ELISA titers were the same both in absence and presence of adjuvant but increased titers against priming immunogen and neutralization was seen with the latter. This indicated that the adjuvant increased the immunogenicity of the particles. Sera from group 4 primed with the stabilized protein b122al-b showed higher neutralization activity against Tier 1 viruses and also showed weak neutralization of the Tier 2, subtype B JRFL strain. Sera from the group primed with empty particles, showed low gpl20 and priming immunogen titers, indicating that priming with the fragment immunogens is beneficial. Competition assays using both SPR and ELISA showed an increased CD4 binding site directed response in week 22 sera from group primed with Qβ VLPs displaying b122al-b compared to other groups

(Table 4, Figure 2B). Collectively the data demonstrate that priming with a CD4bs directed fragment immunogen results in some degree of immune focusing to the CD4bs and improving immunogen stability and bl2 binding affinity resulted in enhanced elicitation of neutralizing antibodies. However, a more native-like boosting immunogen than gpl20 is likely required to elicit a higher proportion of neutralizing antibodies.

Various studies attempting to focus the immune response to a particular epitope on HIV-1 Env have been carried out. Most of the immunogens described in these studies are based on the outer domain of gpl20. An OD construct (OD1) containing residues, 252–482 from YU2 strain of gpl20 was expressed in *Drosophila* S2 cells. This construct was glycosylated and also retained VIV2 and V3 variable loops, but the sera obtained after rabbit immunizations did not neutralize homologous YU2 virus [38]. In another study, mice were immunized with clade C OD as a fusion to human IgGl Fc domain, but failed to elicit any neutralizing response. [39, 40]. Another study described two OD immunogens (monomeric and trimeric) based on group M consensus sequence. The immunogens were able to induce heterologous Tier 1 neutralizing responses although sera immunized with monomeric gpl20 showed higher neutralization titers for all viruses [41]. The OD3 construct based on subtype C strain 1084i having stabilizing disulfides and a cavity filling mutation was able to elicit a Tier 1 and a weak Tier 2 neutralizing response in guinea pig immunization studies [42], In the present work we were able to show elicitation of a strong cross-clade Tier 1 response with Qβ VLPs displaying either OD_{Ec} or the stabilized b122al-b even in the absence of adjuvant. b122a conjugated to Qβ VLP's elicited a similar response when immunized in the presence of adjuvant. Sera elicited by Qβ VLP's displaying the stabilized construct, b122al-b, also elicited a weak Tier 2 response. One possible reason we did not see a better elicitation of antibodies that could neutralize multiple Tier 2 viruses in case of b122a VLP's is that the displayed antigens might not have been conformationally intact. Another reason could be the fact that the valency of antigens displayed on the virus like particles (Table S2) was lower than expected, resulting in reduced immunogenicity. Boosting with more native like trimeric gpl40 [43] or trimeric gpl20 [44, 45] instead of monomeric gpl20 could prove beneficial, and can be used in future immunization studies to improve elicitation of neutralizing antibodies. VLP's are known to enhance cell mediated responses. In this study we focused only on humoral responses and hence cannot comment on elicitation of T cell responses, which have been shown to substantially contribute to control of HIV-1 infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Monoclonal antibodies b12 and VRC01 were obtained from the Neutralizing Antibody Consortium of IAVI. pBR322*bla*-link* vector to determine *in vivo* stability of immunogens was kindly provided by Dr. Linda Foit and Dr. James C. A. Bardwell. The assistance of Nonavinakere Seetharam Srilatha is duly acknowledged for the SPR experiments. MP and SB acknowledge the support of Council of Scientific and Industrial Research, Government of India. This work was funded by the International Aids Vaccine Initiative grant (grant number COATOD00107) and a grant from National Institutes of Health (grant number R01AI118366– 01, DT.15/7/2015) to RV. We also acknowledge funding for infrastructural support from the following programs of the Government of India: DST-FIST, UGC Center for Advanced Study, and the DBT-IISc Partnership Program and of a JC Bose Fellowship from DST to RV.

Abbreviations

CD4bs	CD4 binding site
OD	outer domain
Env	envelope glycoprotein
CP	capsid protein
VLPs	virus-like particles
PBS	phosphate buffer saline
Cys	cysteine
SMCC	succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
RLU	relative luminescence units
RU	response units
MODIP	Modeling Disulfide bridges in Proteins
SPR	surface plasmon resonance
PBST	phosphate buffer saline with 0.05% Tween-20
PBSB	phosphate buffer saline with 0.05% Tween-20 and 3% BSA
ELISA	Enzyme-linked immune sorbent assay

REFERENCES

1. Robertson DL, et al., HIV-1 nomenclature proposal. Science, 2000 288(5463): p. 55–6. [PubMed: 10766634]
2. Buonaguro L, Tornesello ML, and Buonaguro FM, Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. J Virol, 2007 81(19): p. 10209–19. [PubMed: 17634242]
3. Dalgleish AG, et al., The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature, 1984 312(5996): p. 763–7. [PubMed: 6096719]
4. Chan DC and Kim PS, HIV entry and its inhibition. Cell, 1998 93(5): p. 681–4. [PubMed: 9630213]
5. Wyatt R and Sodroski J, The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science, 1998 280(5371): p. 1884–8. [PubMed: 9632381]
6. Richman DD, et al., Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A, 2003 100(7): p. 4144–9. [PubMed: 12644702]
7. Mikell I, et al., Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathog, 2011 7(1): p. e1001251. [PubMed: 21249232]
8. Alter G and Moody MA, The humoral response to HIV-1: new insights, renewed focus. J Infect Dis, 2010 202 Suppl 2: p. S315–22. [PubMed: 20846039]
9. Connor RI, et al., Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. J Virol, 1998 72(2): p. 1552–76. [PubMed: 9445059]
10. Leonard CK, et al., Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope

glycoprotein (gp120) expressed in Chinese hamster ovary cells. J Biol Chem, 1990 265(18): p. 10373–82. [PubMed: 2355006]

11. Bhattacharyya S, et al., Design of a non-glycosylated outer domain-derived HIV-1 gp120 immunogen that binds to CD4 and induces neutralizing antibodies. J Biol Chem, 2010 285(35): p. 27100–10. [PubMed: 20558728]
12. Rathore U, et al., Glycosylation of the core of the HIV-1 envelope subunit protein gp120 is not required for native trimer formation or viral infectivity. J Biol Chem, 2017 292(24): p. 10197–10219. [PubMed: 28446609]
13. Bhattacharyya S, et al., Design of an Escherichia coli expressed HIV-1 gp120 fragment immunogen that binds to b12 and induces broad and potent neutralizing antibodies. J Biol Chem, 2013 288(14): p. 9815–25. [PubMed: 23430741]
14. Astronomo RD, et al., Defining criteria for oligomannose immunogens for HIV using icosahedral virus capsid scaffolds. Chemistry & biology, 2010 17(4): p. 357–70. [PubMed: 20416507]
15. Banerjee D, et al., Multivalent display and receptor-mediated endocytosis of transferrin on virus-like particles. Chembiochem : a European journal of chemical biology, 2010 11(9): p. 1273–9. [PubMed: 20455239]
16. Brown SD, Fiedler JD, and Finn MG, Assembly of hybrid bacteriophage Qbeta virus-like particles. Biochemistry, 2009 48(47): p. 11155–7. [PubMed: 19848414]
17. Sen Gupta S, et al., Accelerated bioorthogonal conjugation: a practical method for the ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjugate chemistry, 2005 16(6): p. 1572–9. [PubMed: 16287257]
18. Pumpens P and Grens E, HBV core particles as a carrier for B cell/T cell epitopes. Intervirology, 2001 44(2–3): p. 98–114. [PubMed: 11509871]
19. Chackerian B, Lowy DR, and Schiller JT, Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. The Journal of clinical investigation, 2001 108(3): p. 415–23. [PubMed: 11489935]
20. Jegerlehner A, et al., A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. Vaccine, 2002 20(25–26): p. 3104–12. [PubMed: 12163261]
21. Maurer P, et al., A therapeutic vaccine for nicotine dependence: preclinical efficacy, and Phase I safety and immunogenicity. Eur J Immunol, 2005 35(7): p. 2031–40. [PubMed: 15971275]
22. Low JG, et al., Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine: results from a double-blinded, randomized Phase I clinical trial in healthy Asian volunteers. Vaccine, 2014 32(39): p. 5041–8. [PubMed: 25045806]
23. Foit L, et al., Optimizing protein stability in vivo. Mol Cell, 2009 36(5): p. 861–71. [PubMed: 20005848]
24. Pokorski JK, et al., Functional virus-based polymer-protein nanoparticles by atom transfer radical polymerization. J Am Chem Soc, 2011 133(24): p. 9242–5. [PubMed: 21627118]
25. Pokorski JK, Hovlid ML, and Finn MG, Cell targeting with hybrid Qbeta virus-like particles displaying epidermal growth factor. Chembiochem, 2011 12(16): p. 2441–7. [PubMed: 21956837]
26. Li M, et al., Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. Journal of virology, 2005 79(16): p. 10108–25. [PubMed: 16051804]
27. Mallajosyula VV, et al., Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. Proc Natl Acad Sci U S A, 2014 111(25): p. E2514–23. [PubMed: 24927560]
28. Creighton TE, Disulphide bonds and protein stability. BioEssays : news and reviews in molecular, cellular and developmental biology, 1988 8(2): p. 57–63.
29. Harrison PM and Sternberg MJ, Analysis and classification of disulphide connectivity in proteins. The entropic effect of cross-linkage. Journal of molecular biology, 1994 244(4): p. 448–63. [PubMed: 7990133]
30. Dani VS, Ramakrishnan C, and Varadarajan R, MODIP revisited: re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins. Protein engineering, 2003 16(3): p. 187–93. [PubMed: 12702798]

31. Sowdhamini R, et al., Stereochemical modeling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis. Protein engineering, 1989 3(2): p. 95–103. [PubMed: 2594728]
32. Indu S, et al., Conformational analysis and design of cross-strand disulfides in antiparallel beta-sheets. Proteins, 2011 79(1): p. 244–60. [PubMed: 21058397]
33. Zhou T, et al., Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature, 2007 445(7129): p. 732–7. [PubMed: 17301785]
34. Li Y, et al., Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. J Virol, 2011 85(17): p. 8954–67. [PubMed: 21715490]
35. Chen L, et al., Structural basis of immune evasion at the site of CD4 attachment on HIV-1 gp120. Science, 2009 326(5956): p. 1123–7. [PubMed: 19965434]
36. Pantophlet R, et al., Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J Virol, 2003 77(1): p. 642–58. [PubMed: 12477867]
37. Bachmann MF and Jennings GT, Virus-Like Particles: Combining Innate and Adaptive Immunity for Effective Vaccination, in Novel Vaccination Strategies. 2004, Wiley-VCH Verlag GmbH & Co. KGaA. p.415–432.
38. Yang X, et al., Characterization of the outer domain of the gp120 glycoprotein from human immunodeficiency virus type 1. J Virol, 2004 78(23): p. 12975–86. [PubMed: 15542649]
39. Chen H, Xu X, and Jones IM, Immunogenicity of the outer domain of a HIV-1 clade C gp120. Retrovirology, 2007 4: p. 33. [PubMed: 17509143]
40. Chen H, et al., Mapping the immune response to the outer domain of a human immunodeficiency virus-1 clade C gp120. J Gen Virol, 2008 89(Pt 10): p. 2597–604. [PubMed: 18796729]
41. Qin Y, et al., Eliciting neutralizing antibodies with gp120 outer domain constructs based on M-group consensus sequence. Virology, 2014 462–463: p.363–76. [PubMed: 25248160]
42. Hu D, et al., Tryptophan 375 stabilizes the outer-domain core of gp120 for HIV vaccine immunogen design. Vaccine, 2017 35(23): p. 3067–3075. [PubMed: 28461065]
43. Torrents de la Pena, A., et al., Improving the Immunogenicity of Native-like HIV-1 Envelope Trimers by Hyperstabilization. Cell Rep, 2017 20(8): p. 1805–1817. [PubMed: 28834745]
44. Jones AT, et al., A Trimeric HIV-1 Envelope gp120 Immunogen Induces Potent and Broad Anti-V1V2 Loop Antibodies against HIV-1 in Rabbits and Rhesus Macaques. J Virol, 2018 92(5).
45. Kesavardhana S, et al., Structure-based Design of Cyclically Permuted HIV-1 gp120 Trimers That Elicit Neutralizing Antibodies. J Biol Chem, 2017 292(1): p. 278–291. [PubMed: 27879316]

Highlights

1. gpl20 fragments with enhanced stability, affinity to CD4bs antibody bl2 were designed.
2. Stabilized fragments were displayed on Qβ VLP's and used in rabbit immunizations.
3. The most stable fragment elicited cross clade Tier 1 and weak Tier 2 neutralization.
4. Priming with the fragments resulted in an epitope focused response to the CD4bs.

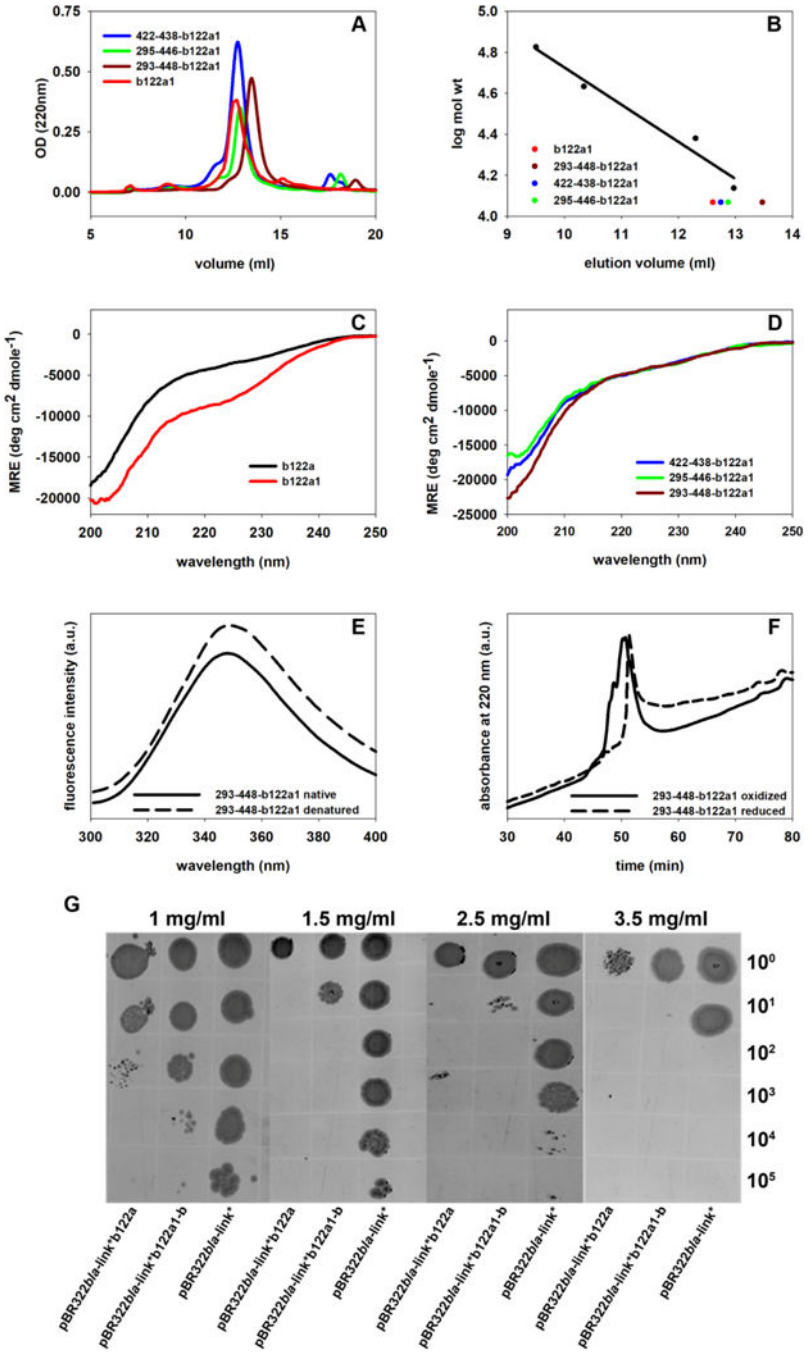

Figure 1:

In Vitro and *in vivo* characterization of designed immunogens. (A) Comparison of the analytical gel filtration elution profiles of b122a1, 422–438-b122a1, 295–446-b122a1 and 293– 448-b122a1 (b122a1-b). The absorbance at 220nm is shown as a function of the elution volume. (B) Calibration curve for Superdex-75 column with standard proteins. The positions of b122a1 and the three disulfide mutants are shown in different colored symbols. The 293 −448-b122a1 (b122a1-b) mutant has the lowest elution volume and is therefore the most compact. (C) Overlay of the CD spectra of b122a and b122a1. The latter shows a more

pronounced dip in the range of 220–230nm, indicating increased secondary structure. (D) Overlay of the CD spectra of 422–438, 295–446 and 293–448 disulfide mutants of b122a1. (E) The intrinsic fluorescence emission spectra of native and guanidine hydrochloride denatured 293–448-b122a1 (b122a1-b). (F) Reverse phase HPLC profiles for 293–448-b122a1 (b122a1-b) in both oxidized and reduced condition. The oxidized protein eluted at a lower acetonitrile concentration than the reduced one, indicating that is more compact. (G) Increased *in vivo* stability of b122a1-b compared to b122a. Cells transformed with the indicated constructs were grown to mid log phase, and the A600 was adjusted to 1.0 in 170 mM NaCl. Five 10-fold dilutions were made for each construct. 2μl of each dilution was spotted on LB agar supplemented with 1.0 mg/ml, 1.5 mg/ml, 2.5 mg/ml and 3.5 mg/ml of PenG. At all concentrations of PenG, pBR322bla-link* b122a1-b show growth at a higher dilution than pBR322bla-link* b122a, indicating increased antibiotic resistance and thus implying increased stability of b122a1-b relative to b122a.

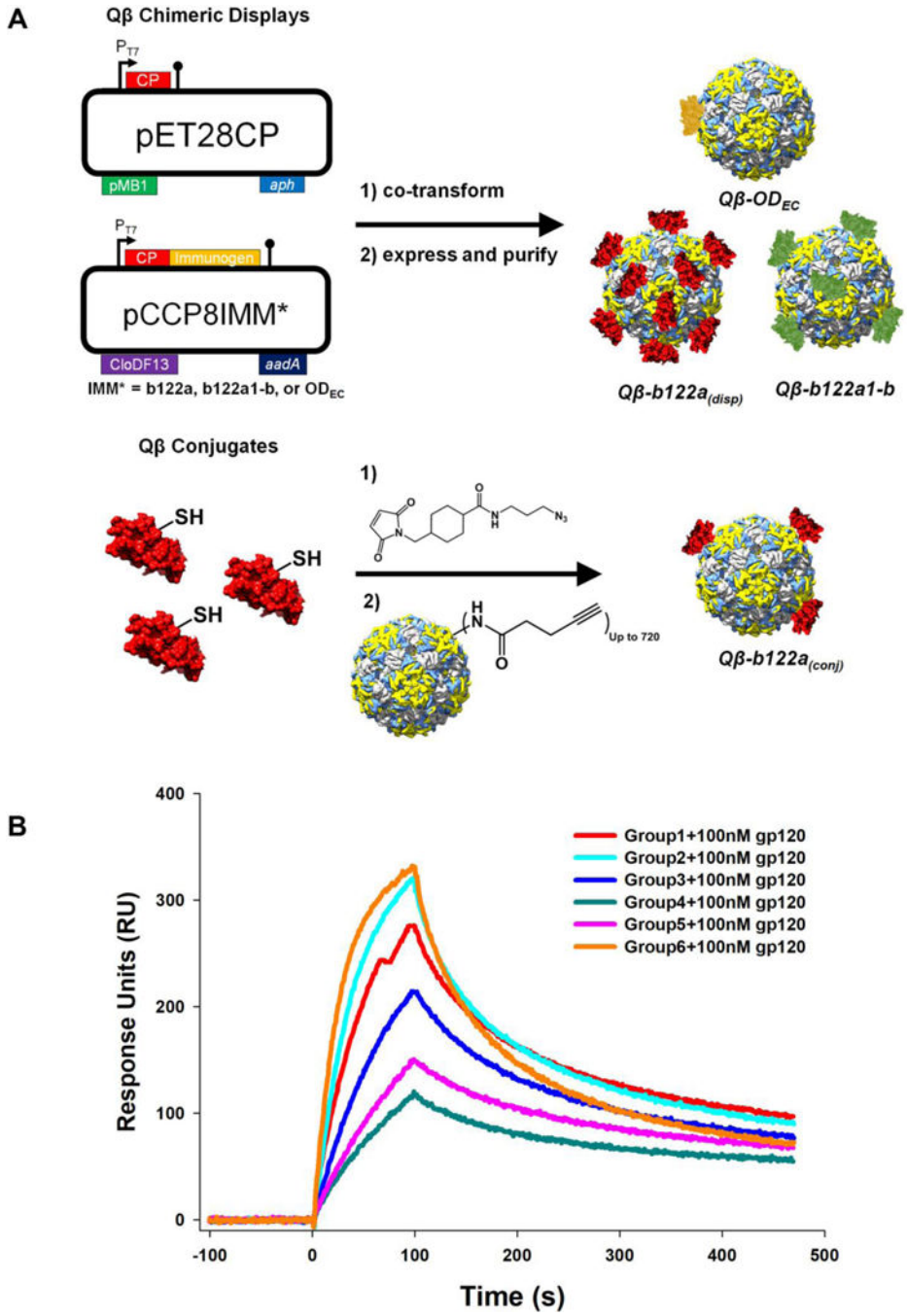

Figure 2:

Generation of VLP-based immunogens and competition binding studies of corresponding sera. (A) Schematic representation of particle design and production. Top, plasmids used for chimeric VLP display; bottom, chemical conjugation to display b122a. (B) Competition assay between sera and monoclonal antibody b12 for binding to CD4bs on gp120. Binding was monitored by surface plasmon resonance. Pre-binding of gp120 to sera from groups 4 and 5 results in reduced binding to b12, compared to other groups indicating presence of

substantial amounts of CD4bs directed antibodies in the former two groups. Sera from all groups were normalized for gp120 titers.

Table 1:

SPR derived kinetic parameters for binding of gp120 and disulfide mutants of b12 binding site constructs to IgG b12

Protein	b12		
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
gp120	$1.4*10^5$	$5.2*10^{-3}$	0.036 ± 0.002
b122a	$5.3*10^2$	$7.5*10^{-3}$	14.2 ± 1.4
b122a1[a]	$4.0*10^3$	$9.6*10^{-3}$	2.4 ± 1.1
b122a1–422C-438C	$8.8*10^2$	$2*10^{-2}$	22.7 ± 3.3
b122a1–295C-446C	$6.4*10^2$	$1.3*10^{-2}$	20.3 ± 1.3
b122a1-b[b] (293C-448C)	$1.2*10^4$	$6.3*10^{-3}$	0.5 ± 0.04
b122a-19iC	$1.9 * 10^3$	$2.2 * 10^{-2}$	11.5 ± 2.2

[a] b122a with K383F mutation

[b] b122a with K383F, 293C-448C mutations

Table 2:

ELISA titers of sera from all groups for study C0913–11 against either gpl20 or the priming immunogen. ‘W’ refers to the week number. Following priming at weeks 0 and 4, all animals were boosted with JRFL gp120 at weeks 12 and 20.

Group	Group Details	Animal ID	W2		W6		W14		W22		W24 Terminal	
			Gp120	[a]b122a/OD_{Ec}	gpl20	[a]b122a/OD_{Ec}	gpl20	[a]b122a/OD_{Ec}	gpl20	[a]b122a/OD_{Ec}	gpl20	[a]b122a/OD_{Ec}
1	particles conjugated to b122a-19iCfor prime, full length JRFL gpl20 for boost	#874	<100	<100	<100	6400	100	400	102400	400	51200	1600
		#875	<100	<100	<100	400	400	<100	102400	<100	51200	<100
		#876	<100	<100	<100	1600	400	400	51200	<100	12800	<100
		#877	<100	<100	100	400	100	<100	204800	<100	51200	<100
2	particles displaying b122a for prime, full length JRFL gpl20 for boost	#878	<100	1600	100	6400	100	1600	25600	1600	12800	1600
		#879	<100	6400	<100	6400	100	1600	51200	400	51200	400
		#880	<100	1600	<100	1600	400	<100	51200	<100	25600	<100
		#881	<100	1600	<100	1600	100	400	25600	400	12800	400
3	particles displaying OD_{Ec} for prime, full length JRFL gpl20 for boost	#882	<100	<100	<100	<100	400	<100	51200	<100	25600	<100
		#883	<100	<100	<100	400	100	<100	102400	<100	51200	<100
		#884	<100	<100	<100	<100	100	<100	51200	<100	25600	<100
		#885	<100	<100	<100	<100	100	<100	102400	<100	25600	<100
4	particles displaying b122al-b for prime, full length JRFL gpl20 for boost	#886	<100	400	<100	1600	1600	<100	409600	<100	102400	<100
		#887	<100	6400	<100	25600	100	1600	102400	1600	25600	400
		#888	<100	400	<100	400	<100	400	102400	400	25600	400
		#889	<100	1600	<100	1600	400	<100	51200	<100	25600	<100
5	particles conjugated to b122a-19iC(ADJUPLEX) for prime. full length JRFL gpl20 for boost	#890	100	1600	100	409600	400	25600	204800	25600	51200	25600
		#891	<100	1600	<100	409600	<100	25600	102400	25600	51200	6400
		#892	<100	1600	<100	102400	100	1600	102400	1600	25600	1600
		#893	<100	<100	<100	409600	400	25600	102400	25600	25600	6400
6	empty particles for prime, full length JRFL gp120 for boost	#894	<100	<100	<100	<100	100	<100	25600	<100	6400	<100
		#895	<100	<100	100	<100	100	<100	51200	<100	25600	<100
		#896	<100	<100	<100	<100	<100	<100	51200	<100	25600	<100
		#897	<100	<100	<100	<100	100	<100	51200	<100	25600	<100

[a] Priming Immunogen titers were determined using b122a for Groups 1,2,4,5,6; OD_{EC} for Group3, 6. For Group 6, titers against both b122a and OD_{EC} were less than 100

Table 3:

Neutralization ID_{50} values obtained with sera from wk0 and and wk22 in a standard TZMbl assay. Pre immune sera failed to neutralize any of the tested viruses.

Group	Group details	Animal ID	Bleed Week	Neutralization ID50				
				SVA-MLV Neg. Ctrl.	MN.3 Clade B Tier 1	JR-FL Clade B Tier 2	SF162.LS Clade B Tier 1	MW965.26 Clade C Tier 1
1	particles conjugated to b122a-19iC for prime, full length JRFL gp120 for boost	#874	22	12	118	<10	62	23
		#875	22	<10	13	<10	39	<10
		#876	22	<10	16	<10	11	11
		#877	22	<10	53	<10	10	13
2	particles displaying b122a for prime, full length JRFL gp120 for boost	#878	22	14	15	11	<10	15
		#879	22	<10	29	<10	14	20
		#880	22	<10	28	<10	18	<10
		#881	22	12	11	<10	<10	10
3	particles displaying ODec for prime, full length JRFL gp120 for boost	#882	22	<10	4502	<10	179	23
		#883	22	<10	21	<10	11	11
		#884	22	<10	56	<10	<10	12
		#885	22	11	12	<10	830	64
4	particles displaying b122a1-b for prime, full length JRFL gp120 for boost	#886	22	12	351	<10	1149	108
		#887	22	<10	427	<10	74	24
		#888	22	12	121	40	615	576
		#889	22	14	19	20	17	<10
5	particles conjugated to b122a-19iC (ADJUPLEX) for prime, full length JRFL gp120 for boost	#890	22	<10	376	<10	238	187
		#891	22	<10	737	<10	35	40
		#892	22	<10	49	<10	18	152
		#893	22	11	14	<10	764	1111
6	empty particles for prime, full length JRFL gp120 for boost	#894	22	<10	10	<10	<10	<10
		#895	22	<10	17	<10	45	<10
		#896	22	<10	17	<10	<10	23
		#897	22	<10	20	<10	19	<10

Table 4:

Sera competition binding studies using ELISA.

Percent competition at 1:50 dilution of week 22 sera[a]					
	b12	**F105**	**VRC-PG04**	**VRC01**	**PGT128**
Group1	10±2	38±1	9±2	14±2	2±1
Group2	12±0.5	26±2	14±1	12±1	4±2
Group3	17±2	28±2	22±1	11±1	14±1
Group4	**42±2**[b]	**57±1**[b]	26±2	21±1	15±1
Group5	21±2	29±1	26±1	18±2	14±1
Group6	19±0.5	18	14±1	18±1	4±1

[a]Data shown represent percent competition from two independent experiments and are represented as mean±SE.

[b]Percent competition for Group 4 sera with antibodies b12 and F105 is significantly higher compared to other groups, $P<0.01$ (individual means compared by Welch's *t*-test).