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Pseudovirion Particles Bearing Native HIV Envelope Trimers Facilitate a Novel Method for Generating Human Neutralizing Monoclonal Antibodies against HIV

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Abstract

Monomeric HIV envelope vaccines fail to elicit broadly neutralizing antibodies or to protect against infection. Neutralizing antibodies against HIV bind to native, functionally active Env trimers on the virion surface. Gag-Env pseudovirions recapitulate the native trimer, and could serve as an effective epitope presentation platform for study of the neutralizing antibody response in HIV-infected individuals. To address if pseudovirions can recapitulate native HIV virion epitope structures, we carefully characterized these particles, concentrating on the antigenic structure of the coreceptor binding site. By blue native gel shift assays, Gag-Env pseudovirions were shown to contain native trimers that were competent for binding to neutralizing monoclonal antibodies. In ELISA, pseudovirions exhibited increased binding of known CD4-induced antibodies following addition of CD4. Using flow cytometric analysis, fluorescently labeled pseudovirions specifically identified a subset of antigen-specific B cells in HIV-infected subjects. Interestingly, the sequence of one of these novel human antibodies, identified during cloning of single HIV-specific B cells and designated 2C6, exhibited homology to mAb 47e, a known anti-CD4-induced coreceptor binding site antibody. The secreted monoclonal antibody 2C6 did not bind monomeric gp120, but specifically bound envelope on pseudovirions. A recombinant form of the antibody 2C6 acted as a CD4-induced epitope-specific antibody in neutralization assays, yet did not bind monomeric gp120. These findings imply specificity against a quaternary epitope presented on the pseudovirion envelope spike. These data demonstrate

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that Gag-Env pseudovirions recapitulate CD4 and coreceptor binding pocket antigenic structures and can facilitate identification of B cell clones that secrete neutralizing antibodies.

1. Introduction

A renewed emphasis on fundamental questions pertaining to a rational approach to HIV vaccine discovery is needed urgently^{1, 2}. Neutralizing antibodies (Abs) are protective in both vaginal and intravenous infection model challenges in non-human primates^{3–6}. Neutralizing Abs are thought to be a crucial component of an appropriate HIV vaccine response⁷. The importance of neutralizing Abs in the response to HIV infection was reviewed recently^{8, 9}. In phase III efficacy trials, gp120-based vaccines failed to induce potent neutralizing Abs against circulating primary isolates of HIV and failed to protect vaccinees from HIV infection¹⁰. Three of the highest current research priorities of the NIAID are to determine why broadly neutralizing Abs are uncommon, to define the specificities of neutralizing Abs, and to characterize humoral immune responses needed to control viral replication¹. Natural virions may display both nonfunctional gp120/gp41 monomers and gp120-depleted gp41 stumps^{11–13}. Monomeric and incomplete trimeric forms of gp120 expressed on the surface of HIV particles may act as immune decoys to help the virus evade neutralization by the immune system¹³. These alternate viral protein structures also may misdirect the immune response toward non-neutralizing epitopes on the gp120 glycoprotein. Recapitulation of the native intact trimeric Env spike presents a major challenge to rational vaccine design.

Although protective Ab responses in infected individuals tend to lag temporally behind the appearance of new viral quasispecies, Abs to gp120 are implicated as agents of selective pressure in the evolution of viral Env sequence variability^{14, 15}. Previous work has shown a relationship between neutralization activity and global antigenic changes in portions of the HIV Env protein¹⁶. Continued stimulation of B cell clones by slightly modified quasispecies viral variants may cause these B cells to undergo repeated rounds of somatic mutation, driving affinity maturation of the immunoglobulin (Ig) genes against the new Env variants. Extensive somatic mutation is important for full maturation of functional Ab responses to other chronic viral infections¹⁷.

Despite difficulties encountered in eliciting broad neutralizing responses through vaccination, there are rare human mAbs (for example 2G12, 2F5, 4E10, b12) that neutralize a broad range of HIV isolates. Env-specific mAbs 2G12 and b12 bind Env trimers or monomers^{18, 19}. The binding of the broadly neutralizing gp41-specific mAbs 2F5 and 4E10, which recognize epitopes in the membrane proximal region, may need trimerization of the gp41/gp120 heterodimer to function optimally. Vaccination with these linear epitope sequences did not elicit Abs with a similar breadth of neutralization²⁰, implying a tertiary or quaternary structural constraint that is important for eliciting broadly neutralizing Abs targeting the gp41 region²⁰. This concept has been illustrated by the recent discovery of new quaternary epitope specific Abs²¹.

Interestingly, some broadly neutralizing Abs possess unusually long CDR3 loops, contain a large number of somatic mutations, or form rare Ab structures, which likely require the use of distinct subsets of Ab variable gene segments^{18, 22}. Selection of Abs able to form these unique structures may be required for effective neutralization of HIV. Repeated rounds of somatic hypermutation may be important for develop neutralizing Abs against quaternary epitopes on trimers of gp120/gp41. Unfortunately, little is known about the molecular features of HIV antigens that lead to induction of high potency anti-HIV Abs or how to induce such Abs by vaccination^{1, 2, 18}.

Solubilized trimers exhibit variable ability to induce neutralizing Abs and may not faithfully reproduce the antigenic structure of native trimerized Env23. In studies examining the importance of quaternary structure of antigen, mice were immunized with monomeric gp140 or oligomers of gp140. The gp140 protein encompasses the extra-membrane portions of gp160 prior to its natural cleavage into gp41 and gp120. Over half of the hybridomas isolated from mice immunized with gp140 oligomers recognized conformational epitopes on the viral Env²⁴. More recently, investigators immunized guinea pigs with three types of pseudovirions (also designated as virus-like particles or VLPs) or soluble gp120. Serum Abs generated by vaccination with Env-containing VLPs generally were reactive against nonfunctional forms of Env on VLP surfaces, possibly gp120/gp41 monomers, and not Env trimers¹¹. These trimers were created either by addition of disulfide bonds or mutation of the natural cleavage site.

VLPs with an uncleaved Gag core stabilize the membrane association of gp41/gp120 heterodimers on the VLP surface. Vaccination of guinea pigs with these HIV VLPs successfully induced a broad intraclade neutralization response, with a significant portion of the response against quaternary epitopes²⁵. Refinement of the production and purification of Gag-Env VLPs allowed selection for completely cleaved gp41/gp120 heterodimers, resulting in a practical platform for presentation of native Env trimers²⁶. Here we utilized fluorescent Gag-Env VLPs (also referred to as pseudovirions) for single-cell sorting of antigen-specific B cells in order to define the potential of these reagents to characterize the HIV-specific B cell response, particularly focusing on quaternary epitopes.

2. Materials and methods

2.1. Human subjects

Whole blood was obtained from subjects having clinical care at the Comprehensive Care Center at Vanderbilt University Medical Center, Nashville, TN or from the HIV Program at Emory Midtown Hospital, Atlanta, GA. Samples were de-identified as to age, gender, and other personal identifying information. For randomly-selected B cell controls, blood from three healthy blood donors was collected by the same method for comparative purposes. The study protocols and consent forms were approved by the Institutional Review Boards of both Vanderbilt University Medical Center and Emory University. Subjects 10055, 10002, and 10076 were chronically infected with a duration of infection from 7–24 years, and with CD4 + T cell counts >500 at the time of B cell sorting.

2.2. Isolation of B cells

Peripheral blood lymphocytes were isolated by centrifugation on 1.078 density lymphocyte separation medium. CD19⁺ B cells were separated using paramagnetic beads according to the manufacturer's instructions (Stemcell Technologies, Vancouver, BC). This procedure yielded ≥ 95% CD19⁺ viable cells when analyzed by flow cytometry (data not shown).

2.3. Generation of green-fluorescent protein-labeled virus-like particles (GFP-VLP) for single-cell sorting of HIV-specific CD19⁺ B cells

PCR fragments of codon-optimized HIV-1 Gag, IRES and HIV-1 BaL Env were introduced sequentially into plasmid pcDNA4/TO (Invitrogen, Carlsbad CA) to generate plasmid pcDNA4/TO Gag-I-Env. Plasmid pcDNA4/TO Gag-I-Env expressing HIV-1 Gag and Env from the tetracycline-controlled cytomegalovirus (CMV) promoter was used to transfect a T-Rex-293 cell line (Invitrogen, Carlsbad CA) that expressed the tetracycline repressor protein TetR. The T-Rex 293 cell line was grown in DMEM containing 5 µg/mL of blasticidin and was transfected using the calcium phosphate method. Forty-eight hours after transfection the cells were split into fresh medium containing 5 µg/mL blasticidin and 200 µg/mL zeocin for stable cell line selection. Single foci were selected and expanded. Clones growing in individual

wells were induced with doxycycline (2 µg/mL) and supernatants were harvested at interval time points to test for tetracycline-inducible HIV-1 Gag and Env expression using p24 and gp120 capture ELISA. Cell clones that exhibited highest Gag and Env expression upon induction and that demonstrated complete cleavage of gp160 to gp41 and gp120 were expanded and characterized further.

For production of GFP-expressing HIV-1 VLPs, the GFP gene was cloned in-frame with the HIV-1 *vpr* gene and cloned into the pcDNA5/TO vector to generate pcDNA5/TO vpr-GFP. PcDNA5/TO vpr-GFP was transfected into a pcDNA4/TO Gag-I-Env stable cell line (designated XC-34), which produced HIV-1 Env VLPs upon doxycycline induction using the method described above. The double-transfected cell lines were selected using 200 µg/mL of hygromycin and 200 µg/mL of zeocin.

GFP-VLPs were harvested from individual clones after three days of 2 µg/mL doxycycline induction and clarified by low-speed centrifugation, filtered through a 0.45 µm filter, and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 × *g* for 3 h, 0 °C). Viral pellets then were resuspended in 1 mL of PBS, and overlaid on linear 20–60% sucrose gradients. Ultracentrifugation was performed at 100,000 × *g* overnight at 4 °C in a Beckman SW41 rotor. Equal fractions were collected and the density of each fraction determined using a refractometer. Samples were subsequently diluted in PBS and pelleted by centrifugation at 45,000 rpm for 2 hours in a R45 rotor. The samples were analyzed by SDS-polyacrylamide gel electrophoresis.

2.4. Labeling of hybridomas and single-cell sorting of HIV VLP-binding CD19⁺ B cells

Hybridoma cells secreting the anti-HIV mAbs F2A3 and 39F were obtained as a kind gift from James Robinson, Tulane. Initially, both hybridomas were enriched by flow-cytometric sorting for surface Ig expression using labeling with anti-Ig-APC. These sorted cells were cultured and maintained as an enriched population of 60–80% surface Ig expressing cells over at least 5 passages. One million hybridoma cells were mixed with 20 µL of concentrated VLP preparation and anti-Ig-APC. Cells were labeled in 2% FBS/PBS and washed twice with 2% FBS/PBS prior to flow cytometric analysis.

For B cell sorting, 2–4 × 10⁶ B cells were stained with anti-CD19-APC (B4-RD1; Coulter, Miami, FL) and 50 µL of concentrated VLP preparation on ice for 30 minutes, similarly to the hybridoma staining. Flow cytometric analysis and single-cell sorting was performed with a FACSAria flow cytometer in a Biosafety Level 3 laboratory, equipped with an automated single-cell deposition unit and aerosol containment accessory (Becton Dickinson, Franklin Lakes, NJ). Single HIV VLP⁺ / CD19⁺ cells were collected one cell per well into 96-well cell culture plates (Costar®, Corning Incorporated, Corning, NY), containing RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% gamma-irradiated heat-inactivated fetal bovine serum, 2 mM L-glutamine, 2.5 µg/mL amphotericin B, 60 µg/mL tylosin, 50 µg/mL gentamicin and 0.1% 2-mercaptoethanol.

2.5. Expansion of single B cells in culture

HIV-specific B cells were cultured to generate small clones and identified as secreting HIV-specific Abs using an approach adapted from a previously described method²⁷. Briefly, 50,000 irradiated (50 Gray) EL4-B5 mouse thymoma cells (kindly provided by Dr. R. H. Zubler) per well of 96-well culture plates were used as feeder cells immediately following single-cell isolation of B cells. A combination of 100 U recombinant human IL-2, 5 ng/mL PMA, and 10% (v/v) of supernatant from pokeweed mitogen-activated human T cells ('T cell replacing factor' or TRF) was added. The culture plates were incubated for 7 days at 37 °C in an atmosphere of 8% CO₂. After 7 days, we removed 100 µL of supernatant and added 10,000

irradiated (50 Gray) fibroblastic L cells stably transfected with human CD154 (CD40L) to each well²⁸. This cell line was kindly provided by DNAX via the ATCC (CRL 12095). We also added 5 ng/mL recombinant human IL-4 in addition to the B cell culture medium described above. The cultures were kept for another two weeks with a second addition of CD154 fibroblasts, cytokines, PMA and TRF on day 14.

2.6. Assays for secreted Ig or HIV-specific Abs

For the detection of total human Ig, Immulon II HB 96-well microplates (Dynex Technologies, Inc., Chantilly, VA) were coated with unlabeled goat anti-human Ig (Southern Biotechnology Associates, Birmingham AL), diluted 1:1,000 in 50 mM sodium carbonate buffer, pH 9, and stored at 4 °C overnight. The plates were blocked with PBS containing 5% (w/v) nonfat milk for 4 hours at 4 °C. We added 100 µL of B cell culture supernatant, incubated for 2 h at room temperature, washed, and detected captured Ig with alkaline-phosphatase conjugated goat anti-human Ig (Southern Biotechnology Associates, Birmingham AL) diluted 1:1,000. Serial dilutions containing 1 ng/mL to 0.1 mg/mL of purified human Ig (Bioscience International, Kennebunk, ME) were included in the assay as quantitative standard controls, and cell culture supernatant from wells lacking B cells but containing all other culture media additives was used as a negative control.

For the detection of antigen-specific B cell clones, several ELISAs were developed. Ninety six-well microplates were coated with GFP-labeled HIV VLPs either incorporating (Gag-Env VLP) or lacking (Gag VLP) the BaL Env protein. An ELISA also was used to assess Ab binding to purified BaL gp120 Env protein (expression vector constructed by Dr. Marvin Reitz; the reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). This reagent was coated at 20 ng/mL at 4 °C overnight. Gp160 from HIV clade B strains MN and LAI (Prospec, Mississauga, ON) were used to screen binding of Fab fragments by coating the antigen at 10 ng/mL at 4 °C overnight. For VLP coating, particles were diluted 1:200 in PBS, pH 7.4, and coated plates were stored at 4 °C overnight.

Soluble CD4 (sCD4-183; Pharmacia, Inc) and the following gp120 mAbs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1: mAb F425 A1g8 from Dr. Marshall Posner and Dr. Lisa Cavacini, mAb 17b from Dr. James Robinson, mAb 2G12 from Dr. Hermann Katinger, and mAb IgG1 b12 from Dr. Dennis Burton and Dr. Carlos Barbas.

Supernatants of B cell clones that were positive in both the Gag-Env VLP and Gag VLP ELISAs were considered non-specific. Those that bound both gp120 and the Env VLP were considered gp120-specific. Those that bound only the Gag-Env VLP but not gp120 protein nor Gag VLPs were considered specific for HIV Env only in the context of VLP presentation. Addition of B cell culture supernatant and Ig capture was performed as described above. Optical density values of supernatants for both the human Ig and the HIV-specific ELISAs $\geq$ twice the background values were considered positive. The sensitivity of the ELISA was demonstrated to be $\leq$ 10 ng of Ig per well in preliminary experiments (data not shown).

2.7. Amplification of V_H and V_L regions

Poly (A⁺) RNA was prepared from ELISA confirmed HIV-specific B cell clones using oligo-dT based mRNA capture (Roche Diagnostics GmbH, Mannheim, Germany). Reverse transcription and a first round of PCR amplification was performed in one step following the manufacturer's protocol using Taq DNA polymerase (Titan One Tube RT-PCR System, Roche Diagnostics GmbH) and a pooled primer mix. The V_H and V_L primer sequences used were based on primers designed to amplify all human V_H and V_L gene segments and were published previously²⁷. Primers incorporated restriction enzyme sites that were introduced for cloning

purposes. For the cDNA reaction and the PCR we used the following thermocycling reaction parameters: 40 cycles of 92.5 °C for 1 min, 45 °C for 2 min, 72 °C for 2 min, followed a final extension at 72 °C for 7 min. First-round PCR products were amplified using V_H and V_L nested primers hybridizing to framework regions (FR) 4 (forward) and FR1 (reverse) with the following thermocycling reaction parameters: 94 °C for 3 min; 30 cycles of 94 °C for 30 sec, 55 °C for 45 sec, 72 °C for 45 sec; and a final extension at 72 °C for 7 min.

2.8. DNA sequence analysis

Extracted V_H and V_L gene PCR products were cloned into the pGEM[®]-T Easy vector system I (Promega Corporation, Madison, WI, USA), transfected into bacterial clones, and then DNA was isolated with a commercial plasmid miniprep kit (Qiagen, Valencia, CA). Plasmid DNA was digested with restriction enzymes ApaI and NcoI for the V_H insert, or with NheI and NotI for the V_L insert to identify clones with proper ligation of V gene regions. The nucleotide sequence of plasmid DNAs that contained a V_H or a V_L insert was determined by dideoxy chain terminator chemistry using fluorescently-labeled dideoxynucleotides and vector-specific primers. The sequence determinations were performed in an ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA). We analyzed V_H or V_L region sequences using MacVector[®] version 10.5 software (MacVector, Inc.) and with the international ImMunoGeneTics (IMGT) database²⁹.

2.9. Construction of Fab expression plasmids

HIV-specific Ab variable genes of interest were PCR-amplified from the pGEM-T Easy cloning vector using transfer primers for the Fab expression plasmid, digested, and HIV-specific V_H and V_L segments were ligated in step-wise fashion into the linearized pComb3XΔ19 bacterial expression vector, as described²⁶. Verified paired expression vectors were transformed into nonsuppressor strain HB2151 *E. coli* cells to produce soluble Fab fragments. Bacterial colonies containing sequence-confirmed pComb3XΔ19 cloned variable gene segments of interest were inoculated into 2XYT broth supplemented with 100 µg/mL ampicillin and grown overnight at 30 °C. The overnight culture then was diluted 1:10 into fresh 2XYT broth supplemented with 100 µg/mL ampicillin. The culture was grown at 30 °C until an OD₆₀₀ of 0.6 was reached. Fab expression was induced by addition of 50 µM IPTG and ampicillin was further supplemented with fresh 100 µg/mL ampicillin. The culture then was grown at 30 °C for 12–15 hours and cells were collected by centrifugation. The periplasm was extracted with TES and analyzed by SDS-PAGE and Western blot to ensure proper formation of the disulfide-bonded product. Fab fragments were purified from the periplasmic extract using a HisTrap column on an AKTA FPLC (GE Healthcare, Piscataway NJ). The eluted purified Fab products were dialyzed against PBS, and analyzed by SDS-PAGE with Coomassie stain or Western blot with goat anti-human Fab (Bethyl Laboratories) and anti-goat Ig HRP conjugate (Sigma, St. Louis, MO) detection to verify purity. The quantity of purified Fab in each preparation was determined by a sandwich ELISA using an unlabeled anti-F(ab')₂ capture and an alkaline phosphatase labeled anti-F(ab')₂ detection (Pierce, Rockford, IL). Concentrations were determined from comparison of experimental OD values to those of varying dilutions of a purified Fab standard at known concentrations.

2.10. Full-length Ab expression

Similarly to Fab cloning, Ab heavy and light chain variable gene segments were cloned into a full-length human IgG expression vector (Lonza) as previously described³⁰. HEK 293 Freestyle cells (Invitrogen, Carlsbad, CA) were transfected transiently with plasmids encoding full-length heavy chain and corresponding light chains per manufacturer's protocol. After 7 days, supernatant was collected and clarified by centrifugation at 400 × g for 10 minutes at 4 °C. Supernatant then was filtered through a 0.2 micron filter and the immunoglobulins were

purified using a 5 mL HiTrap Protein G column on an AKTA FPLC (GE Healthcare, Piscataway NJ). The IgG fraction was recovered from the column by eluting with a citrate buffer, pH 2.6). The eluting solution was neutralized by addition of 100 μ L of Trizma-HCl, 1M, pH 8.0 (Sigma-Aldrich) and then exchanged to Dulbecco's PBS via 3 washes via 20-fold concentrations using an Amicon Ultra-15, PLHK Ultracel-PL Membrane, 100 kDa cutoff (Millipore Corp., Bedford, MA).

2.11 Native blue gel shift assays

The VLPs were incubated with 0.3% DDM or 0.12% Triton X-100 in 1X NativePAGE sample buffer (Invitrogen) and 1 μ L of protease inhibitor cocktail (Sigma, St. Louis MO) on ice for 15 min to gently remove native Env. Centrifugation was performed at $12,000 \times g$ for 30 min at 4 $^{\circ}$ C. One part in five of detergent concentration of G-250 was added to supernatants prior to loading the sample onto 3–12% Native PAGE Bis-Tris gels (Invitrogen, Carlsbad CA). Samples were electrophoresed with 1X NativePAGE running buffer containing 0.002% Coomassie blue as cathode buffer and without Coomassie blue as the anode buffer. Electrophoresis was performed at 4 $^{\circ}$ C at 150 V constant for 90 minutes and then increased voltage of 250 V for 60 minutes. After electrophoresis, the gel was transferred to PVDF membrane using NuPAGE transfer buffer. After transfer, the membrane was incubated in 8% acetic acid for 15 minutes to fix the protein and then proceed with immunodetection. The blot was probed with 1 μ g/mL of each of mAbs 2G12, b12 and 447-52D, as described³¹, followed by Irdye CW800 goat anti-human (LI-COR Biosciences) at 1:10,000 dilution for detection.

2.12. HIV-1 single-round neutralization assay

Neutralization assays were performed similar to previously published protocols³². TZM-bl cells expressing both CD4 and CCR5 and containing a reporter gene for firefly luciferase under control of an HIV-1 LTR were obtained from John Kappes and Xiaoyun Wu through the NIH AIDS Research and Reference Reagent Program. Primary HIV-1 or clade B pseudovirus isolates³³ were incubated with serial dilutions of test samples in duplicate in a total volume of 150 μ L containing 30 μ g/mL of DEAE dextran for 1 hour at 37 $^{\circ}$ C in 96-well flat-bottom culture plates. This mixture then was added to the corresponding well of a 96-well flat-bottom culture plate containing adherent TZM-bl cells that were seeded at 5,000 cells per well in 100 μ L of growth media at 37 $^{\circ}$ C in 5 % CO₂ seeded 24 hours earlier. After a 48-h incubation, 200 μ L of cell lysate was transferred to 96-well black solid plates (Costar, Corning, NY) for measurements of luminescence using Bright Glo substrate solution, as described by the supplier (Promega, Madison, WI). Neutralization titers were reported as the dilutions at which relative light units (RLU) were reduced by 50% compared to those of virus control wells, after subtraction of background RLUs.

3. Results

Pseudovirions (or virus like particles [VLPs]) incorporate Vpr-GFP fusion protein

Gag VLPs self-assemble when the *gag* gene is expressed in a cell line. When *gag* and *env* genes are expressed together, VLPs are created similarly, however, in this case Env protein self assembles on the lipid outer-membrane and forms trimeric Env, thus mimicking a natural HIV virion (herein referred to as Gag-Env-VLP)^{26, 34}.

To more thoroughly explore antigen-Ab interactions using these VLPs, a *vpr-GFP* gene fusion construct that incorporates into the VLP was created. Co-transfection with the *gag* and *env* genes produced fluorescently-labeled Gag-Env-VLPs. Vpr is a late viral protein that incorporates into HIV virions in a p6-dependent manner. Vpr fusion has been the preferred method of incorporating foreign protein expression into intact virions³⁵. The resulting gene

encoding Vpr-GFP fusion protein then was cloned into inducible expression vectors, and stably transduced cell lines expressing Gag, Env, and Vpr-GFP established.

Supernatants from doxycycline-induced cell lines were harvested and examined for the presence of Gag-Env-Vpr-GFP particles. VLPs were examined by sucrose density gradient centrifugation, revealing Vpr-GFP in the peak fraction with Gag and Env, at a density similar to that of native HIV virions (Figure 1, A–B). The purity and stability of the purified VLPs was assessed further by electron microscopy. Although some misshapen particles and microvesicles were observed, the fields examined revealed primarily intact immature particles (Figure 1C).

Uncleaved gp160 has been noted on VLPs produced from overexpression systems. To avoid this potentially undesirable form of Env, we generated Gag-Env/Vpr-GFP cellular clones and examined supernatants by Western blot for cleavage of gp160. We then selected lines for further work that had no detectable gp160. Figure 2A shows that both BaL and JR-FL stably transfected cell lines produced VLPs bearing cleaved gp120 on the surface (BaL cell line, JR-FL cell line lanes). This finding contrasts markedly with VLPs produced by transient transfection (Figure 2A). JR-FL with uncleaved gp160 was constructed by mutations in the native cleavage site between gp41 and gp120 on the gp160, and is included as a control to demonstrate uncleaved glycoprotein. SOSIP is a gp140 soluble form of Env that has gp120 stably linked to the gp41 ectodomain by an intermolecular disulfide bond (SOS) and that contains an isoleucine-to-proline (IP) substitution at position 559 in the N-terminal heptad repeat region of gp41. The SOSIP construct was trimerized by addition of disulfide bonds in the gp41 region of gp140 constructs. In comparison to the selected cell line VLPs, SOSIP or JR-FL VLPs produced via transient transfection demonstrated a significant amount of uncleaved gp160.

Epitope characterization of VLPs by ELISA

VLPs were used as antigens in ELISAs and subsequently probed with Abs to examine their epitopic characteristics. MAb D7324 is an Ab that binds an epitope on the gp120 surface at the interface of gp41 and gp120. This epitope is not available for binding if heterodimers are intact. MAb D7324 did not bind Gag-Env VLPs, indicating maintenance of surface gp120/gp41 heterodimers without D7324 epitopic exposure (Figure 2B). MAb 2G12 is a well-characterized broadly neutralizing Ab that recognizes a glycosylated epitope on the surface of gp120. In ELISA binding assays, mAb 2G12 bound specifically to the Gag-Env VLPs but not Gag VLPs.

Blue native gel electrophoresis assays also were performed to assess the presence of native trimers on the VLPs. Gag-Env VLPs were harvested from producer cell line supernatants and purified through a 20% sucrose cushion. Envelope complexes then were extracted gently with non-ionic detergent, and blue native gel analysis performed. BaL and JR-FL gp41/gp120 trimers were identified by migration near the 480 Kd ferritin marker (Figure 2C). Neutralizing Abs have been demonstrated to shift these trimeric forms specifically in blue native gel analysis, while non-neutralizing antibodies fail to bind to native trimers³¹. As seen in Figure 2C, mAb 2G12 and mAb 2F5 shifted the trimers presented by the BaL Gag-Env VLPs. Similarly JR-FL trimers from JR-FL Gag-Env VLPs also were shifted by broadly neutralizing mAbs, shown here with the anti-CD4 binding site mAb b12 and the anti-gp41 mAb 4E10. Similarly, soluble CD4 and polyclonal HIV-positive serum (data not shown) were capable of producing this shift. These results demonstrate that the Gag-Env VLPs incorporate native, trimeric envelope complexes on their surface as expected.

After binding of CD4 to HIV-1 Env, the gp120 glycoprotein undergoes a conformational change to allow access to the coreceptor binding site. Generally, Abs that exhibit an increase

in affinity after this CD4-induced change bind at or near the coreceptor binding site and have been termed CD4-induced (CD4i) Abs. In a rhesus macaque model, attempts to induce such Abs by vaccination of covalently crosslinked complexes of HIV Env and CD4 elicited a broadly neutralizing Ab response³⁶. However, results from other studies following this approach were less compelling^{37, 38}. Although the importance of CD4i Abs as a potential vaccination goal is controversial, recapitulation of the ability to induce such Abs by a vaccine construct implies a general replication of the native Env structure. To assess if the CD4 binding site and coreceptor binding sites were presented in the Gag-Env VLPs similarly to those in native virions, ELISAs were performed in the absence or presence of recombinant sCD4. The CD4i mAb F425-A1g8 exhibited a low level of ELISA binding that was improved markedly in the presence of recombinant soluble CD4 (Figure 2D). Similar results were obtained with another CD4i mAb, 17b. Notably, no difference was seen in the binding of the known non-CD4i mAb 2G12. These experiments further establish the presence of native envelope structure on the Gag-Env VLPs surface.

Anti-HIV antibody hybridoma cell lines with surface Ig specifically recognize Gag-Env VLPs

To evaluate whether these Gag-Env VLPs could be used as reagents in labeling HIV-specific B-cells during flow cytometry, initial studies were conducted using hybridoma cell lines expressing HIV-specific human mAbs. Cell lines were enriched for Ig cell surface positive cells by flow cytometric sorting. The F2A3 hybridoma expresses a mAb that recognizes the V3 region of gp120. We used an unrelated hybridoma as a control cell line. The mean GFP fluorescent intensity of unlabelled F2A3 hybridoma cells versus labeled F2A3 hybridoma cells was 80 units versus 230 units respectively (Figure 3A). Using these data to guide the setting of the HIV specific B cell sorting gates, we compared the upper limit of labeling between the control and anti-HIV F2A3 hybridoma. Less than 0.02% of labeled control cells fell beyond a difference of one log₁₀ of mean fluorescence intensity (MFI) of the control cell population. The F2A3 hybridoma had 0.08% of cells above this cutoff. Comparing a 0.85 log change between these cells maintained the <0.02% of labeled control cells, but increased the positive labeled population of the F2A3 hybridoma group to 0.37% of cells. This labeling was confirmed to be VLP-specific by competition binding using unlabeled VLPs with a resulting decrease in GFP labeling of the F2A3 hybridoma. Similar results were seen with the 39F hybridoma (data not shown). This finding supported the feasibility of specifically labeling anti-HIV B cells in a flow cytometry based assay using fluorescent VLPs.

Labeling and isolation of HIV-specific B cell clones

In order to study the native Ab repertoire against HIV Env, GFP-labeled HIV-VLP reactive B cells were isolated from patient samples. Samples from chronically infected HIV-positive subjects were obtained and PBLs were collected from either fresh blood draws or from a frozen archive of collected samples from the same subject cohort. To define the appropriate flow cytometric gate of HIV-specific B cells, a cutoff for GFP-VLPs B cells of one log₁₀ difference above the mean GFP fluorescence intensity of the unlabelled B cell population was established. An HIV-negative subject's B cells were labeled with GFP-Gag-Env VLPs (Figure 3 B) and showed no specific population with a log₁₀ difference in MFI above the mean.

VLPs, like native HIV virions, are predicted to incorporate host cell surface molecules that may cause non-specific interactions. A fluorescently labeled Gag VLP (GFP-Gag VLP), which lacks Env protein but is otherwise identical to the Gag-Env VLPs, was created to assess any non-specific binding effects in these assays. HIV-positive subjects had limited nonspecific binding to GFP-Gag VLPs (Figure 3 C). In contrast, GFP-Gag-Env VLPs specifically labeled a subset of circulating B cells in HIV-positive subjects (Figure 3 D). The mean fluorescence intensity of the negative B cell populations from HIV-negative and HIV-positive subjects was similar (Figure 3 B and D). HIV-positive subjects demonstrated 0.2 to 1.0% HIV-specific B

cells at one log₁₀ difference from the mean when labeled by GFP-Gag-Env-VLPs. This frequency of antigen-specific cell numbers is similar to previous estimates of circulating HIV-specific B cells, as determined by ELISPOT assay³⁹, however lower than some other recent estimates⁴⁰.

To determine if the percentage of B cells binding GFP-Gag-Env-VLPs as detected by flow cytometry correlated with other laboratory parameters, a panel of subject samples was analyzed for VLP binding and neutralization of a panel of primary Env isolates. PBLs were obtained and labeled with anti-CD19 and fluorescent Gag VLPs or Gag-Env VLPs. We did not observe a direct linear correlation of serum neutralizing titers to percentage of GFP-Gag-Env-VLPs (the R² correlation coefficients were 0.16 and 0.18 respectively) (see Supplemental Data). However, subjects who had significant serum neutralization of the BaL HIV strain, defined as >1:400 titer, did exhibit a higher percentage of B cells binding to GFP-Gag-Env-VLPs, whereas subjects with BaL neutralizing titers of <1:400 tended to have low to no GFP-Gag-Env-VLP binding. Although this trend is of interest, this study was not yet adequately powered to show a significant correlation (see Supplemental Data).

Isolation of HIV-specific B cell clones and Ab variable gene segments

Prior to selection of an individual's B cells for flow cytometric sorting, serum neutralization assays were performed to identify subjects with circulating neutralizing antibodies against the HIV-VLP parent strain, the clade B strain BaL. Eighteen HIV-positive subjects were screened in this manner with 7 having serum neutralization titers >1:80 for BaL. Individual B cells were sorted as single cells by FACS into wells of a 96-well plate containing cytokine-enriched media and a CD154-expressing feeder cell line. After culture and expansion of single cells into small clonal populations, supernatants were screened for Ig production and antigen specificity using three distinct ELISAs (gp120, Gag-Env VLP, or Gag VLP). Recombinant purified BaL gp120 was used in the gp120 ELISA to screen for monomer reactivity. Table 1 reports the results from 5 B cell sorts performed on three different individuals.

Generally, between 2–10% of B cells that were sorted based on labeling with the fluorescently-labeled HIV-VLPs produced Ig after stimulation. Supernatants then were used to confirm HIV specificity by ELISA. The control Gag VLPs bound to <0.02% of positive cells in the flow cytometric assay. Abs were cloned successfully from subjects 10002 and 10076. Both subjects' sera showed neutralization at >1:80 serum titer against BaL. Additionally, serum from subjects 10002 and 10076 neutralized a number of the standard clade B panel of pseudoviruses (75% and 33% neutralization respectively)³³.

After B cell clones of interest were identified, the cells were lysed and poly-A RNA was collected for RT-PCR. PCR primers recognizing all Ig heavy and light chain variable regions were used²⁷, gene segments were cloned, sequenced, and paired Ab variable gene segments of interest were cloned into Fab and full-length Ab expression vectors.

Homology to a known anti-HIV Ab

Sequence analysis revealed that the majority of the unique Ab clones discovered shared over 90% nucleic acid homology with at least one other Ab clone. When seen, these homologs also shared predicted germline V_H or V_L, D, and J_H or J_L gene segment usage and shared homology of the heavy and light chain CDR3 regions, and are referred to here as clonotypes. Recently, clonotypic populations in HIV-infected subjects have been described using similar methods⁴⁰. Intriguingly, the mAbs 6B8 and 2C6 in a group we designated as clonotype C showed homology in their CDR3 regions to the known anti-HIV CD4i mAb 47e (also designated as 4.7E) (Figure 4A). Comparison of the HCDR3 regions of mAb 47e and clones 2C6 and 6B8 revealed a DY*SDPFY shared amino acid (AA) motif (shared AAs are

underlined). Sulfation of this motif at the first tyrosine (**DY***SDPFY) facilitates mAb 47e binding by allowing mAb 47e to mimic the binding site sulfation of CCR541⁴². IMGT sequence analysis predicted that all three mAbs (47e, 2C6, and 6B8) use the V_H1-69 and J_H6 gene segments. Strikingly, mAbs 6B8 and 2C6 have 43 nucleic acid mutations from germline resulting in 19 AA changes substitutions, while mAb 47e has 11 nucleic acid mutations resulting in only 5 AA substitutions. In the genes encoding mAbs 2C6 and 6B8, mutations were concentrated in the HCDR1, HCDR2 and HFR3 regions (Figure 4B; mAb 2C6 alone is shown for illustration).

Confirmation of supernatant binding and neutralization

Since mAb 47e binds gp120 and shows sequence homology to the mAbs 2C6 and 6B8 (clonotype C), this clonotype also appeared likely to bind to gp120. However, ELISA screening of the mAbs 2C6 and 6B8 showed significant binding activity only to the HIV-VLP and not the gp120 monomer (data not shown). To further study this binding, the paired Ab heavy and light chains of mAb 2C6 were cloned into a Fab expression vector²⁷ and into a IgG full-length expression system³⁰.

After expression and purification, both the expressed 2C6 Fab and the expressed full-length IgG immunoglobulin recapitulated the ELISA binding pattern of the B cell clone supernatants (Figure 5 A and B) by reacting specifically to the HIV-VLP but not the monomeric clade B gp120, monomeric clade B gp160, or the Gag-only-VLP control. Samples were run in duplicate and in minimum 2 different experiments. Lack of binding by the control human rotavirus-specific mAb RV6-26 in turn expressed as both a Fab and full-length IgG showed this activity was specific for mAb 2C6 and not secondary to nonspecific binding of any human Fab expressed in bacteria.

Neutralization assays then were performed to assess the functionality of the mAb 2C6. Upon addition of mAb 2C6 alone, a low level of neutralization activity against the HIV BaL strain was observed at 12.5 µg/mL Fab concentration (Figure 6). Since this clone exhibits sequence homology to the known CD4i antigen-specific Ab mAb 47e, the effect of the addition of sCD4 on neutralizing activity was assessed next. Mab 17b is a prototypical CD4i antigen-specific Ab. As expected, mAb 17b exhibited an increase in neutralization activity with addition of soluble CD4 (Figure 6). Not surprisingly since mAb 2C6 shares homology to the known CD4i mAb 47e, the mAb 2C6 neutralization profile shown here also is consistent with that of a CD4i Ab. Since this mAb 2C6 binds VLPs but not monomers on ELISA and neutralizes as a CD4i antigen-specific Ab, the binding epitope is likely a quaternary structure on the gp120/gp41 trimeric Env spike.

Two additional clade B strains were assessed for neutralization. Strain QH0692, clone 42 (SVPB6) and pREJO4541, clone 67 (SVPB16)³³ also were neutralized by serum from subject 10076, from whom the mAb 2C6 was cloned. However, full-length 2C6 IgG did not neutralize these strains in the presence or absence of CD4.

This finding further indicates that the HIV Gag-Env VLPs recapitulate native HIV virion epitopic structures, particularly the CD4 binding site and the coreceptor binding site, facilitating the efficient identification and sorting of human B cells specific for the native Env trimer.

4. Discussion

The development of immunogens that are capable of eliciting broadly neutralizing antibodies against HIV is a major challenge in the quest for an effective vaccine. Progress in understanding the basis of HIV neutralization is needed in order to design vaccination strategies that might

achieve breadth. A greater understanding of the B cell repertoire against the native Env complex that develops in infected individuals will be beneficial in understanding the basis of neutralization breadth against HIV. Here we generated fluorescent VLPs that possess stabilized surface trimers of gp41/gp120 heterodimers, bind to broadly neutralizing Abs, and label specifically anti-HIV B cell clones. Binding to GFP-labeled VLPs was used to clone out naturally-occurring anti-HIV Abs from chronically infected subjects. One of these Ab clones, the mAb 2C6, shows conformation-dependent binding, and neutralized as a CD4i epitope-specific Ab, yet appeared to bind a quaternary epitope.

It has been proposed that conformationally dependent trimer-specific binding of Abs is a critical component of viral neutralization⁴³. The recent identification of two novel broadly neutralizing monoclonal antibodies illustrates this concept²². In this study, the overwhelming majority of clonal anti-HIV B cell supernatants did not bind to monomeric gp120 or gp41. Vaccination of guinea pigs with VLPs resulted in reciprocal serum neutralizing titers of 300–600²⁵ with up to 50% of activity potentially targeted to quaternary epitopes. We were interested to explore if these VLPs recapitulated a native quaternary Env trimer structure. The cloning of the quaternary epitope-specific mAb 2C6 suggests our VLPs do recapitulate native quaternary Env trimer structures. Stabilization of the trimer on the surface may be due to the use of an immature Gag core that stabilizes the gp120/gp41 interaction and prevents shedding of gp120 even after CD4 binding.

CD4i epitope binding Abs do not necessarily bind directly to the coreceptor binding site, as V3 loop-specific Abs also have been shown to be induced by binding of CD4. However, this finding has been shown with viral subtypes such as JR-FL that tend to have inaccessible V3 loops in the absence of CD4⁴⁴. This finding is not universal for all clade B viruses. In particular, the BaL strain that is the basis for our VLPs displays accessible Env V3 loops in the absence of CD4 binding.

The mAb 2C6 shares significant sequence homology with the mAb 47e, which uses tyrosine sulfation of HCDR3 to improve binding by mimicking CCR5 binding⁴². These and other mAbs that mimic the CCR5 coreceptor structure by utilizing tyrosine sulfation of their HCDR3 have been cloned from long-term HIV-infected subjects. This finding implies either a role for such Abs in long-term control or a need for continued affinity maturation for selection. Interestingly, the sulfated CD4i Abs also neutralize more effectively than the prototypical CD4i Abs 17b and 48d⁴¹. Although mAb 2C6 and mAb 6B8 share the DY motif that is sulfated in CCR5 mimicking Abs, the arginine that abuts this motif in 2C6 usually interferes with sulfation in most contexts. Studies are currently ongoing to explore the ability of 2C6 and 6B8 to act as CCR5 mimics.

We have used this single B cell sorting method successfully in the past to clone human Ab genes that encode productive Abs reactive against rotavirus and respiratory syncytial virus, as well as Abs from randomly-selected B cells^{27, 45–50}. Strikingly, the level of mutation in the variable genes encoding trimer-specific anti-HIV Abs are greater than that in randomly-selected B cells or other virus-specific B cells. Similar to recent reports on anti-HIV Abs⁴⁰, we have also found a significant number of circulating clonal populations of HIV-specific Abs in infected subjects.

GFP-Gag-Env VLPs were able to specifically label 0.2 to 1.0% of circulating B cells in HIV-infected individuals. This frequency was similar to that in other recently published studies³⁵. However, one recent study showed slightly higher frequencies of circulating anti-HIV specific B cells⁴⁰. The differences seen in these studies, although minor, may stem from the fact the latter study enriched for memory B cells prior to analysis. The memory B cell compartment likely is enriched for anti-HIV Abs in infected individuals.

An important aspect of this study was the development and use of separate screening ELISAs to delineate specificity prior to cloning of the Ab gene segments. In this study, clonal supernatants were screened against: BaL-Gag-Env-VLPs (presenting gp120/gp41 as a trimer), BaL-gp120 monomer, and Gag-VLPs which present all of the unrelated lipid membrane associated components that would be on the surface of the BaL-Gag-Env-VLPs but have no gp41 or gp120. Minimal non-specific interaction was detected with the Gag-VLPs that did not contain any form of Env.

The molecular basis for broadly neutralizing Ab responses to HIV is unclear at this juncture, however antibody repertoire studies have potential to address this question⁴⁰. Shotgun cloning of PBLs or analysis of sequences from cells that bind to various HIV antigens during flow cytometry may create confusion if the antigens do not possess the native configuration of epitopes. Such repertoire analysis without Ab expression and confirmation of Ab binding is difficult to interpret. In our studies, only 0.7–10% of the Abs secreted by sorted cells could be shown definitively to bind to viral particles after sorting. Care is needed when drawing conclusions from studies on repertoire usage since the physical sorting process results in potentially a high level of non-specific binding. This study utilized direct B cell labeling by VLPs and confirmed Ab binding by ELISA with the same antigen. This approach, albeit labor intensive, may be the optimal approach to isolate and analyze individual B cells specific for HIV in a natural host.

The ability of these VLPs (or pseudovirions) to recapitulate the native HIV envelope spike makes these constructs ideal tools to further characterize the native antibody response to HIV infection. Fluorescent pseudovirions provide an excellent antigenic platform to identify novel anti-HIV antibodies, particularly those that bind quaternary structures. In doing so, they may assist in elucidating why monomeric Env vaccines have failed and may assist in future improved HIV vaccine development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Soluble CD4 (sCD4-183) from Pharmacia, Inc, the NIH clade B reference panel of HIV isolates, and the following gp120 mAbs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: mAb F425 A1g8 from Dr. Marshall Posner and Dr. Lisa Cavacini, mAb 17b from Dr. James Robinson, mAb 2G12 from Dr. Hermann Katinger, and mAb IgG1 b12 from Dr. Dennis Burton and Carlos Barbas. The TZM-bl cell line also was obtained through this program from John Kappes and Xiaoyun Wu. We thank DNAX for use of the CD154 expressing cell line, Rudolf H. Zubler for the EL4-B5 cell line, the NCI BRB Preclinical Repository, Rockville, MD for recombinant human IL-2. We thank Dr. Carlos Barbas for the original pComb3X vector and Drs. James Robinson and Hermann Katinger for anti-HIV hybridomas. Nucleotide sequencing was performed by the Vanderbilt DNA Sequencing Facility. Flow cytometry was performed in the Vanderbilt-Meharry CFAR Immunopathogenesis Core Laboratory. Electron microscopy was performed with the help of the Robert P. Apkarian Integrated Electron Microscopy Core Laboratory at Emory University. We thank Mohammed Aiyegbo, Frances House, Jordan Willis, Jens Krause, and Valerie Mitchell for technical and organizational support, and Natalie Thornburg for thoughtful comments on this manuscript.

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Figure 1. Production of GFP-labeled Pseudovirions (or VLPs)

The XC-34 cell line was grown to 95% confluence and then induced by the addition of 2 μ g/ml of doxycycline. At 72 hours post-induction, VLP pellets were collected from clarified supernatant by ultracentrifugation through a sucrose cushion. Pellets then were placed on a 20–60% sucrose gradient and spun for 18 hours at $100,000 \times g$. **A**) Gradient fractions were collected, fluorescence intensity (left Y axis) was measured with a scanning cuvette fluorometer, and density (right Y axis) was measured via refractometry. **B**) Western blot analysis of fractions from sucrose gradient probed with mAb D7324 (gp120) and mAb CA183 supernatant (pr55gag), or anti-GFP monoclonal (BD Clontech). **C**) Transmission electron microscopy of VLPs after pelleting through a sucrose cushion was performed. Image shown

was acquired on a Hitachi H-7,500 at a magnification of 120,000 X (the white bar at the lower right indicates 100 nm).

Figure 2. Epitope presentation of Gag-Env-VLPs tested by ELISA and native blue gel shift assays
A) Western blot analysis of HIV Env proteins from purified VLPs comparing stable cell line (BaL and JR-FL) to transient production methods and to control (uncleaved) Env. **B)** Binding of mAbs 2G12 and D7324 was assayed using Gag-Env-VLP or Gag VLP ELISAs. **C)** Blue native gel shift assay of mAbs against the Env protein complexes extracted from VLPs, with subsequent Western blot probing using mAb 7G2 which recognizes an epitope on the V3 loop. Native complexes prior to shift are shown in the leftmost lane for BaL and JR-FL Env complexes. **D)** Binding of mAb 2G12 and CD4i mAbs F425-A1g8 and 17b in the presence or absence of sCD4.

Figure 3. GFP-labeled Gag-Env VLPs bind HIV-specific surface Ig expressing hybridomas and bind HIV-infected subject B cells specifically by flow cytometry

A) MAb F2A3 secreting hybridoma cells (right panel) were compared to cells from a control hybridoma line (left panel). Flow cytometry was used to compare unlabeled cells (white peaks) to labeled cells (grey peaks) after mixing with fluorescent Gag-Env VLPs. Mean GFP fluorescence intensity is marked above each peak, with mAb F2A3 hybridoma having 80 or 230 units and control cells having 68 or 92 units unlabeled versus labeled mean population fluorescence intensity respectively. **B–D)** X axis indicates GFP fluorescence (VLP binding) and y axis shows anti-CD19 labeling of B cell populations after CD19 paramagnetic bead enrichment of B cells from PBLs. **B)** HIV-negative subject B cells were labeled with GFP Gag-

Env-VLP. **C)** HIV-positive subject B cells were labeled with Gag VLPs. **D)** HIV-positive subject (same subject as used in panel C) B cells were labeled with GFP Gag-Env-VLP.

A	Framework 1	CDR1	Framework 2	CDR2	Framework 3	
VH1-69	AEVKKPGSSVKVSCKAS	GGTFSSYA	ISWVRQAPGQGLEWMGG	IIPIFGTA	NYAQKFQGRVTITADESTSTAYMELSSLRSEDATVYYC	
mAb 47e	-----	-----	-----	---V--S-	-----A---T-----F-	
mAb 2C6	T-----R-----	--NFLDFV	V-----A	-V-RV-A-	Q---R---A-F---V-----L-VT-----	
B	Framework 1	CDR1	Framework 2	CDR2	Framework 3	
VK1-27	SVGDRVITTCRAS	QGISNY	LAWYQQKPGKVPKLLIY	AAS	TLQSGVPSRFSGSGSGTDFTLTITSSLPEDVATYYC	
mAb 2C6	-M-----	---S-	-----A-----	G--	-----	
VK1-39	SVGDRVITTCRAS	QSISSY	LNWYQQKPGKAPKLLIY	AAS	SLQSGVPSRFSGSGSGTDFTLTITSSLPEDFATYYC	
mAb 47e	-----	-----	-----V-----	---	-----	
C	HCDR3 AA sequence				D	KCDR3 AA sequence
mAb 47e	KGGEDGDYLSDPFY <u>Y</u> NHGM				mAb 47e	CQQSYSTSH TF
mAb 6B8	RGMIHADYRSDPFYHYMDVW				mAb 6B8	CQETYSVPPWTF
mAb 2C6	RGMIHADYRSDPFYHYMDVW				mAb 2C6	CQSYNSAHF TF

Figure 4. Sequence homology of the mAbs 2C6 and 6B8 to known anti-HIV CD4i mAb 47e

A) Predicted amino acid (AA) sequence alignment of the translated variable heavy chain gene segments of mAb 47e and mAb 2C6 compared to the Ab encoded by the germline VH1-69 gene segment. Sequence comparison begins at IMGT AA 10. **B)** Predicted AA sequence alignment of the translated variable kappa chain gene segments of GenBank partial Ig sequence mAb 47e and mAb 2C6 compared to the germline VK1-27 and VK1-39. Differences between the germline sequences are bolded in the text. **C)** Predicted AA sequence alignment of heavy chain CDR3 regions of known mAb 47e compared to clonotype C mAbs 2C6 and 6B8. **D)** Predicted AA sequence alignment of kappa light chain CDR3 regions of known mAb 47e compared to clonotype C mAbs 2C6 and 6B8.

Figure 5. MAb 2C6 binding recapitulates B cell clonal supernatant screening ELISA results
 Binding specificity of mAb 2C6 and control mAb RV6-26 expressed as **A)** Fabs and as **B)** full-length Ab against the following HIV antigens were assayed by ELISA: BaL gp120 monomer, Gag VLPs, and Gag-Env-VLPs (parental strain of Env was BaL).

Figure 6. Neutralization of Fab 2C6 compared to known CD4i mAb 17b

HIV strain BaL was incubated for 90 min at room temperature with Abs (12.5 $\mu\text{g/ml}$) in the absence or presence of 0.075 $\mu\text{g/ml}$ of soluble CD4. Neutralization was detected 48 hours later. HIV immune patient serum was used as a positive control.

Table 1

Initial Sort Summary: Subject characteristics

Subject (Year infected)	Viral load	Sort	% Gag-Env VLP+ B cells	Gag-Env VLP+ B cells sorted	Ig + wells	% Ig + wells of sorted	HIV specific wells	% HIV specific from Ig +	% HIV specific from + B cells sorted
10076 (1999)	21339	1	0.21	129	47	36	8	17	6.2
		2	0.28	448	70	16	45	64	10.0
10002 (1984)	65	1	0.19	372	17	5	8	47	2.1
		2	0.14	360	28	8	15	53	4.2
10055 (2001)	1000	1	0.13	760	127	17	5	4	0.7