HHS Public Access

Author manuscript

Mol Immunol. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Mol Immunol. 2016 February ; 70: 94–103. doi:10.1016/j.molimm.2015.12.002.

Low frequency of broadly neutralizing HIV antibodies during chronic infection even in quaternary epitope targeting antibodies containing large numbers of somatic mutations

Mark D. Hicar[a,b], **Xuemin Chen**[c,d], **Spyros A. Kalams**[e,f], **Hakimuddin Sojar**[a], **Gary Landucci**[g], **Donald N. Forthal**[g], **Paul Spearman**[d,e,h], and **James E. Crowe Jr.**[e,i,*]

[a]Department of Pediatrics, University at Buffalo, Buffalo, NY 14222

[b]Department of Microbiology and Immunology, University at Buffalo, Buffalo, NY 14222

[c]Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322

[d]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322

[e]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN 37232

[f]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232

[g]Department of Medicine, University of California, Irvine, CA 92668

[h]Children's Healthcare of Atlanta, Atlanta, GA 30329

[i]The Vanderbilt Vaccine Center, Vanderbilt University Medical Center, Nashville, TN 37232

Abstract

Neutralizing antibodies (Abs) are thought to be a critical component of an appropriate HIV vaccine response. It has been proposed that Abs recognizing conformationally dependent quaternary epitopes on the HIV envelope (Env) trimer may be necessary to neutralize diverse HIV strains. A number of recently described broadly neutralizing monoclonal Abs (mAbs) recognize complex and quaternary epitopes. Generally, many such Abs exhibit extensive numbers of somatic mutations and unique structural characteristics. We sought to characterize the native antibody (Ab) response against circulating HIV focusing on such conformational responses, without a prior selection based on neutralization. Using a capture system based on VLPs incorporating cleaved envelope protein, we identified a selection of B cells that produce quaternary epitope targeting Abs (QtAbs). Similar to a number of broadly neutralizing Abs, the Ab genes encoding these

[*]Author to whom correspondence and proofs should be sent: James E. Crowe, Jr., MD, Vanderbilt Vaccine Center, Vanderbilt University Medical Center, 11475 MRB IV, 2213 Garland Avenue, Nashville, TN 37232-0417, USA, Telephone (615) 343-8064, Fax (615) 343-4456, james.crowe@vanderbilt.edu.

Contributions. MDH wrote, performed experiments, and conceptualized this study. XC, HS, and GL performed experiments. SK contributed to study design and cohort access. PS, DF, and JEC wrote and conceptualized the study.

QtAbs showed extensive numbers of somatic mutations. However, when expressed as recombinant molecules, these Abs failed to neutralize virus or mediate ADCVI activity. Molecular analysis showed unusually high numbers of mutations in the Ab heavy chain framework 3 region of the variable genes. The analysis suggests that large numbers of somatic mutations occur in Ab genes encoding HIV Abs in chronically infected individuals in a non-directed, stochastic, manner.

Keywords

HIV; Antibodies; Human; Neutralization; Monoclonal Antibodies; B cells

1. Introduction

An increasing number of broadly neutralizing monoclonal Abs against HIV have been identified in recent years that recognize novel neutralizing epitopes on the envelope (Env) glycoprotein complex. However, broadly neutralizing Ab clones are relatively rare and occur late in infection. Generally, it is thought that the broadly neutralizing epitopes may be relatively poorly immunogenic, difficult to access, and they require extensive somatic hypermutation for targeting [1]. It has been postulated that conformationally dependent Abs against intact native trimers may be necessary to neutralize HIV. This proposal is consistent with studies that show much of the neutralizing activity to HIV is directed to quaternary epitopes [2, 3]. Recent studies show certain constructs of recombinant proteins that seem to have improved specificity for representing neutralizing epitopes [4].

Conformationally or structurally dependent epitopes can take many forms, from discontinuous amino acids in a short sequence, to widely separated amino acids brought together by protein folding, or epitopes present only during the arrangement of multiple protomers, *i.e.* quaternary epitopes. The quaternary epitopes may be formed by portions of a single protein of the complex or quaternary epitopes may be formed *de novo* between the units of the multiple protomers. Structural antigenic determinants that induce quaternary-targeted Abs, have been shown to be important in other infections. For example, less than 1% of dengue virus-specific human mAbs are serotype-specific and potent neutralizing Abs, and these recognize quaternary structures that are found only on virion particles, not on soluble E protein [3–6]. The potent and broad influenza-specific human mAb 1F1 recognizes a quaternary epitope on the hemagglutinin protein head domain [7]. Large-scale efforts to identify potent and broadly neutralizing anti-HIV Abs against HIV using neutralization assays for the screen instead of Ab binding assays have recently isolated the broadly neutralizing Abs PG9 and PG16 [8] that target the same epitope as the quaternary targeting antibody (QtAb) 2909 [11]. PG9, PG16 and 2909 all target a variable loop 1 and 2 dependent epitope present on gp120 monomers [12], although the binding is greatly enhanced by incorporation into the envelope trimeric spike and have been referred to as quaternary-structure-preferring Abs [9, 10]. Other HIV Abs that recognize complex structures have been identified recently [11–13], including the broadly neutralizing QtAb PGT-151 [14, 15]. PGT-151 binds an epitope that is only created between gp120 and gp41 protomers after cleavage and trimeric incorporation. As a class, however, broadly

neutralizing Abs, particularly those that target complex quaternary epitopes, seem to be rare occurrences.

HIV produces a membrane-enveloped virion studded with HIV Env protein. We previously developed a pseudovirion- or virus-like particle (VLP)-based platform for antigen presentation of naturally cleaved Env trimer protein [19]. We subsequently demonstrated that the VLPs presented the epitopes for classical broadly neutralizing HIV mAbs and responded to CD4 binding by increasing access to CD4-induced epitopes [20]. We previously used these VLPs in flow cytometric cell sorting experiments to isolate a neutralizing human mAb to a CD4-induced quaternary epitope [18]. Virus-like particles (VLPs) have been considered as vaccine candidates, however these constructs are not without issue. Production of VLPs can incorporate other host membrane proteins that also induce immune responses [16]. Nonfunctional gp120/gp41 monomers and gp120-depleted gp41 stumps also may be displayed on VLPs as they are on virions [22]. These altered proteins have been proposed to be a component of diversionary tactics to elicit an inefficient Ab response against the virus.

It is poorly understood whether a subset of quaternary epitope-targeting Abs (QtAbs) are neutralizing or whether members of this class of Abs are usually neutralizing. We sought here to address that question by isolating panels of human mAbs that target quaternary epitopes on trimeric HIV Env, without prior screening for neutralization, using our VLPs in flow cytometric B cell capture assays. We identified novel anti-HIV Abs from three different HIV-infected long-term non-progressor subjects. On initial screens, a surprising number of these Abs targeted complex structural epitopes, *i.e.*, quaternary epitopes. As a class, these QtAbs exhibited a very high level of somatic hypermutation in the Ab variable genes. Despite the fact that the majority of Abs isolated by selection with VLPs were QtAbs, none of these Abs exhibited broad or potent HIV neutralizing or Ab-dependent cell-mediated viral inhibition (ADCVI) activity when expressed as full-length immunoglobulins. These QtAbs did display interesting common molecular characteristics, such as the dominant use of particular Ab variable gene segments and extensive mutations away from the inferred germline genes, particularly in the framework 3 region. We sought to examine the idea that extensive somatic mutations and induction of QtAbs would be sufficient to identify broadly neutralizing Abs. This was not the case, however. Mutational and biochemical analysis revealed these framework 3 region mutations accumulated stochastically in a manner that did not contribute to enhanced virus inhibiting activity. The results suggest that although expansion of B cells encoding QtAbs with large numbers of somatic mutations is often necessary for development of neutralizing Abs, these features are not sufficient to confer such activity. Rather, only rare clones of this class of Ab are likely to mediate broad and potent neutralizing activity due to particular patterns of epitope recognition.

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 after informed consent.

Samples were de-identified as to age, gender, and other personal identifying information. The Institutional Review Board of Vanderbilt University Medical Center approved the study protocols and consent forms. Subjects 10002, 10028, and 10076 were infected chronically with HIV, with a duration of infection from 7 to 24 years, and with CD4+ T cell counts >500 at the time of B cell sorting. These subjects were treatment-naïve, as noted in previous publications [23].

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

Stable cell lines were generated as previously described [18]. Briefly, plasmid pcDNA4/TO containing sequence-optimized HIV-1 *gag*, IRES and HIV-1 BaL *env* genes was created in the T-Rex-293 cell line (Invitrogen, Carlsbad CA). The GFP gene was cloned in-frame with the HIV-1 *vpr* gene and cloned into the pcDNA5/TO vector; transfected into the pcDNA4/TO Gag-I-Env stable cell line; and, using blasticidin, hygromyin, and zeocin, selected for stable cell line production. Cell lines were selected for optimal coordinated gag and env production after doxycycline induction and the optimal cell line was designated XC-34.

GFP-labeled 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 x *g* for 2 hours, 4 ºC). Pellets were suspended in PBS. The gp120 and p24 antigen content were measured by antigen-capture ELISA techniques.

2.3. B cell labeling, single-cell sorting of HIV VLP-binding CD19$^+$ B cells

B cell sorting was performed as previously described [18]. 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). Then, 2 to 4 x 10^6 B cells were stained with anti-CD3-PE, anti-CD14-PE, anti-CD19-APC (Becton Dickinson, Franklin Lakes, NJ) and 50 μL of concentrated VLP (containing GFP) preparation on ice for 30 minutes. Flow cytometric analysis and single-cell sorting was performed with a FACSAria III 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 15% 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.

2.4. 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 method [20]. 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 was added. The culture plates were incubated for 7 days at 37 ºC in an atmosphere of 8% CO_2. 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 well and supplemented with the media as described with an additional 5 ng/mL recombinant human IL-4. This cell line was kindly provided by DNAX via the ATCC (CRL 12095). Cultures had an additional supplementation of medium with CD154 cells on day 14. Immunoglobulin production was assessed on withdrawn supernatant on day 14 and 21.

2.5. Assays for secreted immunoglobulin (Ig) or HIV-specific Abs

ELISA assays for clonal interrogation with GFP-labeled HIV VLPs either incorporating (Gag-Env VLP) or lacking (Gag-only VLP) the BaL Env protein and recombinant BaL gp120 Env were previously described [18] Supernatants of B cell clones that were positive in both the Gag-Env VLP and Gag-only VLP ELISAs were considered non-specific (designated phenotype G). Those that bound both gp120 and the Gag-Env VLP were considered gp120-monomer specific (designated phenotype M). Those that bound only the Gag-Env VLP but not gp120 protein nor Gag-only VLPs were considered specific for HIV Env only in the context of VLP presentation and defined as quaternary targeting Abs (designated phenotype Q or QtAbs). Addition of B cell culture supernatant and Ig capture was performed as described above. Positive optical density values for both the human Ig and the HIV-specific B cell supernatant screening ELISAs ≥ were defined as 1.5 times the background signal, after the background signal had been subtracted from all test sample results. The sensitivity of the ELISA was demonstrated to be ≤ 1 ng of Ig per well in preliminary experiments (data not shown) and in a 1 to 5 ng/mL range for the VLP-specific ELISA. Additional reagents used in ELISAs and functional assays were soluble CD4 (sCD4-183; Pharmacia, Peapack, NJ), HIV-1 gp41 recombinant protein (Prospec, Ness-ziona Israel), the mAbs 2G12, 2F5 and 4E10 from Dr. Hermann Katinger through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.6. Heavy or light chain variable region amplification, DNA sequence analysis, and Ab expression

The isolation of variable chain sequences and subsequent Ab production follows procedures previously described [18] as adapted from previous methods [20]. Sequence determinations were performed in an ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA). Resulting variable region sequences were analyzed using MacVector® version 10.5 software (MacVector, Inc.) and with the international ImMunoGeneTics (IMGT) database [25]. Heavy chain cDNAs were paired with the light chain cDNA from the same cell, or a highly related clone, as shown in Table 3. 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. Supernatants were clarified by centrifugation and filtered prior to isolation using a 5 mL HiTrap Protein G column on an AKTA FPLC (GE Healthcare, Piscataway NJ), citrate buffer elution and washes.

2.7. Ab functional assays

Neutralization tests were performed with a conventional pseudovirion assay and TZM-bl cells, as previously described [22, 23]. Assays were performed in duplicate in a total volume of 150 μL containing 30 μg/mL of DEAE dextran for 1 hour at 37ºC 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 μL of growth media at 37 ºC in 5% CO_2 seeded 24 hours earlier. After a 48-hour incubation, 200 μL 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. Ab-dependent Cell-mediated Virus Inhibition (ADCVI) was measured using methods similar to those we described previously [24]. Briefly, target cells were prepared by infecting CEM.NKr.CCR5 cells (AIDS Reagent Program) with HIVUS657 for 72 hours. After washing, target cells were mixed with fresh human PBMC effector cells at an effector:target ratio of 10:1 in the presence of test monoclonal Abs at a final concentration of 100 μg/mL. Wells were washed at day 3 to remove Ab and replaced with fresh medium. Supernatant fluid was assayed at day 7 post-infection for p24 by ELISA (Zeptometrix, Buffalo, NY). Percent inhibition (compared to negative control) was calculated as previously described [24].

2.8. Replacement to Silent mutational analysis

Clones with ambiguous binding phenotypes were not used for this analysis. Since screening of antigen specificity was determined by ELISAs performed using supernatants from single B cell derived clones, the level of protein expression may be highly variable. Notably, 10028-Q1-2H3 and 10028-Q1-7B4 (with gp120 ELISA ODs of 0.379 and 0.300 with background signal 0.120) were borderline positive on gp120 screening. MAb 2D9 (part of the 10028-Q2 group) had an OD of 0.436 with background of 0.150 and the 8F10 clone (part of the 10028-M1 group) was negative in the VLP screen (OD of 0.350 with background OD of 0.150). 10028 Q4-3A9 and Q8-4H10 clones barely achieved positive values in the VLP ELISA, and Q7-4D11 bound to VLPs but also showed borderline gp120 binding values (0.297 ODs with a gp120 background OD of 0.110). Borderline assigned samples such as these were not used in further statistical analysis.

With these exceptions noted, the numbers of nucleotide mutations and those that lead to amino acid change were tabulated using IMGT database. Regions that lacked silent mutations were given the conservative denominator value of 1 for purposes of calculation. Each clonal group was counted as a single event (using the average of values for each group) for statistical calculations, to avoid skewing to those clonal groups that possessed more clones. This analysis was not performed on the HCDR3 region since an inferred germline sequence cannot be determined for those sequences because of non-templated N-additions in the junctions. GraphPad Prism Software (La Jolla, CA) was used for graphing and linear regression.

Results

To explore Ab responses to membrane associated HIV Env, we used a fluorescent VLP and single B cell cloning Ab system. We previously showed it is possible to isolate single HIV-specific B cells that recognize quaternary epitopes using flow cytometric sorting by labeling B cells with fluorescent VLPs displaying a stabilized form of the BaL strain Env protein[18]. Here, we studied in detail the B cells from three HIV-infected individuals who exhibited viral control and maintenance of adequate peripheral blood T cell counts without taking antiviral drug therapy, so-called long-term non-progressor subjects.

These individuals were known to exhibit moderate breadth of neutralizing activity in serum for clade B virus strains, as reported previously [23]. Using the NIH clade B reference neutralization panel, serum from Subject 10028 neutralized 59% of strains, serum from Subject 10002 neutralized 47% of isolates, and serum from Subject 10076 neutralized 18–24% of strains. Prior to performing sorting experiments, we repeated the serum neutralization assays for these donors using the HIV-1 BaL strain, since BaL Env was the basis of our VLP labeling reagents (Table 1). The neutralization profiles observed were consistent with those previously published. These subjects had donated plasma specimens over a 2–4 year period for determination of viral loads, which varied for each individual. Viral loads shown as RNA genome copies per mL plasma varied in the subject samples as follows: 10076 from 5,205 to 21,339; 10028 from 3,051 to 29,345; and 10002 from 65 to 1,217. Cells were isolated from blood samples by density gradient purification and sorted fresh or following PBMC cryopreservation in some cases.

We identified single Ab-secreting B cell derived clones that were HIV-specific using three different ELISA tests. ELISA screen positivity was defined as a signal 1.5 times the background signal, after the background signal had been subtracted from all test sample results. Figure 1 shows an example of results from one BaL-VLP ELISA screening plate; five wells containing clones derived from single VLP+ B cells secreted a level of HIV-specific Ab that was considered positive. On each plate, we included testing of a positive control Ab (mAb 2F5 shown here) at 25 ng/mL or 5 ng/mL in separate wells. The signal from the 2F5 control mAb at 5 ng/mL concentration was near the level of detection for the VLP ELISA. The contents of wells with positive signal then were harvested for RNA extraction and RT-PCR amplification of Ab heavy and light chain variable regions. The nucleotide sequences of the Ab gene cDNAs were determined and then analyzed using the international ImMunoGeneTics (IMGT) database at www.imgt.org [25].

Table 1 shows the results from the five cell-sorting experiments used to clone the Abs analyzed in this study. Direct B cell labeling with fluorescent VLPs resulted in enrichment of HIV-specific B cells. Of those clones that secreted Ab in the supernatant, 32 to 51 % were positive in HIV antigen screening ELISAs. The other Ab secreting non-HIV specific clones selected by sorting were likely B cells that nonspecifically bound the VLPs. Alternatively, since the immunoglobulin ELISA screening had greater sensitivity than the HIV antigen ELISAs, these clones may have simply had too low of an avidity to exceed the positive cutoff in the HIV antigen ELISAs. Overall, Ab producing anti-HIV Env B cells were enriched to between 3.1 % and 9.2 % of wells into which single B cells were sorted.

For specific sequence analysis, we chose to analyze only clones that were both physically sorted with VLPs and that secreted HIV-specific Ab after stimulation, to ensure the specificity of the results. Ab genes were cloned from a small number of the cultures that secreted Ig that did not bind to HIV antigens (Figure 2, 10028 controls). These clones possessed near-germline Ab gene sequences, likely representing nonspecific IgM producing clones since, being CD19 positive, these are more likely to have bound non-specifically in the sorting process. All confirmed Ab sequences were submitted to GenBank (accession numbers are provided in Supplemental Table 1).

Within the HIV specific clones, sequence analysis revealed a high proportion of shared or highly similar sequences among the clones. We previously have defined clonality based on shared V_H genes, a shared V_H-D-J_H junction, and a sequence with shared somatic mutations [26]. Since the Abs discovered in this present study were so highly mutated (likely due to recurrent antigenic exposure during chronic infection), we developed more stringent definition to maximally reflect the most recent of antigen binding events that would influence clonal stimulation against the HIV quasispecies strains. We defined clonality as having same heavy chain CDR3 region length, >90% CDR3 nucleotide identity, and having overall amino acid sequence homology of 90%. IMGT defined variable gene usage in both heavy and light chains, when available, was supportive of these assignments. An example of analysis of the relatedness of Subject 10076 clones is shown in Table 2; numbering of amino acids was assigned by IMGT analysis [25].

In this example of clones from individual 10076, clones were first organized according to shared heavy chain variable gene segments, in this case V_H1-02. As shown, the difference in HCDR3 length immediately assigned clone 2A6 to its own clonal group (designated Q8, with “Q” indicating recognition of a quaternary epitope, VLP but not monomeric gp120 binding). Clonal groups then were defined by overall similarity in the variable gene region, in this case highlighted by allele assignment within V_H1-02 and by J segment usage. From this analysis, clonal group Q2 (1B1 and 1F7) and group Q11 (4E4) were resolved easily. MAbs 6F5, 6F11, and 7C6 were not assigned a final grouping until the outlined strict homology criteria were applied. The relationship within the 6F11/7C6 pairing and the differences from 6F5 were supported similarly by shared mutations within the CDR3 region of the associated light chains, and the light chain gene family assignment (V_K1-39). Shared mutations within the full variable gene sequences agreed with these overall assignments, as did independent intraclonal Clustal alignments (data not shown). We cannot completely eliminate the possibility that these clones derived from a single clonal event very early in derivation of these clones. The shared overall V_H and V_L assignments and length of CDR3s of clones 1B1, 1F7, 4E4, 6F5, 6F11 and 7C6 may imply such a distant relation.

Figure 2 shows the unique clones assigned to their clonal groups with the B cell supernatant ELISA result for binding to gp120 (red), HIV Env-VLP (green) or Gag-only VLP ELISA (blue). Gag-only VLPs were used to assess non-Env or non-specific interactions with VLPs. Binding to Gag-only VLPs was observed only in supernatants from cells sorted from Subject 10076. Overall, the majority of reactive clones targeted what appeared to be trimer-specific, or quaternary epitopes (designated by label “Q”).

Although containing minor sequence differences, the Abs assigned to clonal groups by sequence similarity, (as above) also exhibited common patterns of binding to the three antigens tested (VLP with Env, VLP lacking Env, or monomeric gp120 -Figure 2). There were only minor disparities (discussed in methods). The majority of the clonal populations used only three different heavy chain variable gene segments (V_H1-69, V_H3-30, or V_H3-23). The predominant clones that were specific in binding to trimeric antigens used only V_H1-69.

Many of the isolated clones possessed a remarkably large number of somatic mutations, comparable to the mutation frequency seen in some of the most broadly neutralizing Abs in the literature. These mutations were located predominantly in HCDR 1, 2 and Framework 3 (Supplemental Figure 1-A). Figure 3 (left panel) shows the number of somatic mutations for multiple individual Abs organized into phenotypic binding groups (binding Gag; binding gp120 monomer; or quaternary epitope targeting Abs [QtAbs]). For comparative purposes, we show heavy chain mutation data from previously published studies (Figure 3 right panel). The level of mutation in the variable genes encoding trimer-specific anti-HIV Abs (QtAbs) was greater than that in randomly-selected B cells [30], or rotavirus-specific B cells [28]. A similar study using a gp140 trimer reported over 300 HIV-specific Abs, however of the Abs that targeted the trimeric gp140, none found were specific for quaternary epitopes [29]. The average number of mutations of this collection of Abs (labeled gp140 binding Abs, Figure 3) was comparable to rotavirus-specific B cells, to our clones that also recognized gp120 monomer or Gag proteins, and to the average in HIV-specific clones in a general survey [30]. Strikingly, the average number of mutations in variable heavy chain genes in our collection of QtAbs was 43, which is similar to the extensive number of mutations seen in individual broadly neutralizing Abs (Figure 3; right panel- mAb PG16: 43 [31]; mAb b12: 45 [32]; and mAb 2G12: 51 [33]).

We fully characterized using recombinant expression of cloned heavy and light chains a collection of ten QtAbs from Subject 10076 that represented a total of 8 sequence similarity clonal groups (Table 3)(reviewed in Table 2). Table 3 also reflects the excessive mutations as percentage of homology to germline. Since these clones recognized potential quaternary epitopes, and many possessed high numbers of somatic mutations comparable with that in many broadly neutralizing Abs (Figure 2), it was reasonable to think that many of these mAbs might exhibit neutralizing activity. Surprisingly, these full-length recombinant Abs did not exhibit neutralization to the BaL strain used to manufacture the VLP. The addition of CD4 and complement also failed to improve neutralization of the BaL strain. MAb 2C6 had been shown previously to have modest CD4 induced neutralizing activity when expressed as a Fab [18]. Notably, when 2C6 was expressed as a full-length immunoglobulin there was a modest enhancement of neutralization in the presence of CD4 (data not shown), but this activity also was weak (50% neutralization activity >50 μg/mL). These Abs also failed to neutralize a selection of viruses from a Clade B panel [34] or HIV strain SF162 (data not shown). We also tested the Abs in a standardized assay for Ab-dependent cell-mediated virus inhibition; none of the Abs exhibited any inhibitory activity (data not shown).

Due to limited volume of B cell supernatant, we did not perform screening for gp41 specific Abs in initial screens. It is well known that gp41 is highly immunogenic [38]. It has also been described that gp120-depleted gp41 stumps also may be displayed on VLPs as they are

on virions [22]. To explore if these potential QtAbs were directed toward gp41, a series of ELISA assays with gp41 protein (Prospec, Israel) and VLPs were performed, and EC_{50} values were calculated using Prism 6 software (Graphpad, La Jolla, California). The recombinant full-length Abs fully replicated the specific quaternary epitope VLP ELISA targeting patterns observed with the original primary B cell supernatant ELISA screening, as shown in Figure 1. ELISAs with the recombinant gp41 protein performed on a subset of QtAbs all showed inferior epitope targeting, with 8B10 in particular showing no binding at 1 μg/mL of Ab. Additional epitope mapping confirmed the structurally influenced nature of these epitopes ***(Hicar et al, in review).*** Lack of functional activity seen with these QtAbs is not be explained by poor binding activity, since the EC_{50} results were in the nanogram/mL concentration range (Table 3).

Due to their large number of mutations, yet poor neutralizing activity, we further evaluated these Ab sequences to attempt to identify the molecular basis for poor neutralizing activity. Since long Ab HCDR3s have been proposed to be enriched in populations of HIV neutralizing Abs [35], we evaluated the heavy chain complementarity determining region 3 (HCDR3) length distribution within our clones. The mean amino acid length of HCDR3s in the variable genes in human B cells in circulation is roughly 16 amino acids [40]. The HCDR3 length of HIV Env QtAbs analyzed here was on average 16.6 (Supplemental Figure 1B). The HCDR3 lengths were not distributed normally, implying a bias against average length CDR3s. When examined on an individual basis, it was clear that most of the Abs possessed either short or long HCDR3s, with the overwhelming majority being either <13 or ≥ 17 amino acids long. Despite their non-neutralizing functional profile, there was no apparent bias against these QtAbs having long HCDR3s (Supplemental Figure 1B).

We further evaluated structural elements focusing on the location of targeting of somatic hypermutation. Replacement to silent (R/S) mutation ratios can be used to suggest positions altered by somatic hypermutation that are selected preferentially based on antigen binding. An R/S ratio of >2.96 indicates targeted somatic hypermutation and selection of those mutations for further propagation. The sequences of Ab variable regions can be compared to known germline gene sequences for this analysis. Typically, the CDR regions, since they tend to interact directly with antigen, accumulate a majority of mutations and often have higher R/S ratios. The majority of heavy chains of the QtAbs here exhibited signs of antigen-driven selection (*i.e.*, elevated R/S ratios) within their HCDR 1 and 2 regions (Figure 4C and E). This finding is typical of most antigen-specific responses, however the extent of targeting to HCDR1 here is notable. Targeting of framework 1, 2 or 3 seemed to be a more unusual occurrence, with only a minority of clones analyzed showing targeting in the frameworks (Figure 4B, D, and F).

Despite the very large number of mutations in these Ab sequences, and particularly in the framework 3 regions (Supplemental Figure 1A), antigen-driven selection for mutations outside of the CDRs was not a generalizable characteristic of these QtAbs. To assess the influence of potential CDR3 length bias, these data were analyzed in the context of CDR3 length (Figure 4). Interestingly, the data for framework 3 suggested increased targeting of this region for selected mutations as the HCDR3 region became shorter (Figure 4F). This finding suggests that increased numbers of selected clones with large numbers of somatic

mutations, particularly in the framework 3 region, may compensate for the lack of complexity in Abs with shorter HCDR3s. This finding may be due to the structural interplay of a central portion of framework 3 that is near the CDR pocket [41].

This correlation only had an R^2 value of 0.281 and appeared to be driven primarily by only two clonal groups (Figure 4F). We interpret this finding to mean that many of the framework 3 mutations, and subsequently the excessive numbers of mutations seen in these Abs, are accumulating stochastically, rather than driven by antigen-specific responses. As a proof of concept, we chose an Ab with a low R/S ratio in framework 3 to further study the effect of mutations in this region. The heavy chain of 76-Q3-2C6 (*i.e.*, mAb 2C6) contains a 21 amino acid long CDR3 region and has an R/S ratio of 1.45 in framework 3. A recombinant Ab with near germline sequence in the framework 3 region (labeled 2C6-FR3Δ, sequence comparison Figure 5B) was created and compared to the original 2C6. On comparing binding kinetics, EC_{50} values on three separate experiments did not show a significant difference in binding; a typical binding curve comparison is shown in Figure 5A. These data support the idea that the extensive mutations accumulated in this set of Abs were not driven by major affinity improvements that enhanced epitope targeting.

Discussion

This work shows that HIV-infected individuals make a surprisingly large number of highly mutated non-neutralizing Abs to quaternary epitopes on HIV Env. The reason that we were able to identify such a dominant non-neutralizing population of Abs when previous studies have not done so may stem from a number of factors. We chose subjects on the basis of their clinical non-progression, but did not choose any for broad neutralization activity of their serum as other studies have done. Additionally, identified clones for study by binding to VLPs, without specifically screening for neutralizing activity. Most previous studies of HIV B cell repertoire would not have identified such non-neutralizing quaternary epitope-specific clones because the screening was done by binding to monomeric gp120 or to gp140 trimers that may not efficiently present quaternary epitopes, or alternatively clones were identified by a neutralization assay. Since other studies selected clones for study based on for neutralizing activity, there is perhaps a false assumption that many or most Abs to HIV that recognize quaternary epitopes are neutralizing. Some groups have suggested that any Ab that bound a quaternary epitope or recognized the trimeric structure of Env properly would be neutralizing [38–40]. Certainly, some potently neutralizing mAbs recognize quaternary epitopes. However, our data suggests that such potent QtAbs are rare Abs from rare individuals, isolated by large-scale screening, and that the typical QtAb that dominates the response to complex Env epitopes in most individuals is not neutralizing.

The Abs that we isolated revealed a number of interesting molecular and genetic features, suggesting that they are a dominant feature of the B cell response in the infected individuals studied. Many of the sequences were highly related, suggesting they derived from a single B cell or alternatively from a strong process of convergent evolution with highly related clones. Regardless of whether the individual sequences derived from a single common ancestor within an individual or not, the striking feature is that clonal families with these highly over-represented Ab genes exhibited identical binding patterns, suggesting a limited

number of immunodominant antigens driving the response. Furthermore, the fact that similar genes were used in Abs from differing individuals suggests that the over-representation of these gene sequences reflects a common dominant response across individuals. The correspondence of the common genes with QtAb binding patterns also suggests that these genes likely encode Abs that are structurally optimal for initiating responses to quaternary epitopes in the germline unmutated configuration.

A second feature in these clones that was quite interesting was the pattern of somatic hypermutation. Many of the sequences possessed either a long HCDR3 or a high degree of somatic mutation in the framework 3 region. However, the data suggests these mutations generally accumulated in a stochastic fashion. Long HCDR3s, common in HIV Env-specific Abs, are a proposed mechanism to penetrate the glycan shield to interact with otherwise hidden amino acids on Env [35]. In many cases, long structured HCDR3s additionally interact with the glycan shield [1].

Short HCDR3s intrinsically possess less structural complexity and sequence diversity than Abs with longer HCDR3s, and this limitation may restrict their ability to bind to complex quaternary epitopes with high affinity. We initially hypothesized that, during somatic hypermutation, antigen directed targeting of framework 3 would compensate for short CDR3s. It is known that in some cases the framework 3 region interacts with antigens; indeed some have referred to portions of framework 3 as HCDR4 [41]. In this collection of highly mutated Abs however, these framework 3 mutations appear to accumulate in a stochastic fashion that did not enhance Ab affinity or function. A caveat to this conclusion is that we did not use autologous virus strains from the individuals studied. Ideal studies would follow virion and Ab development in concert. Our conclusion is consistent with the recent studies showing neutralizing Abs can retain much of their neutralizing activity, even with significant reversion of Ab sequence toward the inferred unmutated ancestor gene [41].

Other than complete functional trimers, natural virions may display gp120/gp41 monomers, gp120-depleted gp41 stumps, and other incomplete or false oligomeric forms that may act as immune decoys [19, 42, 43]. These alternate viral protein structures 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. Recent data suggests SOSIP forms are superior to other trimeric gp140 forms in replicating native trimers [4] and cleavage of Env is particularly crucial [48]. With this in mind, it is significant that the VLPs used in this current study react both in flow cytometry assays and ELISA. Identifying excessively mutated Abs against complex epitopes on VLPs from infected individuals implies that during natural infection, HIV virions are repeatedly stimulating similar Ab responses. The question remains whether vaccines that attempt to mimic the native envelope structure also will enhance for these complex non-neutralizing epitopes.

The results from this study have direct relevance to current strategies for developing new experimental vaccines for HIV. Much of the current focus in the field is appropriately aimed at recapitulating Env antigens with conformational fidelity to the Env form found in virions. While this objective is logical, our data suggest that it may be desirable to develop a

reshaped Env antigen that retains conserved neutralizing determinants but obscures immunodominant quaternary epitopes that frequently induce non-neutralizing Abs. Our data also suggests that the repetitive vaccination strategy to drive multiple mutations may have limitations. Newer structure-based vaccine antigens designed using emerging computational methods might provide a way forward for development of optimized Env proteins [49]. Further characterization of immunodominant epitopes that are undesirable in an optimal HIV immunogen is of interest to those engaged in rational vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by U01 AI-078407 (NIAID, NIH), R01 AI-102715 (NIAID, NIH), P30 AI-054999 (NIAID, NIH; the Vanderbilt-Meharry Center for AIDS Research), and P30-AI50409 (Emory Center for AIDS Research). MDH was supported by a Pediatric Infectious Diseases Society-St. Jude Children's Research Hospital Fellowship Program in Basic Research and NIH K08 AI 083078 and portions of this work were performed as the Young Investigator Award recipient of the IDSA ERF and NFID sponsored by Wyeth Vaccines Research.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: MAbs: 2G12, 2F5, and 4E10 from Dr. Hermann Katinger; the TZM-bl cell line from John Kappes and Xiaoyun Wu; SF162 gp140 trimer protein from Dr. Leo Stamatatos; soluble CD4 (sCD4-183) from Pharmacia, Inc.; The CEM.NKR-CCR5 cell line from Dr. Alexandra Trkola and the NIH clade B Env reference panel of HIV isolates (Drs. Montefiori, Gao, Li, Hahn, Kothe, Wei, Shaw, Salazar-Gonzalez, Kappes and Wu).

References

1. Burton DR, Stanfield RL, Wilson IA. Antibody vs. HIV in a clash of evolutionary titans. Proc Natl Acad Sci U S A. 2005; 102(42):14943–8. [PubMed: 16219699]
2. Sanders RW, et al. A next-generation cleaved, soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog. 2013; 9(9):e1003618. [PubMed: 24068931]
3. Fibriansah G, et al. A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. EMBO Mol Med. 2014; 6(3):358–71. [PubMed: 24421336]
4. Messer WB, et al. Dengue virus envelope protein domain I/II hinge determines long-lived serotype-specific dengue immunity. Proc Natl Acad Sci U S A. 2014; 111(5):1939–44. [PubMed: 24385585]
5. de Alwis R, et al. Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. Proc Natl Acad Sci U S A. 2012; 109(19):7439–44. [PubMed: 22499787]
6. Fibriansah G, et al. A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. Nat Commun. 2015; 6:6341. [PubMed: 25698059]
7. Tsibane T, et al. Influenza human monoclonal antibody 1F1 interacts with three major antigenic sites and residues mediating human receptor specificity in H1N1 viruses. PLoS Pathog. 2012; 8(12):e1003067. [PubMed: 23236279]
8. Walker LM, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science. 2009; 326(5950):285–9. [PubMed: 19729618]
9. Steimer KS, Haigwood NL. Importance of conformation on the neutralizing antibody response to HIV-1 gp120. Biotechnol Ther. 1991; 2(1–2):63–89. [PubMed: 1726963]
10. Steimer KS, et al. Neutralization of divergent HIV-1 isolates by conformation-dependent human antibodies to Gp120. Science. 1991; 254(5028):105–8. [PubMed: 1718036]
11. Pietzsch J, et al. Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity. J Virol. 2010; 84(10):5032–42. [PubMed: 20219932]

12. Louis JM, et al. Binding of HIV-1 gp41-directed neutralizing and non-neutralizing fragment antibody binding domain (Fab) and single chain variable fragment (ScFv) antibodies to the ectodomain of gp41 in the pre-hairpin and six-helix bundle conformations. PLoS One. 2014; 9(8):e104683. [PubMed: 25105806]
13. Gustchina E, et al. Complexes of neutralizing and non-neutralizing affinity matured Fabs with a mimetic of the internal trimeric coiled-coil of HIV-1 gp41. PLoS One. 2013; 8(11):e78187. [PubMed: 24244293]
14. Blattner C, et al. Structural delineation of a quaternary, cleavage-dependent epitope at the gp41-gp120 interface on intact HIV-1 Env trimers. Immunity. 2014; 40(5):669–80. [PubMed: 24768348]
15. Scharf L, et al. Antibody 8ANC195 reveals a site of broad vulnerability on the HIV-1 envelope spike. Cell Rep. 2014; 7(3):785–95. [PubMed: 24767986]
16. Hammonds J, et al. Advances in methods for the production, purification, and characterization of HIV-1 Gag-Env pseudovirion vaccines. Vaccine. 2007; 25(47):8036–48. [PubMed: 17936444]
17. Hicar MD, et al. Emerging studies of human HIV-specific antibody repertoires. Vaccine. 2010; 28(Suppl 2):B18–23. [PubMed: 20510738]
18. Hicar MD, et al. Pseudovirion particles bearing native HIV envelope trimers facilitate a novel method for generating human neutralizing monoclonal antibodies against HIV. J Acquir Immune Defic Syndr. 2010; 54(3):223–35. [PubMed: 20531016]
19. Crooks ET, et al. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. Virology. 2007; 366(2):245–62. [PubMed: 17580087]
20. Weitkamp JH, et al. Generation of recombinant human monoclonal antibodies to rotavirus from single antigen-specific B cells selected with fluorescent virus-like particles. J Immunol Methods. 2003; 275(1–2):223–37. [PubMed: 12667686]
21. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008; 36:W503–8. Web Server issue. [PubMed: 18503082]
22. Mascola JR, et al. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. J Virol. 2005; 79(16):10103–7. [PubMed: 16051803]
23. Montefiori DC. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol. 2009; 485:395–405. [PubMed: 19020839]
24. Forthal DN, et al. Rhesus macaque polyclonal and monoclonal antibodies inhibit simian immunodeficiency virus in the presence of human or autologous rhesus effector cells. J Virol. 2006; 80(18):9217–25. [PubMed: 16940533]
25. Sather DN, Stamatatos L. Epitope specificities of broadly neutralizing plasmas from HIV-1 infected subjects. Vaccine. 2010; 28(Suppl 2):B8–12. [PubMed: 20510750]
26. Krause JC, et al. Epitope-specific human influenza antibody repertoires diversify by B cell intraclonal sequence divergence and interclonal convergence. J Immunol. 2011; 187(7):3704–11. [PubMed: 21880983]
27. Clark LA, et al. Trends in antibody sequence changes during the somatic hypermutation process. J Immunol. 2006; 177(1):333–40. [PubMed: 16785529]
28. Tian C, et al. Evidence for preferential Ig gene usage and differential TdT and exonuclease activities in human naive and memory B cells. Mol Immunol. 2007; 44(9):2173–83. [PubMed: 17196657]
29. Scheid JF, et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature. 2009; 458(7238):636–40. [PubMed: 19287373]
30. Huang CC, et al. Structural basis of tyrosine sulfation and VH-gene usage in antibodies that recognize the HIV type 1 coreceptor-binding site on gp120. Proc Natl Acad Sci U S A. 2004; 101(9):2706–11. [PubMed: 14981267]
31. Pejchal R, et al. Structure and function of broadly reactive antibody PG16 reveal an H3 subdomain that mediates potent neutralization of HIV-1. Proc Natl Acad Sci U S A. 2010; 107(25):11483–8. [PubMed: 20534513]

32. Zwick MB, et al. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol. 2001; 75(22):10892–905. [PubMed: 11602729]
33. Kunert R, Ruker F, Katinger H. Molecular characterization of five neutralizing anti-HIV type 1 antibodies: identification of nonconventional D segments in the human monoclonal antibodies 2G12 and 2F5. AIDS Res Hum Retroviruses. 1998; 14(13):1115–28. [PubMed: 9737583]
34. 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. J Virol. 2005; 79(16):10108–25. [PubMed: 16051804]
35. Pejchal R, et al. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. Science. 2011; 334(6059):1097–103. [PubMed: 21998254]
36. Briney BS, Willis JR, Crowe JE Jr. Human peripheral blood antibodies with long HCDR3s are established primarily at original recombination using a limited subset of germline genes. PLoS One. 2012; 7(5):e36750. [PubMed: 22590602]
37. Bond CJ, et al. A structure-based database of antibody variable domain diversity. J Mol Biol. 2005; 348(3):699–709. [PubMed: 15826665]
38. Fouts TR, et al. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. J Virol. 1997; 71(4):2779–85. [PubMed: 9060632]
39. Sattentau QJ, Moore JP. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. J Exp Med. 1995; 182(1):185–96. [PubMed: 7540648]
40. Parren PW, et al. Neutralization of human immunodeficiency virus type 1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. J Virol. 1998; 72(5):3512–9. [PubMed: 9557629]
41. Georgiev IS, et al. Antibodies VRC01 and 10E8 neutralize HIV-1 with high breadth and potency even with Ig-framework regions substantially reverted to germline. J Immunol. 2014; 192(3): 1100–6. [PubMed: 24391217]
42. Lin G, Nara PL. Designing immunogens to elicit broadly neutralizing antibodies to the HIV-1 envelope glycoprotein. Curr HIV Res. 2007; 5(6):514–41. [PubMed: 18045109]
43. Moore PL, et al. Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. J Virol. 2006; 80(5):2515–28. [PubMed: 16474158]
44. Ringe RP, et al. Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. Proc Natl Acad Sci U S A. 2013; 110(45):18256–61. [PubMed: 24145402]
45. Schief WR, Ban YE, Stamatatos L. Challenges for structure-based HIV vaccine design. Curr Opin HIV AIDS. 2009; 4(5):431–40. [PubMed: 20048708]

Highlights

- We isolated a large panel of human mAbs to HIV that were identified by binding to VLPs.
- Many of these mAbs possess large numbers of somatic mutations and recognized quaternary (Qt) epitopes.
- Surprisingly, most highly mutated QtAbs did not exhibit a high level of neutralizing activity.

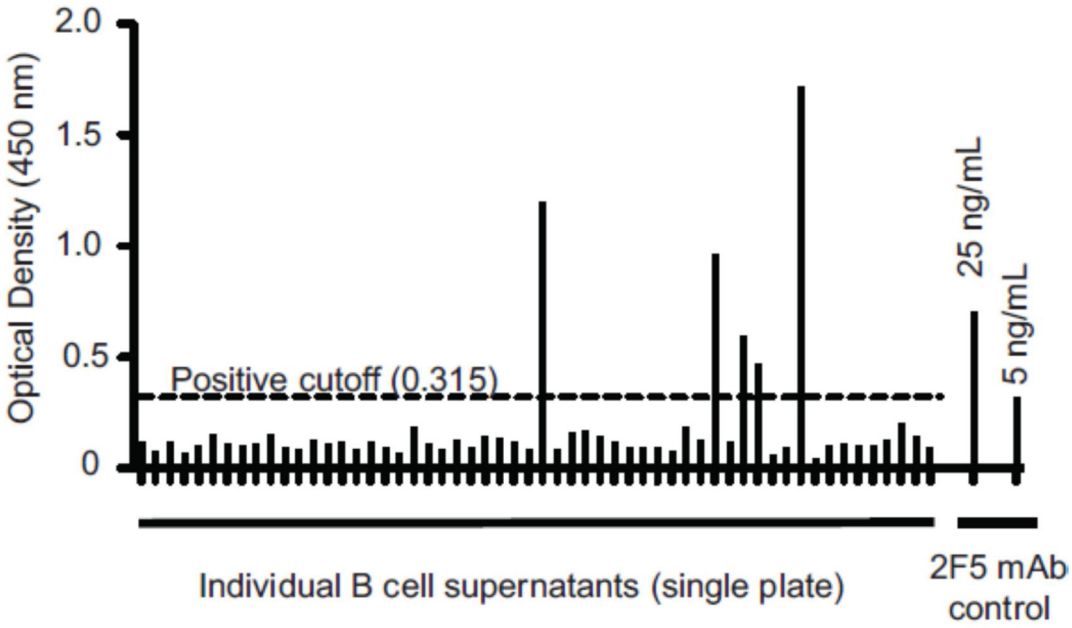


Figure 1. HIV VLP ELISA screening of stimulated B cell supernatants

Example of results from one strain BaL VLP ELISA screening plate. Secondary was conjugated to HRP and absorbance was read at 450 nanometers after acid stop. Five wells containing clones derived from single VLP+ B cells secreted a level of HIV- specific Ab that was considered positive. This positive cutoff was defined as 1.5x background above the background level. A control mAb known to bind VLP was included on each plate. In this example, mAb 2F5 was the positive control measured at 25 ng/mL and 5 ng/mL, with 5 ng/mL reading a level just below positive cutoff.

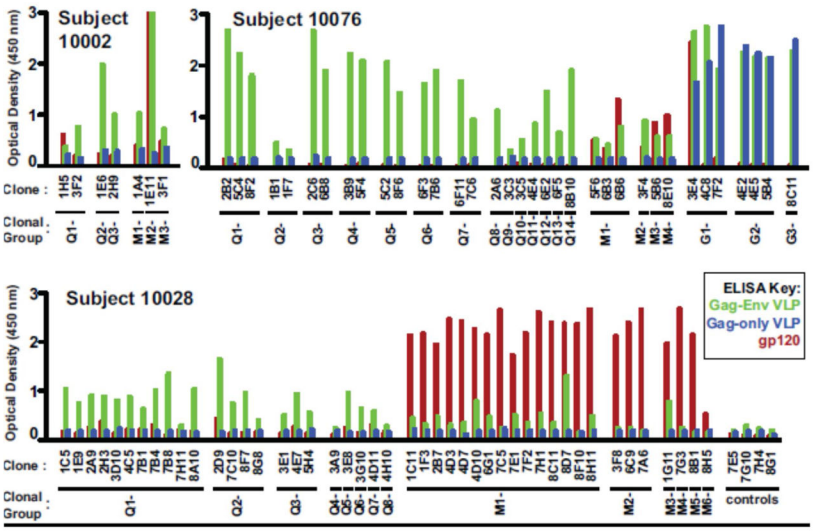


Figure 2. Cell culture supernatants from physically sorted HIV virus-like particle (VLP)-binding single B cells contain a high frequency of quaternary epitope-targeting Abs

Summary of individual B cell supernatant screening ELISAs: results of binding to Gag-Env VLPs (green) or Gag-only VLPs lacking Env (blue), or recombinant gp120 (red). Each ELISA plate contained internal positive and negative controls (see Figure 1 for an example). Clonal groups were assigned by sequence homology and are labeled with Q for quaternary binding or M for monomeric gp120 binding. Gag-only VLP ELISA screening was used to assess potential non-Env binding to Gag-Env VLPs, with clones showing this reactivity labeled G for Gag-only VLP binding.

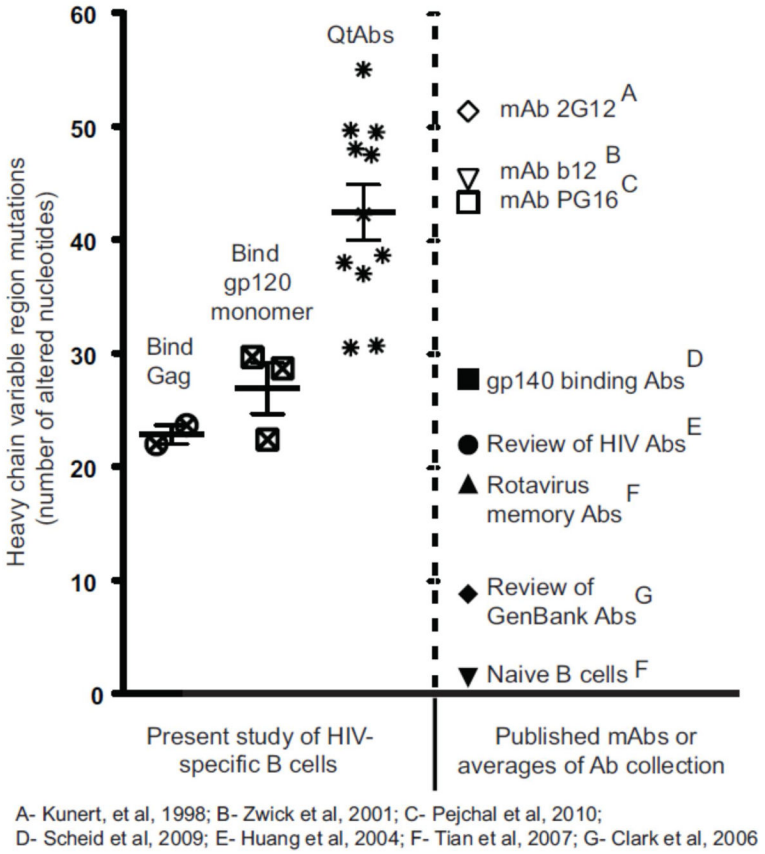


Figure 3. The occurrence of quaternary epitope-targeting Abs correlates with the presence of large numbers of somatic mutations in Ab variable region genes

Clonal groups of Abs from this study are shown on the left. Each point represents multiple related Abs that form a clonal group, and these are separated by antigen targeting as labeled (*checked circle* - bind to Gag, *checked square* -bind to gp120 monomer, and *starred* - quaternary epitope-targeting Ab). On the right (shaded symbols) are average mutations from various published studies, as labeled. Data for the broadly neutralizing mAbs 2G12, b12 and PG16 also are included on the right (open symbols, data from single Abs). References: A-[33]; B-[35]; C-[34]; D-[32]; E-[33]; F-[31]; G-[30].

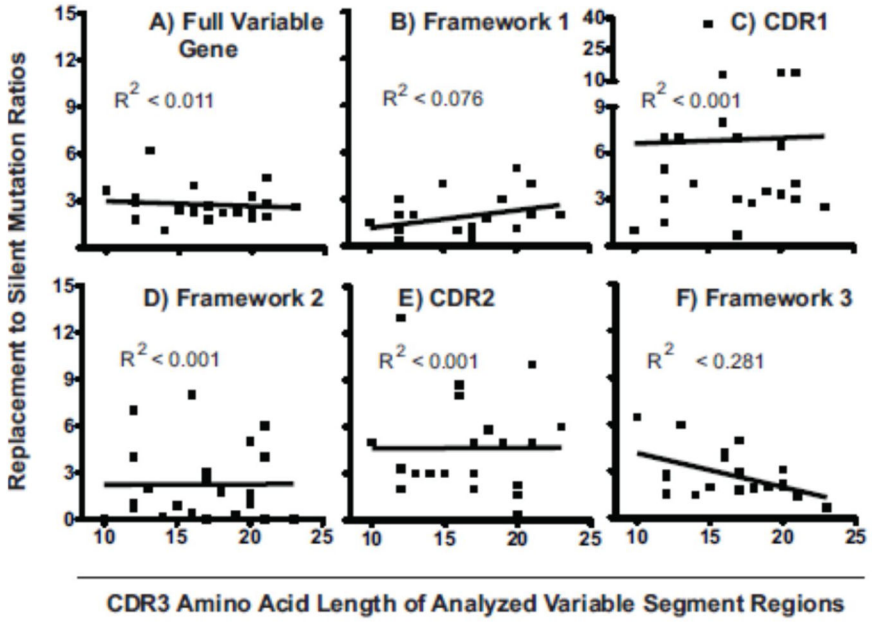
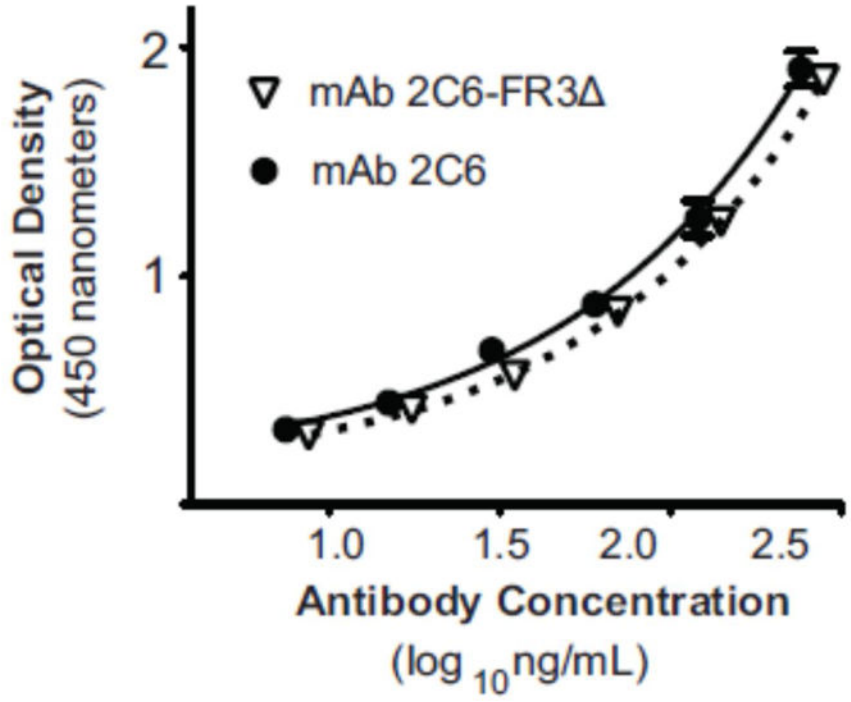


Figure 4. Heavy Chain Variable Region Replacement to Silent Mutation Ratios Correlated to CDR3 Length

CDR3 amino acid length and replacement and silent mutation data were obtained from IMGT analysis. Tabulated replacement to silent ratios (R/S) for the whole variable gene segment (A) or for each subregion of the variable gene segment excluding CDR3 (B-F, regions as labeled, closed circles), were plotted and correlated to amino acid length of the CDR3 region of each variable gene segment. R-squared correlations were calculated using Prism (GraphPad Software) and are included within the figure for each panel.

A



B

Gene or Clone	Heavy Chain Variable Region Amino Acid Residues (position 66-95 of FR3)
V_H 1-69*01F	NYAQKFQGRVTITADESTSTAYMELSSL
mAb 2C6	Q---R----A-F---V------L-VT--
mAb 2C6-FR3Δ	----------------A---T-------

Figure 5. Framework 3 Mutation Influence on QtAb Binding

A) Binding of twofold dilutions of mAbs 76-Q3-2C6 or mutated 76-Q3-2C6 (or 2C6 and 2C6-FR3Δ as shown) to HIV trimeric Env are compared in ELISA format. B) Sequence alignment of amino acids in mAbs 2C6 and 2C6-FR3Δ compared to germline V_H1-69F, as determined by IMGT analysis. Differences from germline are shown and dashes represent no change from germline amino acid.

Table 1

Summary of donor characteristics and yield of HIV VLP-specific B cell sorting and stimulation experiments

Subject (*year infected*)	Serum neut. titer to VLP strain (BaL. 26)	Plasma viral load (copies/mL)	CD4 count (cells/μL)	FACS sorts	% Gag-Env VLP+ B cells by FACS	Immunoglobulin (Ig) ELISA + wells from total sorted cells (%)	HIV antigen ELISA+ wells from Ig ELISA + wells (%)	HIV antigen ELISA + wells from total sorted cells (%)
10076 (*1999*)	84	21,339	700	2	0.21–0.28	19.6	46.9	9.2
10002 (*1984*)	669	65	1,026	2	0.14–0.19	6.1	51.1	3.1
10028 (1992)	748	7,783	1,004	1	0.44	27.6	31.9	4.8

VLP= Virus-like particles; Ig= immunoglobulin; neut. = neutralization; FACS= fluorescence-activated cell sorting

Table 2

Representative assignment of clonal groups based on differences between Ab sequences

Antibody		Heavy chain variable gene sequence				Light chain variable gene sequence			
Clonal Group	**Clone**	**V_H1-02 allele**	**J_H gene**	**HCDR3 amino acids (aa)**	**CDR3 length (aa)**	**V_K1-39 allele**	**J_K gene**	**CDR3 length (aa)**	**LCDR3 amino acids**
Q2	1B1 1F7	02 *05	5 *02	CARGSITARWLDPW -----T-T------	12 12	nd nd	nd nd	nd nd	nd nd
Q7	6F11 7C6	02 *05	4 *02 or 4 *03	CGRTSIASRHLDSW ----------	12 12	01	2 *02	10 10	CQETYSVPPWTF
Q13	6F5	02 *05	4 *02 or 4 *03	CARVQMAGRDADLW	12	01	1 *01	10	CQQTYASPPWTF
Q11	4E4	02 *04 or 02 *05	3 *01 or 3 *02	CARGEIAGRRLDLW	12	01	2 *01 or 2 *02	10	CQHIYATPPFTF
Q8	2A6	02	4 *02	CARASPDGYNYW	10	nd	nd	nd	nd

In this example, seven clones from one individual (subject 10076) sharing the initial same heavy chain variable gene segment (V_H1-2) organized into 2 principal groups based on HCDR3 length (10 vs. 12 amino acids). The inferred V_H1-2 allele and J_H gene segment assignment and the HCDR3 sequences further separated the 12-amino length HCDR3 clones into 4 groups by homology. Hyphen (−) indicates identity to the clonal group sequence above. “nd” indicates not determined, *i.e.*, the light chain sequence could not be recovered.

Table 3

Antigen binding and variable genes used to produce recombinant full-length QtAbs that were analyzed functionally in this study

Antibody	Antigen Binding EC_{50} [ng/mL] (95% CI)		Heavy chain variable gene sequence						Light chain variable gene sequence				
Clone	BaL-VLP ELISA binding	gp41 ELISA binding[1]	V_H gene	nucleotide % homology[2]	D_H gene	J_H gene	HCDR3 amino acids (aa)	CDR3 length (aa)	V_K gene	nucleotide % homology[2]	J_K gene	LCDR3 amino acids (aa)	CDR3 length (aa)
Q3-2C6	0.87 (0.75–1.0)	>1,000	1-69	86	4-17	6	CARGMIHADYRSDPFYHYYMDVW	21	1-27	95	3	CQSYNSAHFTF	9
Q4-5F4	2.0 (1.8–2.2)	500–1,000	1-69	83	3-10	5	CANSRLYYEGPLLTGVGYFDPW	20	Light chain from Q5-5C2 clone below[3]				
Q5-5C2	2.5 (2.3–2.8)	316 (171.2–583.0)	1-69	82	3-16	1	CAGSRMYYEGGLLTGVGYFDPW	20	1-5	90	1	CQQYENYPRTF	9
Q5-8F6	2.6 (2.1–3.2)	nd	1-69	82	3-16	1	CAGSRMYYEGGLLTGVGYFDPW	20	1-5	90	1	CQQYENYPRTF	9
Q6-7B6	2.6 (2.4–2.8)	nd	1-69	85	3-10	5	CSNSRLYYEGGLLTGVGWFGPW	20	1-5	93	1	CQQYHDYPRTF	9
Q7-6F11	10.8 (8.7–13.3)	nd	1-2	83	6-6	4	CGRTSIASRHLDSW	12	Light chain from Q7-7C6 clone below[3]				
Q7-7C6	4.4 (3.6–5.5)	9.2 (6.5–13.1)	1-2	80	6-6	4	CGRTSIASRHLDSW	12	1-39	87	2	CQETYSVPPWTF	10
Q11-4E4	11.2 (10.1–14.1)	nd	1-2	88	2-15	3	CARGEIAGRRLDLW	12	Light chain from Q13-6F5 clone below[4]				
Q13-6F5	20.6 (16.0–26.0)	500–1,000	1-2	83	1-26	4	CARVQMAGRDADLW	12	1-39	91	1	CQQTYASPPWTF	10
Q14-8B10	1.8 (1.4–2.3)	>1,000	4-59	92	4-23	4	CARVRKAMSTVAFDSW	14	Light chain from G1-3E4 clone, a V_H4-59 utilizing Ab[5]				

[1] gp41 binding was performed using commercially available recombinant gp41 protein; “nd” indicates not determined; EC_{50} calculations with wide confidence intervals were estimated as 500–1,000 or >1,000 ng/mL.

[2] Variable gene percent homology to the inferred germline variable gene, as predicted by IMGT.

[3] Related light chains with 100% identical CDR3 and >90% overall homology.

[4] Antibody clone Q11-4E4 was a chimeric antibody using the 76-Q11-4E4 heavy chain co-expressed with the 76-Q13-6F5 kappa chain, since the 76-Q11-4E4 light chain could not be recovered from the single B cell derived culture.

[5] Antibody clone Q14-8B10 was a chimeric antibody using the 76-Q14-8B10 heavy chain co-expressed with the 76-G2-3E4 kappa chain. 76-Q13-3E4 similarly used the V_H gene 4-59 in the heavy chain and the natural Q14-8B10 heavy and light chain pairing did not express well in culture.