Cooperation of B Cell Lineages in Induction of HIV-1-Broadly Neutralizing Antibodies

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SUMMARY

Development of strategies for induction of HIV-1 broadly neutralizing antibodies (bnAbs) by vaccines is a priority. Determining the steps of bnAb induction in HIV-1-infected individuals who make bnAbs is a key strategy for immunogen design. Here, we study the B cell response in a bnAb-producing individual and report cooperation between two B cell lineages to drive bnAb development. We isolated a virus-neutralizing antibody lineage that targeted an envelope region (loop D) and selected virus escape mutants that resulted in both enhanced bnAb lineage envelope binding and escape mutant neutralization—traits associated with increased B cell antigen drive. Thus, in this individual, two B cell lineages cooperated to induce the development of bnAbs. Design of vaccine immunogens that simultaneously drive both helper and broadly neutralizing B cell lineages may be important for vaccine-induced recapitulation of events that transpire during the maturation of neutralizing antibodies in HIV-1-infected individuals.

INTRODUCTION

The development of a successful HIV-1 vaccine has been stymied by the inability to induce broadly neutralizing antibodies (bnAbs) to regions of the HIV-1 envelope glycoprotein (Env) (Burton et al., 2012; Mascola and Haynes, 2013) that include the CD4-binding site (CD4bs); the membrane proximal external region; and glycans and amino acid residues in the regions of the first (V1), second (V2), and third (V3) loops (Burton et al., 2012; Kwong and Mascola, 2012; Sattentau and McMichael, 2010; Stamatatos, 2012; Walker et al., 2011; Walker et al., 2009; Zhou et al., 2010). To date, all bnAbs isolated have one or more unusual characteristics: high levels of somatic hypermutations, long heavy chain third complementarity determining regions (HCDR3), or poly- or auto-reactivity to non-HIV-1 antigens (Haynes et al., 2005; Haynes et al., 2012; Kwong and Mascola, 2012; Mouquet and Nussenzweig, 2012; Scheid et al., 2009)—all antibody traits influenced by various host tolerance mechanisms (Haynes et al., 2012; Mascola and Haynes, 2013; Mouquet and Nussenzweig, 2012). As a consequence of these antibody traits, bnAbs appear to be disfavored and difficult to induce with traditional immunization regimens (Haynes et al., 2012; Mascola and Haynes, 2013; Mouquet and Nussenzweig, 2012). We and others have suggested strategies whereby immunogens are selected to react with bnAb lineage members at multiple stages in their development in an effort to drive otherwise unfavored antibody pathways (Haynes et al., 2012; Liao et al., 2013a; Mascola and Haynes, 2013).

One approach to dissect the mechanisms underlying bnAb development is to identify the drivers that are responsible for the sequential stimulation of HIV-1 reactive B cell lineages in chronically infected individuals over time (Bonsignori et al., 2011; Corti et al., 2010; Gray et al., 2011; Hraber et al., 2014;
Klein et al., 2012; Lynch et al., 2012; Moore et al., 2009; Moore et al., 2011; Tomaras et al., 2011; Walker et al., 2011). We have recently identified an African individual (CH505) in whom HIV-1 infection was established by a single subtype C transmitted/founder (T/F) virus and mapped the coevolution of CD4bs bnAbs (the CH103 bnAb B cell lineage) and CH505 T/F virus over time (Liao et al., 2013a). The T/F Env continuously diversified over time under the selection pressure of bnAbs and, concurrently, the inferred unmutated common ancestor (UCA) of the CH103 B cell lineage accumulated somatic mutations leading to gradual acquisition of bnAb activity (Liao et al., 2013a). While the minimally mutated early members of this lineage neutralized only the T/F virus, the later, more mature members of the CH103 clonal lineage potently neutralized both the CH505 T/F and 55% of multiclade heterologous HIV-1 strains (Liao et al., 2013a). These data engendered interest in determining the autologous virus Env variants that stimulated the development of this broadly neutralizing CH103 antibody lineage. Cocrystal structure of the CH103 antibody and the HIV-1 Env revealed antibody contacts in the V5, CD4-binding loop, and loop D regions in Env, and analysis of the env gene sequences obtained by single-genome amplification demonstrated additional early mutations in the V1 and V4 loop regions (Liao et al., 2013a).

In this study, we have probed the mechanisms of selection of early CH505 Env mutations and found that amino acid changes in the V1, V4, V5, and CD4-binding loops resulted in escape from neutralization by the CH103 lineage (V1, V5, CD4-binding loop) or from cytotoxic T cell pressure (V4). Surprisingly, however, the mutations in the Env loop D increased neutralization sensitivity to the CH103 bnAb lineage. We demonstrated a mechanism of bnAb induction wherein a second antibody lineage targeted a bnAb contact site, thus selecting Env variants with enhanced binding and neutralization sensitivity for bnAb lineage antibodies. These results demonstrated that cooperation between two B cell lineages early in HIV-1 infection can facilitate the induction of broadly neutralizing CD4bs antibodies.

RESULTS

Early CH505 Env Mutations in V1, V4, V5, and the CD4bs Were Associated with Escape from CH103 bnAbs or T Cell Responses

To study the interplay between HIV-1 Env variants and bnAb development in the CH505 individual, we determined neutralization susceptibility of 124 Env pseudoviruses (~18 per time point) from seven time points after HIV-1 transmission (weeks 4, 14, 20, 30, 53, 78, and 100) to members of the CH103 bnAb lineage. The CH103 UCA and intermediate antibody (IA8-1) only neutralized the CH505 T/F virus. Over time (weeks 4–100), CH505 viruses gradually became more resistant to subsets of the CH103 bnAbs and mature Abs (V1, V5, CD4-binding loop) or from cytotoxic T cell pressure (V4). Surprisingly, however, the mutations in the Env loop D increased neutralization sensitivity to the CH103 bnAb lineage. We demonstrated a mechanism of bnAb induction wherein a second antibody lineage targeted a bnAb contact site, thus selecting Env variants with enhanced binding and neutralization sensitivity for bnAb lineage antibodies. These results demonstrated that cooperation between two B cell lineages early in HIV-1 infection can facilitate the induction of broadly neutralizing CD4bs antibodies.

![Figure 1. Neutralization Activity of CH103 Clonal Lineage Antibodies against Autologous CH505 Viruses](image-url)

Heat map analysis of neutralization data generated from 124 pseudoviruses (row) and 13 CH103 lineage mAbs (column). The neutralization potency (IC50) is shown in different shades of colors as indicated in the histogram, from white (>50 μg/ml) to dark red (0.079 μg/ml). The Env pseudoviruses were generated for the CH505 T/F virus and variants from weeks 4–100 and were all assayed against the unmutated common ancestor (UCA), intermediate antibodies (IA8-1), and mature bnAbs (CH103-106) in the TZM-bl cell-based neutralization assay. The resistant viruses (blue brackets) were defined as those with the average IC50 values 1.8-fold higher than that of the T/F pseudoviruses for later IAs (IA3-IA1) and all mature Abs. The week 30 viruses that escaped from the early IAs (IA3-IA1) are also indicated by blue bracket. See also Table S1 and Figures S1 and S4.
of the CH103 antibody lineage (UCA and IA8-4). For lineage members that exhibited increased heterologous neutralization (IA3-IA1 and mature CH103, CH104, CH105, and CH106 CD4bs bnAbs), escape was less complete, with a spectrum of sensitive and resistant autologous virus variants isolated from each time point (Figure 1 and Table S1). Thus, pseudoviruses were categorized into sensitive and resistant groups and analyzed for location of accumulated mutations in the env gene (Figure 1).

Env sequence analysis showed that all but 1 of the 20 viruses resistant to early IAs (IA8-IA4) at week 30 contained insertions (3–12 amino acids) in V1 (Figure 2A). Importantly, the V1 insertions also added 1–4 potential N-linked glycosylation (PNLG) sites (Figures 2A and S2). Although V1 was not seen in the Env-CH103 cocrystal structure (Liao et al., 2013a), a recent cryo-EM structure of the fully glycosylated Env trimer showed that V1/V2 could significantly affect the binding and neutralization of CD4bs bnAbs (Lyumkis et al., 2013). Docking the CH103 bnAb and CH505 Env sequences on the cryo-EM structure showed that an enlarged V1 loop with potential extra glycan(s) might push V2 into positions incompatible with early IA binding (Figures S2A and S2B). All but one of the 16 viruses resistant to later IAs (IA3-IA1) and mature CH103 mAbs had a two or seven amino acid insertions in V5, which is a major Env contact site for the CH103 bnAb heavy chain (Liao et al., 2013a). All resistant viruses from weeks 53, 78, and 100 had insertions in V5 (Figures 1 and S1). The two amino acid (Asp and Thr) insertions could push loop V5 and the glycan at position 461 into a possible clashing position with the light chain of CH103 antibodies (Figure S2C). These results indicated that early insertions in the V1 and V5 loops as well as site mutations in the CD4-binding loop resulted from CH103 bnAb mediated selection pressure.

Mutations in V4 were found early on at week 7 and persisted throughout later time points, suggesting that they were strongly selected (Figure S1). Viruses with mutations in a nine amino acid region in V4 were predominant (78%) as early as week 7 and completely replaced the T/F virus population from week 14 onward (Figures S1 and S3A). The N279K mutation in loop D was detected at as early as week 4 (10%), peaked at week 7 (57%), and disappeared from week 14 onward (Figures 3A and S1). In addition, the V281A and V281G mutations were detected at week 7 and week 9, respectively, and both became predominant in later time points together with other mutations in a nine amino acid region in loop D (Figure 3A). Since the mutations in both regions occurred early and their patterns were typical for T cell escape mutations, we performed ELISpot analysis using autologous overlapping peptides to determine if those mutations were driven by CD8+ T cell responses.

A CD8+ T cell response was detected for a putative T cell epitope (NSTRTITIHC) in V4 (Figure S3B and Table S2). The same peptides containing a T415K mutation, which were detected at week 14, could not be recognized by CD8+ T cells. Four mutants containing individual V4 mutations, including the T415K, had similar neutralization susceptibility to CH103 mAbs as the T/F virus (Figure S3C). These results demonstrated that the predominant mutations in V4 were driven by CD8+ T cell responses. In contrast, no T cell responses were found targeting the loop D or V1, V3, and V5 regions (Table S2).

Selection of the Loop D Mutations in CH505 Envelope by Neutralizing Non-CH103 Abs

Since the Env loop D region is a binding site for CH103 lineage bnAbs (Liao et al., 2013a), we next asked if loop D mutations might be due to the selection by CH103 lineage CD4bs antibodies. To test this hypothesis, we introduced loop D mutations at positions 275, 279, 280, and 281, individually or in combination as they occurred in vivo, into the T/F env gene to determine their effect on Env pseudovirus sensitivity to neutralization by the autologous CH103 lineage mAbs. Unexpectedly, all loop D
Figure 3. Mutations in Loop D Rendered the Env Mutants More Sensitive to CH103 Lineage bnAb Neutralization with Enhanced Env Binding

(A) Alignment of nine amino acids in loop D of Env. The amino acid sequences from week 4 to week 160 were compared to the CH505 T/F sequence. The number and frequency of each variant in loop D are shown at the right of the alignment. The amino acids that interact with the CH103 light chain are indicated in yellow. The loop D mutations that occurred early or predominated are indicated in red, and Env mutants containing those mutations are indicated by red arrows.

(B) Neutralization susceptibility of the loop D mutants to the CH103 lineage mAbs. Heatmap analysis was performed for the neutralization data of all CH103 lineage mAbs (column) against the CH505 T/F virus and the loop D variants (row). The neutralization potency (IC50) is shown in different colors as indicated, from white (>50 μg/ml) to dark red (0.12 μg/ml).

(C) Neutralization activity of the nAb CH235 were compared to that of the bnAb CH103 against the CH505 T/F virus and loop D mutants.

(D) The fold difference in binding to loop D mutant Envs versus the CH505 T/F Env by both CH103 and CH235 lineage Abs. Seven loop D mutant Envs (M6V281A, M10V281G, M11N279D/V281G, M7E275K/N279D/V281S, M8N280S/V281A, M9E275K/N279D/V281G, and M21N280T/V281A) and the CH505 T/F Env were serially diluted and the log area under the curve (AUC) values for all members of the CH103 and CH235 lineage mAbs were determined by ELISA. The fold difference in log AUC between each loop D mutant Env versus the CH505 T/F Env is shown.

See also Figures S3, S4, and S6; Tables S2, S3, and S6.
mutations rendered the mutant Env pseudoviruses 4.5-fold (range, 0.4–20) more sensitive than the T/F virus to neutralization by CH103 lineage bnAbs (Figure 3B and Table S3). In addition, when compared to the CH505 T/F virus, four loop D mutants (M5N279K, M6V281A, M7E275K/N279D/V281S, and M10V281G) with one or three mutations were less fit than the T/F virus (Figure S3D). These results demonstrated that the loop D mutations were selected by an antibody lineage other than the CH103 bnAb lineage.

To isolate the antibodies responsible for the loop D mutations, we established limiting dilution cell cultures from peripheral blood memory B cells collected at week 41 in CH505 (Bonsignori et al., 2011). We chose week 41 to study because neutralization of heterologous tier 2 viruses was first detected at week 41, 21 weeks after the detection of the first autologous neutralization activity. We identified one mAb, CH235 (VH1-46, V\kappa3-15), that neutralized the CH505 T/F, but belonged to a clonal family distinct from CH103 lineage (VH4-59, V\lambda3-1). mAb CH235 neutralized the CH505 T/F virus ~7-fold more potently than antibody CH103 (Figure 3C). However, CH235 poorly neutralized loop D mutant M11\N279D/V281G and could not neutralize five other loop D mutants (M7E275K/N279D/V281S, M8N280S/V281A, M9E275K/N279D/V281G, M20N280S/V281G, and M21N280T/V281A). In contrast, CH103 neutralized the same five loop D mutants ~10-fold better than the CH505 T/F virus (Figure 3C).

To identify antibody members of the CH235 lineage, we analyzed limiting dilution memory B cell cultures from week 41 and identified four additional CH235 lineage members (CH236, CH239, CH240, and CH241). The frequency of CH235 clonal lineage memory B cells at week 41 posttransmission was 0.018%, which was similar to that (0.014%) of the CH103 bnAb lineage. The four CH103 lineage antibodies isolated at week 41 were of similar mutation frequencies as inferred antibodies of the neutralization arm of the CH103 lineage (Figure S4). To confirm the relevance of the inferred IAs of the CH103 lineage, we characterized these newly isolated four natural IAs (CH186, CH187, CH188, and CH200) of the CH103 bnAb lineage from week 41 and demonstrated that their neutralization specificity was similar to that of the inferred IAs with only neutralization of the autologous CH505 T/F virus and no neutralization of heterologous viruses (Figure S4).

We inferred the CH235 lineage UCA and IAs, and expressed all CH235 lineage members as IgG1 recombinant antibodies (Liao et al., 2013a) (Figure S5). We then determined the ability of the CH235 lineage antibodies to neutralize the CH505 T/F and its variants. Like CH103 lineage mAbs, the CH235 UCA did not neutralize the CH505 T/F virus, with neutralization capacity acquired at IA3 (Figure 4 and Table S4). The CH235 lineage mAbs could partially neutralize week 30 viruses but could not neutralize the majority of viruses from weeks 53–100 after these viruses acquired loop D mutations. These results demonstrated that CH235 lineage mAbs had an autologous neutralization profile distinct from the CH103 bnAb lineage, in that they potently neutralized early autologous viruses and then at week 53 selected viruses that completely escaped CH235 lineage neutralization.

To determine whether the escape from CH235 lineage mAbs was indeed due to loop D mutations, we determined the ability of CH235 lineage mAbs to neutralize the CH505 loop D mutants. CH235 lineage antibodies neutralized the early loop D mutants (M5N279K, M6V281A, M7E275K/N279D/V281S, and M10V281G) that occurred before week 30 equally well or better than the T/F virus (Figure 5 and Table S5). These mutants have only one mutation at position 279 or 281. However, the CH235 lineage mAbs only partially neutralized loop D mutants M19V281D and M11N279D/V281G that were first
Mutations in Loop D Enhance Interactions between Env gp120 and CH103 bnAbs

Based on our previous cocystal structure of Env and CH103 (Liao et al., 2013a), the T/F Env favored the interaction with the CH103 UCA (Figure 6A). However, the loop D mutant M7E275K/N279D/V281S that contained three mutations favorably bound the mature CH103, which contained three CDR L2 mutations (G50E, D51N and S52Y) compared to the UCA (Figure 6B). The E275K mutation in M7 rendered the Loop D positively charged at one side and this change was complementarily accommodated by a CDR L2 Q50E mutation in the mature CH103. Similarly, the N279D mutation made the other side of loop D more negatively charged to better complement the CDR L2 Lys53. Computational reversion of critical mutations in loop D more negatively charged to better complement the CH103 UCA through IA4 (Figure 3D). All seven loop D mutant Envs also bound to four natural IAs (CH186, CH187, CH188, and CH200) of the CH103 bnAb lineage better than the T/F Env (Figure S4D). Thus, it is likely that CH505 Env loop D mutant viruses drove the maturation of the CH103 lineage by targeting early lineage members through mutations at amino acid positions 281 and 279, and late CH103 lineage members by a combinations of mutations at amino acid positions 280, 281, 279, and/or 275 (Figures 3A and 3D).

In contrast, when compared to the T/F Env, mature CH235 mAbs and IA3-Ia1 bound loop D mutant Envs at least 100-fold lower than the T/F Env, except that they bound to M6V281AEnv better than the T/F Env (Figure 3D). While none of the loop D mutants or the CH505 T/F Env bound the CH235 UCA or IA4 by ELISA, the T/F Env did weakly react with the CH235 lineage mAbs selected the loop D mutant Envs that had lower binding to the CH235 lineage mAbs, but higher binding to, and enhanced neutralization by, the CH103 lineage mAbs (Figure 7).

DISCUSSION

One fundamental question in HIV-1 vaccine design is how immunogens can be optimized to drive the maturation of bnAbs in vivo. By studying the HIV-1 quasispecies evolution in an individual (CH505) with a single TF virus, and by mapping the neutralization susceptibility of early quasispecies members to the autologous CH103 lineage, we have shown that the maturation of the CD4bs bnAb lineage was driven by cooperation of two
neutralizing antibody B cell lineages. This observation came from the surprising finding that one of the contact sites of the CH103 bnAb light chain (the Env loop D) contained mutations that did not lead to escape from the CH103 bnAb lineage, but, in contrast, resulted in enhanced binding and neutralization of loop D mutant viruses by the CH103 bnAb lineage. These data demonstrated that the CH103 bnAb lineage members did not select the loop D mutants in CH505 Env, but rather suggested the existence of antibodies that could neutralize the CH505 T/F virus, but not neutralize loop D mutant viruses. Thus, these observations led to the isolation of the CH235 lineage, and the demonstration that this neutralizing lineage indeed selected CH505 transmitted/founder virus loop D escape mutants.

The concept of bnAbs evolving from autologous neutralizing antibody lineages has been recently put forth (Doria-Rose et al., 2014; Liao et al., 2013a; Moore et al., 2012; Wibmer et al., 2013). Moore and colleagues demonstrated that autologous polyclonal plasma neutralizing antibodies targeted at subsequent sites of V1V2 bnAbs could be documented (Moore et al., 2012; Wibmer et al., 2013). Our studies differ from these studies in that we have used recombinant antibody techniques to isolate entire clonal lineages to directly demonstrate their cooperation in induction of CD4bs bnAbs. While it is clear that bnAbs develop heterologous broad neutralizing capacity by first neutralizing autologous virus Env mutant viruses (Doria-Rose et al., 2014; Liao et al., 2013a), our study directly demonstrates a mechanism of how this can happen at the B cell lineage level. These observations of two lineages cooperating to drive a bnAb lineage provide a view of how one lineage can be affected by another to accommodate autologous Env variation. Thus, the cooperation between the CH235 and CH103 lineages represents a novel molecular mechanism of bnAb development, wherein one neutralizing lineage (CH235) selected escape mutations in an Env contact site (loop D) that led to increased binding and neutralization of the other bnAb lineage (CH103), ultimately driving the maturation and development of broadly cross-reactive neutralizing antibodies (Figure 7). It has been unclear how bnAbs can acquire heterologous breadth of neutralization in response to evolving T/F variants. Our studies show that one mechanism for achieving this is via cooperating lineages that select virus mutants with more bnAb lineage neutralization in sensitivity than the T/F virus, thus potentiating bnAb affinity maturation. It has recently been demonstrated that CH103 antibody lineage mutations also resulted in bnAb conformational shifts that led to accommodations of mutational insertions in Env V5 (Fera et al., 2014).

The contrast in selection of autologous escape mutants by the CH103 bnAb lineage (Figure 1) and the CH235 nAb lineage (Figure 4) is striking and suggested a difference in their biology. Whereas the CH235 nAb lineage led to total escape from identified lineage members after 30 weeks of infection (Figure 4), the CH103 bnAb lineage differed in that it was comprised of two components, the early autologous-only nAbs (UCA through IA4) and the more mature antibodies (IA3 through CH103, CH104, CH105, and CH106 bnAbs) with neutralization breadth (Figure 1). The early CH103 autologous-only nAbs also selected total escape by week 53, but the later CH103 antibodies with neutralization breadth did not. Rather, the more mature CH103
bnAbs retained the ability to neutralize select autologous variants through week 100 (Figure 1). These data suggested that a component of bnAb development is the retention of ability to neutralize autologous variants (Figure 1). Thus, the cooperation between the CH235 nAb lineage and the CH103 bnAb lineage demonstrated the first step in bnAb lineage development in which CH103 lineage bnAbs retained the ability to neutralize autologous virus variants as they matured to neutralize heterogeneous viruses. It is critical to determine if the types of cooperating lineages as seen in CH235-CH103 interactions are the key initiators of bnAb breadth, or if other additional Env-reactive B cell lineages are required. Against this latter notion was the observation that multiple loop D Env mutants can likely drive all stages of the CH103 lineage (Figure 3).

It will be important to determine whether the interaction between CH235 and CH103 lineages only occurs during a short window early in viral evolution, or over a longer period of time. We note in this context that the heavy chain of CH235 derived from VH1-46, which has also been observed to produce broadly neutralizing CD4bs antibodies like 1B2530 and 8ANC131 (Scheid et al., 2011).

It is also important to note that the CH103 bnAb is a loop binding CD4bs bnAb in contrast to the VRC01 class of CD4bs bnAb that recognizes the CD4-binding site in a manner similar to CD4 (Zhou et al., 2013). Whereas VRC01 class mAbs derive from restricted VH1-2 paired with a VxJx with a five amino acid LCDR3, loop binding CD4bs bnAbs have been isolated, which utilize multiple VxJx/VxJx pairs (Bonsignori et al., 2014; Corti et al., 2010; Liao et al., 2013a). It will be key to determine, with immunization of CH505 Envs, if either CH103-like VH4-59, VxJx-1 CD4bs lineage Abs are induced or if other VH usage can be induced with CD4bs bnAb signatures. These findings have considerable importance for HIV-1 vaccine design for induction of CD4bs bnAbs. First, mapping of individual bnAb lineages over time in those individuals who make them may not be sufficient for obtaining the information needed for the design of protective immunogens for bnAb development. Rather, mapping of multiple neutralizing antibody lineages may be required for optimal choice of immunogen candidates. Second, these data suggest that induction of one or more neutralizing antibody lineages to select Env variants with enhanced affinity for bnAb antibody members may be required to induce CH103-like CD4bs HIV-1 neutralizing antibodies. For example, for CH103-like lineage induction, priming with the transmitted/founder Env and additional Envs variants such as M6V281A with enhanced binding to CH235 lineage antibodies, followed by boosting with Env mutants with enhanced binding to CH103 lineage members, is likely to be important for experimental vaccine design.

**EXPERIMENTAL PROCEDURES**

**Generation of Pseudoviruses**

Env pseudoviruses were produced as described (Kirchherr et al., 2007).

**Neutralization Assay**

Neutralization activity was measured as a reduction in luciferase activity after a single round infection of TZM-bl cells as previously described (Li et al., 2005; Montefiori, 2004).

**IFN-γ ELISpot Assay**

The IFN-γ ELISpot assay was performed according to previous descriptions (Cox et al., 2006). The responses were considered positive if > 50 SFC per 10⁶ PBMCs were detected.

**Site-Directed Mutagenesis**

Mutants of CH0505.T/F envelope gene were constructed using the Quick Change II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). All final env mutants were confirmed by sequencing.
Viral Fitness Assay
The fitness of the Env loop D mutants was determined by comparing to their cognate CH505 T/F virus in a competitive fitness assay as previously described (Cai et al., 2007; Song et al., 2012).

Envelope Glycoprotein Expression
The codon-optimized CH505 transmitted/founder and loop D mutant env genes were generated by de novo synthesis (GeneScript, Piscataway, NJ) or site-directed mutagenesis in mammalian expression plasmid pCDNA3.1/ hygromycin (Invitrogen, Grand Island, NY) as described (Liao et al., 2013a), and stored at ~80°C until use.

B Cell Culture
IgG+ memory cells were isolated from PBMCs using a previously described protocol (Bonsignori et al., 2011). Cell culture supernatants were screened for binding to autologous CH505 T/F gp140 and neutralization of the autologous CH505 w4.3 Env pseudovirus. Culture supernatants that neutralized CH505 w4.3 were then screened for differential neutralization of the CH505 T/F and M10 mutant viruses.

Isolation of Immunoglobulin V(D)J and VL Gene Segments and Expression of Recombinant Antibodies
RNA from positive cultures was extracted by using standard procedures (RNasey minikit; Qiagen, Valencia, CA), and the genes encoding Ig V,D,Ju and V,I,Ju rearrangements were amplified by RT and nested PCR without cloning by use of a previously reported method (Liao et al., 2009). Sequence base calling was performed by using Phred. V, D, and J region genes and mutations were analyzed by using the SoDa information system (Volpe et al., 2008). The genetic information of the Ig V,D,Ju and V,I,Ju were annotated using the method as described (Liao et al., 2013a). For further characterization, the isolated V,D,Ju/V,I,Ju genes of the observed antibodies from CH505 and infected V,D,Ju/V,I,Ju for UCAs and intermediate antibodies were synthesized (GeneScript, Piscataway, NJ) and cloned into pCDNA3.1 plasmid (Invitrogen, Grand Island, NY) for production of purified recombinant IgG1 antibodies in 293F cells by transient transfection as described previously (Liao et al., 2011).

Inference of UCA and IAs of CH235 Lineage
The five members of the CH235 antibody lineage were used to infer the UCA and IAs of both heavy and light chains simultaneously using methods described in (Kepler, 2013).

Direct-Binding ELISA
Direct-binding ELISAs were performed in 384-well plates as previously described (Bonsignori et al., 2011).

Surface Plasmon Resonance Affinity and Kinetics Measurements
Binding Kd and rate constant (association rate k+a, dissociation rate k+d) measurements of mAbs to the autologous CH505 gp140 were carried out on Biacore 3000 or Biacore T200 instruments as described (Alam et al., 2007; Alam et al., 2009; Liao et al., 2013a).

Structural Alignment and Loop Modeling
To visualize effects of evolutionary mutations in CH505 variants, such as sequence insertions and addition of potential glycosylation sites, in the trimeric viral spike context, the antibody CH103-gp120 complex structure (PDB ID: 4JAN) was aligned to the structure of BG05 SOSIP.664 HIV-1 Env trimer in complex with VRC-PG04 (PDB ID: 3JSM) by superposing the outer domains of gp120 in each structure using program package CCP4 (Winn et al., 2011). Based on the superposed CH103 gp120 structure, possible conformations of loop insertions in the HIV-1 V1, V2, and V5 loop were modeled with program Loopy (Xiang et al., 2002). Relative positions and potential clashes between the HIV-1 V1, V2, and V5 loop and the gp120-bound CH103 were depicted with program PyMOL (http://www.pymol.org).

Calculation of Changes in Binding Affinity upon Mutation
To evaluate the effects of evolutionary mutations in loop D of CH505 gp120 and affinity maturation mutations in antibody CH103 on binding affinity, we computationally estimated the changes in binding affinity caused by mutations on either loop D of gp120 or CHR L2 of antibody CH103 with the program BeAtMuSiC (Dehouck et al., 2013; Moretti et al., 2013). This program uses the known structure of a protein-protein complex to evaluates the change in binding affinity between two proteins caused by single-site mutations in their sequence; we observed a high concordance in prediction by this program and binding data for HIV-1 gp120-antibody complex structures for which binding data for single-site mutations had been previously determined experimentally (Zhou et al., 2010). Single mutations were modeled using the structure of mature antibody CH103 in complex with gp120 (PDB ID: 4JAN) while keeping the protein backbone rigid. Calculations were carried out by reverting respective amino acid at each position (275, 279, and 281 in the HIV-1 loop D and 50-52 in CH103 CDR L2) to its counterpart in the T/F virus or germline antibody.

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SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.06.022.
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