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Targeted selection of HIV-specific antibody mutations by engineering B cell maturation

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Competing interests: K.O.S. and B.F.H. are inventors on International Patent Application PCT/US2018/020788 submitted by Duke University, that covers the composition and use of CH848 HIV-1 envelopes for induction of HIV-1 antibodies. D.C.M., C.L., K.O.S., K.W., and B.F.H. are inventors on International Patent Application PCT/US2018/034777 submitted by Duke University, that covers the composition and use of CH505 HIV-1 envelopes for induction of HIV-1 antibodies.

Data and materials availability: Antibody sequences have been deposited to GenBank under accession numbers MN643173 through MN643554. The cryo-EM maps and refined coordinates were deposited in the EMDB and RCSB PDB databases, respectively, under the following accession numbers: DH270 UCA (EMD-20817 and PDB ID 6UM5), DH270.6 (EMD-20818 and PDB ID 6UM6), and DH270.mu1 (EMD-20819 and PDB ID 6UM7). The ARMADILLO program is available for download at <http://sites.duke.edu/ARMADILLO>. All flow cytometry data are available upon request. All other data are in the main and supplementary figures and text. The DH270UCA V_H/V_L KI mice and CH235UCA V_H/V_L KI mice are available from F.W.A.'s laboratory under a standard material transfer agreement with Boston Children's Hospital. 3M-052 stable emulsion adjuvant is available from M. Tomai and S. Reed under a material transfer agreement with 3M Company (St. Paul, MN) and Infectious Disease Research Institute (Seattle, WA), respectively.

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Abstract

INTRODUCTION—A major goal of HIV-1 vaccine development is the design of immunogens that induce broadly neutralizing antibodies (bnAbs). However, vaccination of humans has not resulted in the induction of affinity-matured and potent HIV-1 bnAbs. To devise effective vaccine strategies, we previously determined the maturation pathway of select HIV-1 bnAbs from acute infection through neutralizing antibody development. During their evolution, bnAbs acquire an abundance of improbable amino acid substitutions as a result of nucleotide mutations at variable region sequences rarely targeted by activation-induced cytidine deaminase, the enzyme responsible for antibody mutation. A subset of improbable mutations is essential for broad neutralization activity, and their acquisition represents a key roadblock to bnAb development.

RATIONALE—Current bnAb lineage-based vaccine strategies can initiate bnAb lineage development in animal models but have not specifically elicited the improbable mutations required for neutralization breadth. Induction of bnAbs requires vaccine strategies that specifically engage bnAb precursors and subsequently select for improbable mutations required for broadly neutralizing activity. We hypothesized that vaccination with immunogens that bind with moderate to high affinity to bnAb B cell precursors, and with higher affinity to precursors that have acquired improbable mutations, could initiate bnAb B cell lineages and select for key improbable mutations required for bnAb development.

RESULTS—We elicited serum neutralizing HIV-1 antibodies in human bnAb precursor knock-in mice and wild-type macaques vaccinated with immunogens designed to select for improbable mutations. We designed two HIV-1 envelope immunogens that bound precursor B cells of either a CD4 binding site or V3-glycan bnAb lineage. In vitro, these immunogens bound more strongly to bnAb precursors once the precursor acquired the desired improbable mutations. Vaccination of macaques with the CD4 binding site–targeting immunogen induced CD4 binding site serum neutralizing antibodies. Antibody sequences elicited in human bnAb precursor knock-in mice encoded functional improbable mutations critical for bnAb development. In bnAb precursor knock-in mice, we isolated a vaccine-elicited monoclonal antibody bearing functional improbable mutations that was capable of neutralizing multiple HIV-1 global isolates. Structures of a bnAb precursor, a bnAb, and the vaccine-elicited antibody revealed the precise roles that acquired improbable mutations played in recognizing the HIV-1 envelope. Thus, our immunogens elicited antibody responses in macaques and knock-in mice that exhibited the mutational patterns, structural characteristics, or neutralization profiles of nascent broadly neutralizing antibodies.

CONCLUSION—Our study represents a proof of concept for targeted selection of improbable mutations to guide antibody affinity maturation. Moreover, this study demonstrates a rational strategy for sequential immunogen design to circumvent the difficult roadblocks in HIV-1 bnAb induction by vaccination. We show that immunogens should exhibit differences in affinity across

antibody maturation stages where improbable mutations are necessary for the desired antibody function. This strategy of selection of specific antibody nucleotides by immunogen design can be applied to B cell lineages targeting other pathogens where guided affinity maturation is needed for a protective antibody response.

Graphical Abstract

Overcoming somatic mutation roadblocks to advance broadly neutralizing HIV-1 antibody (bnAb) development

Vaccination of animal models with engineered HIV-1 immunogens generated antibodies that acquired functional improbable mutations critical for virus neutralization. The lack of envelope selection of improbable mutations is a roadblock for bnAb development. Vaccine-elicited antibodies exhibited neutralization activity similar to that of intermediate-stage bnAbs. Structural studies showed a vaccine-elicited neutralizing antibody bound to HIV-1 envelope in a manner similar to that of a mature bnAb.

The design of immunogens to direct antibody maturation is a major goal for vaccine development. One roadblock preventing HIV-1 vaccine design is the need for broadly neutralizing antibodies (bnAbs) to acquire somatic mutations rarely made by activation-induced cytidine deaminase (AID). We designed immunogens that bind with higher affinity to antibodies with improbable mutations compared to unmutated precursor antibodies. In knock-in mice, such immunogens engaged unmutated bnAb precursors, selected for functional improbable mutations, and induced neutralizing antibodies. Structural studies revealed how bnAb precursors interact with the envelope protein (Env) and the functions of the elicited improbable mutations. In macaques, the CD4 binding site–targeting immunogen induced potent CD4 binding site–neutralizing antibodies. Our immunogen design strategy may allow for the delineation of sequential immunogens to direct bnAb development for HIV-1.

To date, HIV-1 vaccination has not resulted in the induction of high titers of potent HIV-1 broadly neutralizing antibodies (bnAbs) (1, 2). bnAbs are disfavored by immune tolerance mechanisms because of their unusually long complementarity-determining regions (CDRs), autoreactivity, and polyreactivity (3, 4). In addition, bnAbs have high frequencies of somatic mutation resulting from extended rounds of affinity maturation (5–7). Antibody somatic mutation is mediated by activation-induced cytidine deaminase (AID), the enzyme that deaminates cytidine to uridine and can lead to nucleotide substitution during DNA repair (8). As a result of the preferential targeting of AID to specific sequence motifs, mutability varies greatly among positions within an antibody sequence (9). We recently used the computational program Antigen Receptor Mutation Analyzer for Detection of Low-likelihood Occurrences (ARMADiLLO) to determine that bnAbs are enriched for somatic mutations that occur at variable region sequences not routinely targeted by AID or that require multiple changes to the germline codon (10). A subset of these improbable mutations are required for broad neutralization activity and therefore represent key roadblocks for the development of bnAbs (10–12). These obstacles have led to the hypothesis that vaccine strategies are needed that direct the immune system to expand B cell lineages that are usually rare and select for disfavored antibody traits (4). At the foundation of this vaccine strategy is the specific engagement of germline precursors of neutralizing antibodies and the selection of improbable mutations by strong antigenic selection (10, 11, 13). Recent studies have reported variable results in induction of V3-glycan bnAb B cell lineage precursors, either starting from mutated precursors (14) or induction of precursors in macaques (15); however, to date, there are no reports of vaccination specifically eliciting improbable mutations above the frequency with which they would be expected to occur in the absence of selection (10, 11). Learning the rules to precisely select for specific antibody somatic mutations would improve the design of vaccines or therapeutics for infectious diseases, autoimmunity, inflammatory diseases, and malignant diseases (16, 17).

One of the major bnAb epitopes on HIV-1 Env is the V3-glycan site, which consists of the base of the third variable (V3) loop and surrounding glycans (18–21). The DH270 bnAb B cell lineage, isolated from an HIV-infected individual, targets the V3-glycan epitope on HIV-1 Env (18). We computationally inferred the clonal history of the DH270 lineage, including the naïve B cell V(D)J rearrangement of the DH270 lineage termed the unmutated common ancestor (UCA) (18). The computational reconstruction of the DH270 lineage showed that the lineage initially progressed from the UCA precursor antibody to an early intermediate antibody (IA) designated DH270 IA4 (18). This initial affinity maturation step for the DH270 lineage included the acquisition of four amino acid changes [Gly³¹ → Asp, Met³⁴ → Ile, Ser⁵⁵ → Thr, and Gly⁵⁷ → Arg (G57R)] and one amino acid change [Ser²⁷ → Tyr (S27Y)] in the heavy and light chain variable regions, respectively (18). The DH270 IA4 antibody bearing these mutations had the ability to neutralize a subset of HIV-1 isolates representative of the globally circulating virus population (heterologous viruses) (18). Two of the five amino acid changes in DH270 IA4 were the result of mutations at AID hot spots, and their acquisition did not contribute to heterologous HIV-1 neutralization (18). In contrast, the G57R amino acid change alone is sufficient for heterologous HIV-1 neutralization and is the result of a nucleotide mutation within a disfavored AID cold spot, making its acquisition highly improbable (10, 18). Thus, immunogens are needed that can

specifically select for the G57R amino acid change in order for vaccines to elicit DH270-like V3-glycan antibody responses.

The CD4 binding site bnAb lineage CH235 was isolated from the CH505 HIV-1-infected individual (22). Unlike most potent CD4 binding site bnAbs that mimic CD4, the CH235 lineage does not require difficult-to-elicited insertions or deletions for broad neutralization (22, 23). Instead, the most potent bnAbs in the CH235 lineage had many improbable mutations, which suggested that the primary obstacle in CH235 lineage development is accumulation of improbable mutations (10). When the CH235 clonal lineage was reconstructed, a key early improbable functional mutation, Lys¹⁹ → Thr (K19T) in the heavy chain variable region, was identified. The K19T amino acid change improved binding to Env by the CH235 bnAb precursor and was necessary for neutralization of HIV-1 by CH235 bnAbs (10). The generation of an immunogen capable of eliciting the K19T change would overcome the first roadblock in the development of the CH235 lineage, and it could be applicable to other antibodies in the CH235 (also called 8ANC131) bnAb class because they all encode K19T changes as well.

Here, we designed immunogens that select for specific functional improbable nucleotide mutations required for bnAb development, thus demonstrating proof-of-concept for directing DH270 V3-glycan and CH235 bnAb B cell lineage affinity maturation. These immunogens for two different bnAb lineages can overcome the two initial roadblocks—engagement of the bnAb precursor and selection of functional improbable mutations—and are key components of a sequential HIV-1 vaccine regimen.

Engagement of V3-glycan bnAb precursor B cell receptors

We previously determined that recombinant HIV-1 Env gp120s produced from sequences isolated from the HIV-1-infected individual who developed the DH270 bnAb lineage were incapable of binding to the UCA (18). Computational modeling of the structure of the bnAb DH270.6, the most potent DH270 bnAb lineage member, when bound to trimeric Env showed steric hindrance by an N-linked glycan present at amino acid 137 in the first variable region (V1) of HIV-1 Env (fig. S1A). Using an autologous Env from CH848, we removed glycosylation sites near position 137—the Asn¹³³ and Asn¹³⁸ (N133 and N138) glycans. The DH270 UCA neutralized the CH848.D949.10.17 virus with the N133 and N138 glycans removed (fig. S1C; 50% inhibitory concentration IC₅₀ = 1.7 µg/ml), but the same virus with the glycans present was not neutralized (Fig. 1A). Removing the V1 glycans from JR-FL was not sufficient for DH270 UCA neutralization sensitivity (fig. S1B). This difference in neutralization sensitivity between JR-FL lacking V1 glycans and CH848 10.17 lacking V1 glycans indicated that V1 glycan removal alone was not sufficient for making all viruses sensitive to DH270 UCA neutralization (fig. S1B).

For immunizations, soluble, well-folded CH848 gp140 SOSIP Env trimers with (10.17) and without (10.17DT) the N133 and N138 glycans present were produced (fig. S1C and figs. S2 to S4). We initially generated stabilized SOSIPs using three different published strategies (24–26) but found them to have very similar trimer conformation and antigenicity (fig. S2). We selected the SOSIPv4.1 version with the lowest non-neutralizing antibody binding for

further study. The CH848 10.17DT Env trimer bound to the DH270 UCA antigen-binding fragment (Fab) with an equilibrium binding constant (K_D) of 532 nM (Fig. 1B). The presence of V1 glycans reduced the binding affinity by a factor of 5 between the DH270 UCA and CH848 10.17DT-stabilized SOSIP Env (fig. S1D). The K_D of CH848 10.17DT improved to 118 nM for the DH270 IA4 Fab, although IA4 differed from the UCA by only five amino acids (Fig. 1B). The difference in binding affinity suggested that 10.17DT could select these five amino acid changes. The selection of these amino acids would advance antibody affinity maturation toward neutralization breadth. When the DH270 UCA immunoglobulin M (IgM) was expressed as an IgM B cell receptor (BCR) on the surface of a Ramos B cell line, 10.17DT Env trimers bound with sufficient avidity to cross-link the receptor and induce calcium flux (Fig. 1C). Removal of the N133 and N138 V1 glycans resulted in an increase in the percentage of high-mannose glycans at Asn³³² on the CH848 10.17DT Env trimer (fig. S3). Because DH270-lineage antibodies bind the Asn³³² glycan and are high mannose-reactive, the increase in high mannose likely contributed to the improved binding to the DH270 UCA (figs. S1D and S3). Therefore, V1 glycans were inhibitory for DH270 antibody binding to envelope trimers, and their removal generated an envelope capable of interacting with soluble and membrane-bound DH270 UCA.

Structural characterization of DH270 UCA bound to CH848 10.17DT Env

To provide atomic-level information on the interaction between DH270 UCA and the CH848 10.17DT Env, we determined the structure of the antigen-binding fragment (Fab) of DH270 UCA in complex with CH848 10.17DT SOSIP by cryo-electron microscopy (cryo-EM) to an overall resolution of 4.2 Å (Fig. 1, D to H, figs. S5 and S6, and table S1). In cryo-EM reconstructions, we observed that the Env trimer was engaged by three Fabs in a 1:1 Env protomer/Fab ratio (Fig. 1D). The DH270 UCA Fab bound a proteoglycan epitope composed of variable loops 1 (V1) and 3 (V3) and surrounding glycans at positions 301 and 332 (Fig. 1, E to H). Of the ~2212 Å² total surface area buried at the Env-antibody interface, ~57% was contributed by interactions of the antibody with Env glycans. Whereas glycan 301 contacted only the light chain (total buried interface area ~250 Å²), glycan 332 made contacts with both the heavy and light chains, burying a total of 1047 Å² at its interface, with about two-thirds of the interactive surface contributed by the heavy chain (Fig. 1F).

DH270 UCA contacted the V3 loop by means of its HCDR1, HCDR2, and HCDR3 loops. The interaction was focused on the conserved V3 GDK/R (Gly-Asp-Ile-Lys/Arg) motif, with the side chain of HCDR1 Tyr³³ in the fitted coordinates placed favorably to engage in a potential hydrogen bond with the side chain of the conserved Asp³²⁵ of the GDK sequence (Fig. 1G). The N133, N138-deleted V1 loop was stacked against the V3 loop and adopted a β hairpin-like structure with the tip of the hairpin stabilized by interactions with the HCDR2 loop. In contrast to the N156 glycan (Fig. 1E), the presence of a glycan at position 138 in the V1 loop would have created steric clashes with the HCDR2 loop of the bound DH270 UCA (Fig. 1, E, G, and H). The N133D mutation was distal from the binding site of DH270 UCA but may modulate the conformation of the V1 loop. In summary, the structure of the Env-DH270 UCA complex provided an atomic-level understanding of the interaction of the DH270 UCA with the Env CH848 10.17DT by revealing key contacts with conserved

elements of the V3 loop and the surrounding glycans, and by showing how the engineered V1 loop engaged the DH270 UCA.

Generation and characterization of DH270 UCA knock-in mice

To evaluate the ability of the CH848 10.17DT Env to bind to the DH270 UCA BCR on primary B cells and to test CH848 10.17DT Env immunogenicity, we developed a humanized mouse model with the V_HDJ_H and $V_\lambda J_\lambda$ regions of the DH270 UCA knocked in (fig. S7A). As shown in fig. S7B, the DH270 UCA $V_H + V_L$ heterozygous knock-in (KI) mice had reduced numbers of B cells relative to T cells, which served as an internal reference. In control mice without KI genes, the ratio of B cells to T cells was 1.4; this was reduced to about 0.4 in DH270 UCA heterozygous KI mice. An obvious increase in $IgM^{+} IgD^{lo}$ immature B cell populations was observed in the spleen of DH270 UCA KI mice relative to control mice (figs. S7C and S8C), suggesting a slowdown or block in B cell maturation. To determine the proportion of B cells expressing DH270 UCA V_H , we examined allotypic markers of the two IgH alleles: DH270 UCA V_H was expressed as IgM^a , whereas mouse heavy chains were expressed as IgM^b . As shown in fig. S7C, about 30% of splenic B cells expressed IgM^a and more than 50% of B cells expressed IgM^b . In theory, expression of a pre-rearranged V_H exon should preclude the rearrangement of the other IgH^b allele (27). However, KI heavy chains of autoreactive antibodies can be deleted via V_H replacement (28–31), thereby freeing the other IgH allele for expression. DH270 UCA V_L was expressed as a κ light chain, but the KI mice expressed 2.5 times as many $Ig \lambda^{+}$ B cells as normal mice, which is indicative of receptor editing (32–34). Overall, the B cell phenotype of the DH270 UCA KI mice is consistent with the concept that the expression of some bnAbs is under negative selection by tolerance control mechanisms (35). However, the extent of B cell deletion in the DH270 UCA model is less severe than in some previously reported bnAb KI mouse models (36–38). The DH270 UCA BCR was functional, as the 10.17DT-stabilized SOSIP trimer induced calcium flux in splenic B cells from each of the three homozygous DH270 UCA ($V_H^{+/+}, V_L^{+/+}$) KI mice tested (fig. S9). Calcium flux was not detectable when the 10.17-stabilized SOSIP was used as the antigen (fig. S9). Because tolerance control mechanisms may also curtail DH270 precursor expression in humans, this DH270 $V_H + V_L$ heterozygous mouse provides a relevant and challenging model to test our immunogens.

Nanoparticle immunization of DH270 UCA KI mice

Multimeric immunogens have been reported to be superior to soluble monomers for inducing humoral responses (15, 39–46). Therefore, we sought to generate a nanoparticle immunogen arraying the 10.17DT SOSIPv4.1 version of the Env trimer. The 10.17DT Env was chosen over the wild-type 10.17 on the basis of 10.17DTs stronger binding affinity (by a factor of 5) for the DH270 UCA (fig. S1D), detectable induction of calcium flux (fig. S9), and more frequent elicitation of heterologous-neutralizing serum antibodies in DH270 UCA KI mice (fig. S10). To ensure that well-folded, native-like Env trimers were present on a multimeric Env trimer nanoparticle, we purified well-folded trimers and used sortase A to site-specifically ligate them to ferritin nanoparticles (Fig. 2A and fig. S11). HIV-1 Env trimers on the nanoparticle had the same bnAb-specific antigenic profile as the soluble Env

trimer, in that neutralizing antibodies but not non-neutralizing antibodies bound to the trimer (Fig. 2B and fig. S11). The CH848 10.17DT Env trimer nanoparticles cross-linked the BCR on mature and immature splenic B cells from homozygous DH270 UCA ($V_H^{+/+}$, $V_L^{+/+}$) KI mice and induced intracellular calcium flux (Fig. 2C).

Next, we immunized heterozygous DH270 UCA ($V_H^{+/-}$, $V_L^{+/-}$) KI mice with CH848 10.17DT Env trimer nanoparticles. In this mouse model, about 12% of splenic follicular B cells bind CH848 10.17DT, making this model more physiologic than previous KI model systems where most B cells express the KI variable regions (14) (Fig. 2D and fig. S8C). Mice that received the Env-trimer nanoparticle had significant higher numbers of germinal center B cells and follicular helper T cells relative to adjuvant only-immunized mice (Fig. 2E). The Env-trimer nanoparticle induced N332-directed antibodies and tier 2 autologous neutralizing antibodies against CH848 10.17DT and CH848 10.17 viruses after two or three immunizations, respectively (Fig. 2F and fig. S12). Heterologous neutralizing antibodies were also detectable in four of five CH848 10.17DT Env-immunized mice, but not in those animals that received adjuvant only (fig. S12). Thus, in an in vivo setting where DH270 UCA precursors represented a minority of B cells, high-quality Env nanoparticles induced serum V3-glycan antibodies.

Selection of improbable mutations by CH848 10.17DT immunization

We next determined whether 10.17DT vaccination could select for improbable somatic mutations that occurred during the natural evolution of DH270 in the HIV-1 infected individual. 10.17DT-stabilized envelope trimer bound to the DH270 UCA with the improbable G57R mutation with affinity improvement by a factor of 4 relative to the DH270 UCA ($K_d = 132$ nM and 558 nM, respectively; figs. S13, A and B, and S14, A to C). We hypothesized that this improvement in binding affinity would provide the necessary affinity difference to select for the G57R mutation. Indeed, next-generation sequencing of heavy chain variable regions (V_H NGS) from the vaccinated mice demonstrated that the G57R mutation occurred more frequently in CH848 10.17DT nanoparticle-immunized DH270 UCA KI mice than in adjuvant only-treated mice (Fig. 2G, top). Immunization with the CH848 10.17DT nanoparticle increased the median frequency of the G57R mutation within DH270 sequences by a factor of 200 after nanoparticle immunization (Fig. 2G, bottom).

In addition to the G57R improbable mutation, DH270 IA4 encodes a light chain variable region (V_L) S27Y improbable mutation (Fig. 1B and fig. S13C). Addition of the S27Y mutation to the DH270 UCA enabled accommodation of V1 glycan, as manifested by more potent neutralization of CH848 10.17 virus with the V1 glycans present (fig. S13A). We isolated single memory B cells that bound to the CH848 10.17 Env trimer from Env nanoparticle-vaccinated mice (fig. S15). Of the 10.17 Env trimer-reactive antibodies, 66% were derived from the DH270 antibody B cell lineage and 99% of these DH270 antibodies were somatically mutated (Fig. 3, A and B, and fig. S16). The somatic mutations included improbable and probable mutations (figs. S16 to S18). Among the DH270 antibodies from the nanoparticle-immunized mice, antibody DH270.mu1 encoded both V_H G57R and V_L S27Y mutations (Fig. 3D and fig. S18). The DH270.mu1 antibody neutralized autologous tier 2 CH848 viruses as well as heterologous HIV-1 6101, Q23, and 92RW020 viruses with

titers nearly identical to antibody DH270 IA4 (Fig. 3E). DH270.mu1 neutralized 16 of 23 heterologous isolates tested, including heterologous tier 1B and tier 2 viruses (Fig. 3F). Hence, vaccination induced a heterologous tier 2 neutralizing DH270 antibody that possessed the G57R and S27Y improbable mutations.

Accumulation of additional bnAb mutations in vaccine-elicited antibodies

In addition to S27Y and G57R, the V_H and V_L amino acid sequences of the 134 nanoparticle-induced DH270 antibodies included other somatic mutations present in DH270.1 (figs. S16 and S18). More specifically, 16 of 19 somatic amino acid changes present in DH270.1 were also present among the vaccine-induced antibodies (Fig. 3G). However, no single vaccine-induced antibody had acquired all 16 observed V_H mutations in combination; instead, they were distributed among the 134 antibodies. Although 90% of the vaccine-induced antibodies shared two somatic mutations with DH270.1, the highest number of shared mutations accumulated on a single vaccine-induced DH270 V_H sequence was six; this occurred in 6% of the observed monoclonal DH270 antibody V_H sequences (Fig. 3H). Similar to the V_H sequences, 6 of 10 amino acid changes present in DH270.1 light chain were elicited by 10.17DT nanoparticles. Of the three V_H mutations that were not sampled, none were improbable, and of the four V_L mutations that were not sampled, two were improbable (figs. S17 and S19).

To examine more deeply the accumulation of low V_H somatic mutations, we performed V_H next-generation sequencing on splenic B cells. We examined DH270 sequences for the co-occurrence of R98T and G57R because R98T has also been shown to be critical for DH270 interaction with glycan (47). The co-elicitation of both R98T and G57R was observed for all mice immunized with CH848 10.17DT nanoparticle at a higher frequency than adjuvant alone (fig. S20). The highest percentage of DH270 sequences with both mutations from any one mouse was 3.7% of DH270 sequences (fig. S20). We also analyzed DH270 sequences from the mice for the occurrence of all four DH270 IA4 V_H mutations in single antibodies. All mice immunized with CH848 10.17DT nanoparticle generated DH270 sequences containing all four somatic mutations present in DH270 IA4, but administration of adjuvant alone did not generate such sequences (fig. S20). Thus, the CH848 10.17DT nanoparticle selected for limited combinations of mutations present in the initial stages of the DH270 lineage maturation, but again did not elicit antibodies with all 19 somatic mutations observed in DH270.1. These data indicate the importance of sequential boosts to select for the improbable mutations required for full bnAb capacity.

We hypothesized that without strong antigenic selection, acquiring all improbable mutations observed in DH270.1 (18) in one antibody would be a highly unlikely event. Under a scenario in which only four improbable mutations are required (fig. S19, A and B), and given the assumption of a constant rate of one improbable mutation per 12 weeks (the duration of immunization in nanoparticle-immunized mice), it would take 120 weeks of biweekly immunization of mice to reliably acquire four improbable mutations (fig. S19C), or multiple decades of immunization in humans given the age relation between mice and humans (48). Although B cell evolution does not proceed under a constant mutation rate (7, 49), these data suggest that boosting immunogens designed to specifically select for

combinations of key improbable mutations will be necessary to accelerate neutralizing antibody lineage maturation to broad neutralization.

To determine whether the V1 glycan deletions were important for optimal selection of the G57R mutation, we compared elicitation of G57R mutations in mice immunized with CH848 10.17 versus CH848 10.17DT in DH270 UCA KI mice. We found that the 10.17DT SOSIP trimer more consistently elicited higher frequencies of the G57R improbable mutation than did the CH848 10.17 trimer (fig. S14D). Additionally, to determine whether the selection of the G57R mutation by CH848 10.17DT Env was a result specific to this immunogen, we analyzed previously published antibody sequences from KI mice that encode the same human V_H1–2*02 gene as DH270 but were immunized with a different HIV-1 Env immunogen (50, 51). Analysis of next-generation sequencing of V_H regions from V_H1–2*02 KI mice immunized with eODGT8 nanoparticles showed that this immunogen did not elicit the G57R mutation (50, 51). Thus, the selection of the desired G57R mutation is not merely a product of activation of V_H1–2*02-bearing B cells and is therefore the result of immunization of the DH270 UCA KI mice with the CH848 10.17DT Env.

Structural comparison of DH270.mu1 and DH270.6

To provide information on the interaction between the nanoparticle-induced antibody DH270.mu1 (containing two key improbable mutations, G57R and S27Y) and the 10.17DT Env, we determined the structure of the DH270.mu1 Fab in complex with 10.17DT SOSIP trimer by cryo-EM to an overall resolution of 3.5 Å. We also determined the structure of the affinity-matured V3-glycan bnAb DH270.6 (18) in complex with CH848 10.17DT SOSIP to an overall resolution of 4.3 Å (Fig. 4, A and B, figs. S21 and S22, and table S1). By comparing the DH270.mu1 complex to the DH270 UCA and the matured DH270.6 bnAb complexes, we were able to assess the structural evolution of the vaccine-induced DH270.mu1 antibody. DH270 UCA, DH270.mu1, and DH270.6 all bound at a 1:1 Env protomer/Fab ratio with similar overall orientations (Fig. 4, A to D, and figs. S6, S21, and S22). All three antibodies used the 20-amino acid HCDR3 to contact the base of the V3 loop by reaching past the N332 glycan (Fig. 1E and Fig. 4, C and D). The most striking difference was observed in the conformation of the V1 loop (Fig. 1E and Fig. 4, C to H). In the DH270 UCA-bound complex, the tip of the short V1 loop was oriented toward the antibody paratope with the HCDR2 loop making van der Waals contacts with the V1 tip (Fig. 1, E, G, and H, and Fig. 4E). In contrast, the V1 loop in the DH270.6-bound complex was positioned roughly perpendicular to the antibody paratope, with the tip of the V1 loop separated by about 14 Å between the DH270 UCA-bound and DH270.6-bound conformations (Fig. 4, E to H). The V1 loop in the DH270.mu1-bound complex adopted a conformation similar to that in the DH270.6-bound complex, and distinct from the V1 loop conformation observed in the DH270 UCA-bound complex (Fig. 4, E to H). The altered disposition of the V1 loop, and its movement away from the HCDR2 loop in the DH270.6 and DH270.mu1-bound complexes, allowed access to the conserved GDIK motif in the V3 loop by the improbable mutation G57R, via hydrogen bonds between the side chain of Arg⁵⁷ of the HCDR2 loop and the main-chain carbonyl of residue Gly³²⁴ in the GDI(R/K) motif of the V3 loop (Fig. 4, G and H, and figs. S21B and S22B). We also examined the LCDR1 loop, which is the site of the improbable S27Y tyrosine mutation that

was acquired in both the mature DH270.6 bnAb and the elicited DH270.mu1 antibody. The tyrosine substitution facilitated an interaction with the glycan 301 (Fig. 4, I to K). Thus, the three cryo-EM structures revealed that the V1 loop lacking N133 and N138 glycans was conformationally flexible, adopted two distinct conformations when bound to the DH270 UCA versus the DH270.6 mature bnAb, and, when bound to the elicited DH270.mu1 antibody, resembled the V1 loop conformation in the DH270.6-bound complex.

Induction of precursors of broad and potent CD4 binding site CH235-class neutralizing antibodies with improbable mutations

To generalize the selection of improbable mutations to additional bnAb B cell lineages, we designed immunogens for the CD4bs bnAb lineage CH235 that was derived from the CH505 HIV-1-infected individual (22). The CH235-lineage bnAbs have many improbable mutations, including a functional improbable mutation, K19T in V_H (10). The K19T mutation is sufficient on its own to expand neutralization breadth against autologous CH505 viruses by the CH235 UCA (Fig. 5A). However, only low-affinity autologous envelopes have been shown to bind to the CH235 UCA (fig. S25A) (23). In recent work, LaBranche and colleagues showed that the CH505 M5 envelope with a G458Y (M5.G458Y) mutation was bound by the CH235 UCA with high affinity (52). The apparent affinity was improved by a factor of 9 by generating the envelope in GnTI^{-/-} cells, which restricted glycan processing beyond Man₅GlcNAc₂ (Man, mannose; GlcNAc, *N*-acetylglucosamine) (52). M5.G458Y-stabilized SOSIP gp140 bound with an apparent affinity of 9 nM for the CH235 UCA and 0.1 nM for the CH235 early bnAb that included the K19T change (52). Thus, we identified a high-affinity Env immunogen that had the potential to select for the critical K19T change.

Generation and characterization of CH235 UCA knock-in mice

To evaluate immunogen selection of the K19T mutation, we generated CH235 UCA heterozygous (V_H^{+/-}, V_L^{+/-}) KI mice in the same way as the DH270 UCA model. In this mouse line, the ratio of splenic B cells versus T cells was close to normal (fig. S23A). The IgM/IgD profile of splenic B cells was comparable to that in control mice, with elevation of the IgM⁺IgD^{lo} population by less than a factor of 2 (figs. S23B and S24C). There was no detectable population of surface Ig^{lo} B cells, which are usually anergic. About 30% of B cells expressed IgM^a, the IgH allotype associated with CH235 UCA V_H, and nearly 60% of B cells expressed IgM^b from the IgH^b allele (fig. S23C). Again, as with the DH270 KI model, the CH235 UCA V_H was presumably deleted through V_H replacement in IgM^{b+} B cells. With regard to light chain expression, the proportion of Igλ⁺ B cells (2%) was below normal. The profile suggests that the KI V_L inhibited the expression of endogenous mouse light chain and was not subject to active deletion by receptor editing. Together, these data suggest that expression of CH235 UCA is subject to negative selection at the heavy chain level, likely at the B cell progenitor stage, where V_H replacement takes place. B cells that have passed through this initial hurdle progress normally into mature B cells in peripheral lymphoid tissues.

Heterozygous ($V_h^{+/-}$, $V_l^{+/-}$) CH235 UCA KI mice were immunized five times with M5.G458Y-stabilized SOSIP trimer glycosylated with heterogeneous glycans or enriched for $\text{Man}_5\text{GlcNAc}_2$ glycans (Fig. 5B). In this model, approximately 10% of follicular B cells bind the CH505 M5.G458Y envelope, making it a relatively stringent model for testing engagement of bnAb precursors (fig. S24). $\text{Man}_5\text{GlcNAc}_2$ -enriched and heterogeneously glycosylated M5.G458Y Env trimer elicited comparable serum neutralizing antibody titers (Fig. 5C). Serum neutralization could be knocked out by a N280D mutation indicative of a CD4bs epitope-targeting response (Fig. 5C). Serum antibodies from either group of trimer-immunized mice were capable of neutralizing the tier 2 autologous CH505 M5 virus (fig. S25B), which is resistant to the CH235 UCA, suggesting maturation of the CH235 antibody response (Fig. 5A).

By next-generation sequencing of the CH235 V_H region, we determined that M5 G458Y trimer-immunized mice generated CH235 sequences with the K19T improbable mutation (Fig. 5D). To determine the effects of immunogen affinity on K19T selection, we immunized CH235 UCA mice with low-affinity CH505 M5 gp120 that bound to the UCA with 4 μM affinity (fig. S25A). The highest-affinity immunogen elicited the highest frequency of K19T-encoding antibodies, and the lowest-affinity immunogen elicited the lowest frequency of K19T-encoding antibodies (Fig. 5, D and E). To determine the function of K19T-encoding antibodies, we isolated 57 CH235 monoclonal antibodies by sorting antigen-specific B cells from M5.G458Y trimer-immunized mice, 86% of which were somatically mutated (Fig. 5F). Three antibodies (termed CH235.mu1, 2, and 3) possessed the K19T mutation (Fig. 5G and figs. S25 and S26). In addition to the K19T mutation, CH235.mu1 acquired the improbable somatic mutation D99N, which is also present in CH235 (figs. S25 and S26). These three vaccine-induced antibodies more potently neutralized $\text{Man}_5\text{GlcNAc}_2$ -enriched or heterogeneously glycosylated M5.G458Y virus than did the UCA (Fig. 5H and fig. S25D). These antibodies were specific for the CD4bs, as judged by their sensitivity to a N280D Env mutation (Fig. 5H). Vaccine-induced antibodies also neutralized heterogeneously glycosylated CH505 M5 and $\text{Man}_5\text{GlcNAc}_2$ -enriched CH505 TF, which were both resistant to CH235 UCA neutralization (Fig. 5H, right). Therefore, vaccination stimulated these somatically mutated antibodies to progress beyond the neutralization phenotype of the CH235 UCA. Thus, we have demonstrated that the design of UCA-targeting Envs that bind with high affinity to B cells bearing key improbable mutations can overcome rare mutations as a maturational bottleneck in both V3-glycan and CD4bs bnAb B cell lineages.

Induction of potent neutralizing CD4bs antibodies in macaques

We immunized rhesus macaques to determine whether $\text{Man}_5\text{GlcNAc}_2$ -enriched M5.G458Y SOSIP gp140 could elicit CH235-like CD4 binding site antibodies in outbred primates with wild-type antibody repertoires. Given previous successes of Env nanoparticle immunogens in macaques (15, 39) and in the DH270 UCA KI mouse studies performed here, we generated a nanoparticle displaying the $\text{Man}_5\text{GlcNAc}_2$ -enriched M5.G458Y SOSIP gp140 (Fig. 6A) using the two-step conjugation method described above (fig. S11). Macaques were immunized three times with M5.G458Y SOSIP gp140 nanoparticles formulated in the adjuvant 3M-052 in stable emulsion (Fig. 6B). Plasma IgG-binding antibodies to the $\text{Man}_5\text{GlcNAc}_2$ -enriched M5.G458Y SOSIP gp140 were detectable after a single

immunization and continued to increase with the two subsequent boosts (Fig. 6C). Serum autologous tier 2 neutralizing antibodies against Man₅GlcNAc₂-enriched M5.G458Y virus were detected after the second immunization and were boosted by the third immunization (Fig. 6D). In previous studies, the elicitation of autologous tier 2 neutralizing antibodies in all vaccinated animals has been difficult to achieve, requiring osmotic pumps and fractionated immunizations over short time intervals (53, 54). However, Man₅GlcNAc₂-enriched M5.G458Y nanoparticles successfully elicited potent neutralizing antibodies in all four macaques with two monthly immunizations (Fig. 6, D to H). Neutralizing antibodies were not restricted to Man₅GlcNAc₂ enrichment, as the heterogeneously glycosylated M5.G458Y virus produced in 293T cells was potently neutralized by all four animals also (Fig. 6E).

Next, we examined whether the neutralizing antibodies in the macaque serum had the same neutralization signature as CH235 antibodies early in bnAb development. The CH235 UCA plus the K19T mutation potently neutralized M5.G458Y, weakly neutralized M5, and showed undetectable neutralization toward CH505 TF virus (Fig. 5A). The macaque serum showed a similar neutralization signature, as the CH235 UCA plus the K19T mutation in that the serum neutralized Man₅GlcNAc₂-enriched M5.G458Y potently, M5 moderately, and CH505 TF weakly (Fig. 6, F and G). To map the neutralizing antibodies to the same CD4bs epitope as CH235, we examined neutralization of M5.G458Y virus with a knockout mutation in the CD4bs at position 280 (N280D), a knockout mutation characteristic of CH235-class bnAb precursors (52). After two and three immunizations, all four macaques generated N280-dependent neutralizing antibodies (Fig. 6, F and G). As a group, the macaques showed a factor of 34 decrease (range: >9 to 114) in serum neutralization potency against Man₅GlcNAc₂-enriched M5.G458Y when the CH235 epitope was eliminated (Fig. 6, H and I). Thus, the CD4bs immunogen that is capable of selecting improbable mutations in the CH235 lineage elicited CD4bs serum neutralizing antibodies in primates with neutralization signatures similar to that of the CH235 bnAb.

Concluding remarks

Improbable somatic mutations in HIV-1 bnAb variable regions are often required for their neutralization activity and represent obstacles to the induction of bnAbs by vaccination (10). We have shown that immunogens can be designed to select for specific antibody nucleotide mutations in the setting of vaccination. Our immunogen design approach was to design an immunogen that could bind to the initial precursor of the bnAb lineage with sufficient affinity to activate it, and could bind with higher affinity to the affinity maturation intermediates with improbable mutations. Our results show that the immune system, when provided with an affinity gradient between two clonally related antibodies, can be manipulated to generate HIV-1 bnAb improbable mutations. In addition to the CH235 and DH270 bnAb lineages, we envision that this vaccine design strategy will be applicable to other HIV-1 bnAb lineages.

We succeeded in eliciting CD4bs neutralizing antibodies in primates with a neutralization phenotype similar to CH235 intermediate antibodies. The next step for vaccine design will be to guide these antibodies to develop neutralization breadth. Unlike bnAbs such as

PGT121 or VRC01, the development of the two bnAb lineages investigated here is not limited by rare insertion or deletion events. Instead, these antibodies encode improbable nucleotide mutations needed for neutralization activity (18, 22, 23). Thus, we hypothesize that the elicitation of DH270 V3-glycan or CH235 CD4bs types of bnAbs will require a mutation-guided vaccine design approach that specifically selects for improbable mutations (10). Among the 26 bnAbs for which we analyzed the number of improbable mutations, CH235.12 had the most V_H improbable mutations (10). Although the CH505 M5 G458Y Env trimer selected for K19T at a sufficiently high frequency for isolation of monoclonal antibodies encoding this mutation, the functionally important W47L improbable mutation (10) was not elicited with the current immunogen. The absence of the W47L mutation in the 57 monoclonal antibodies demonstrated that immunogen engagement of the UCA is a required step to initiate affinity maturation of the antibody lineage, but engagement alone is not sufficient to select for all improbable mutations. Not all amino acids encoded by improbable nucleotide mutations are required for neutralization breadth. Thus, defining the amino acids required for neutralization breadth comparable to CH235.12 or DH270.6 would provide a simplified maturation pathway for vaccine-induced antibody responses. This functionally required subset of mutations should be inducible with a vaccine, given our observation that the 134 vaccine-induced single DH270 antibodies encoded 16 of 19 V_H mutations present in the DH270.1 mature bnAb—including the four improbable mutations of Arg¹⁹, Arg⁵⁷, Thr⁹⁷, and Thr⁹⁸ (Fig. 3G and figs. S18 and S20). All 16 mutations did not occur on the same antibody; rather, they were scattered among the 134 antibodies (figs. S16, S18, and S19). Without proper antigen selection, it will take multiple decades of vaccination to elicit a DH270-like bnAb. This timeline could be accelerated by the design of sequential immunogens that select for the required combination of functional antibody somatic mutations (13, 14, 55, 56).

It is important to consider the status of glycans on 293 cell line–produced Envs that we have used as immunogens. In Go *et al.*, multiple stabilized Envs forms were produced in 293, CHO, or CD4 T cell lines and their site-specific glycans were profiled (57, 58). Remarkable similarity was found among all SOSIP trimers regardless of the cell line used for Env expression (57, 58). Comparison of virion Env and recombinant SOSIP trimer glycans has also demonstrated glycosylation similarities (59). Thus, both 293 cell–produced and CHO-produced stabilized trimers have glycosylation patterns of predominant high-mannose glycans similar to those found on native virion Env. Evaluation of the CH848 10.17DT immunogen used in this study was consistent with these published glycosylation patterns.

Our study emphasizes the difficult nature of induction of broadly neutralizing antibodies, in that the design of sequential immunogens must be precise, and the simultaneous use of sequential immunogens for multiple bnAb types also runs the risk of leading some bnAb B cell lineages off track. Thus, multiple rounds of iterative immunogen design will be needed to develop complex sequential immunogens that can induce a polyclonal bnAb response.

Finally, this study demonstrates pro-of-of-concept for targeted selection of improbable mutations to improve antibody affinity maturation. Although HIV-1 was the focus of this study, this strategy of selection of specific antibody nucleotides by immunogen design can

be applied to B cell lineages targeting other pathogens where guided affinity maturation is needed for a protective antibody response.

Methods

Animals and immunizations

DH270 UCA V_H and V_L KI mice were generated by introducing rearranged $V_H(D)J_H$ and $V_\lambda J_\lambda$ exons into mouse J_H and J_κ loci of an F_1 (129/Sv $\times$ C57BL/6) ES cell line, respectively. The IgH alleles from the two mouse strains are distinguishable by allotypic difference. The DH270 UCA $V_H(D)J_H$ exon was integrated via homologous recombination into the J_H region of the IgH allele (IgH^a) from 129/Sv mouse strain (fig. S7A). The IgH^b allele from C57BL/6 strain was not modified. The integration replaces a 2255-bp region containing mouse J_H1-4 (starting from 877 bp upstream of J_H1 to 57 bp downstream of J_H4) with an expression cassette consisting of a promoter upstream of mouse V_H81X segment and rearranged $V_H(D)J_H$ exon encoding the V_H of DH270 UCA. Similarly, the DH270 UCA $V_\lambda J_\lambda$ exon was introduced into the J_κ region of one Ig κ allele. The other Ig κ allele was not altered and the two Ig κ alleles are not distinguishable by allotypic difference. The V_L KI replaces a 1762-bp region containing mouse $J_\kappa1-5$ (starting from 114 bp upstream of $J_\kappa1$ to 286 bp downstream of $J_\kappa5$) with an expression cassette consisting of a promoter upstream of mouse $V_\kappa3-1$ segment. Correct V_H and V_L KI have been verified with Southern blotting. The ES clones were injected into Rag2 deficient blastocysts to generate chimeric mice, which were subsequently bred with 129/Sv mice for germline transmission. Germline mice were used for the immunization experiments. The CH235 UCA V_H and V_L KI mice were generated in the same way.

DH270 UCA heterozygous heavy chain variable and light chain variable region double KI mice ($V_HDJ_H^{+/-}$, $V_\lambda J_\lambda^{+/-}$ KI strains) were immunized six times 2 weeks apart with 25 μ g of protein immunogens with 5 μ g of the TLR4 agonist-based, IDRI proprietary adjuvant system GLA-SE. Immunizations were performed via intramuscular injection (200 μ l). Heterozygous CH235 UCA ($V_HDJ_H^{+/-}$, $V_\kappa J_\kappa^{+/-}$) KI mice were immunized similarly. Control groups were administered in parallel with 5 μ g of GLA-SE (adjuvant only). Blood samples were collected 10 days after each immunization for immune profiling.

Indian-origin rhesus macaques were housed and treated in AAALAC-accredited institutions. The study protocol and all veterinarian procedures were approved by the Duke University IACUC and were performed based on standard operating procedures developed by Bioqual (Rockville, MD). Macaques were immunized intramuscularly in the quadriceps every 4 weeks. Each macaque was administered 100 μ g of CH505 M5.G458Y SOSIP gp140 ferritin nanoparticles adjuvanted with 30 μ g of 3M-052 stable emulsion. 3M-052 stable emulsion was formulated as described (60). Whole blood and serum were drawn on the day of vaccination and 2 weeks after each immunization to examine immune responses.

Recombinant antibody and Fab production

Antibody and Fab was produced as described (61, 62). Briefly, recombinant proteins were expressed in Expi293 cells (Life Technologies) by transient transfection with Expifectamine

(Invitrogen). Five days after transfection, cell culture media was cleared of cells and protein A (ThermoFisher) or KappaSelect (GE Healthcare) affinity chromatography was used to purify IgG or Fab, respectively. Purified protein was buffer exchanged into PBS with successive rounds of centrifugation, filtered, and stored at -80°C .

Recombinant SOSIP envelope production

HIV-1 Env SOSIP gp140 trimers were designed as chimeric SOSIP trimers (62). CH848 SOSIP gp140 trimers were stabilized with a 201C-433C disulfide bond (24). CH505 SOSIPs were stabilized with E64K and A316W mutations (25). Freestyle 293 (Life Technologies) cells were cultured in Freestyle 293 media below 3×10^6 cells ml^{-1} . On the day of transfection, cells were diluted to 1.25×10^6 cells ml^{-1} with fresh media and 1 liter of cells was transfected with 293Fectin (Life Technologies) complexed with 650 μg of envelope-expressing DNA and 150 μg of furin-expressing plasmid DNA. Cells were cultured for 6 days in shaker flasks. Cell culture supernatant was cleared of cells by centrifugation for 30 min at 3500 rpm and subsequently 0.8- μm filtered. The cell-free supernatant was concentrated to less than 100 ml with a single-use tangential flow filtration cassette and 0.8- μm filtered again. Trimeric Env protein was purified with PGT145 affinity chromatography. PGT145 IgG1 antibody (100 mg) was conjugated to 10 ml of CnBr-activated sepharose FastFlow resin (GE Healthcare). Coupled resin was packed into a Tricorn column (GE Healthcare) and stored in PBS supplemented with 0.05% sodium azide. Cell-free supernatant was applied to the column at 2 ml/min in PBS supplemented with 0.05% sodium azide using an AKTA Pure (GE Healthcare). The column was washed, and protein was eluted off of the column with 3 M MgCl_2 . The eluate was immediately diluted in 10 mM Tris pH 8, 0.2- μm filtered, and concentrated down to 2 ml for size-exclusion chromatography. To produce biotinylated CH0848 10.17DT SOSIP gp140s, the envelope sequence was expressed with a C-terminal avidin tag (AviTag: GLNDIFEAQKIEWHE). After antibody affinity chromatography and eluate concentration, the envelope was dialyzed for 1 hour in 10 mM Tris pH 8. Envelope was biotinylated with the BirA biotin-protein ligase standard reaction kit (Avidity). The ligation reaction was done by agitating 25 μM of SOSIP trimer at 900 rpm at 30°C for 5 hours. The biotinylated protein was then concentrated to 2 ml for size-exclusion chromatography. Size-exclusion chromatography was performed with a Superose6 16/600 column (GE Healthcare) in 10 mM Tris pH 8, 500 mM NaCl. Fractions containing trimeric HIV-1 Env protein were pooled together, sterile-filtered, snapfrozen, and stored at -80°C .

Deglycosylation of CH848 10.17DT SOSIP Envs

Env samples were either partially or fully deglycosylated depending on the type of analysis. For disulfide analysis, samples containing 10 μg of the CH848 10.17DT SOSIP Envs were alkylated with a 10-fold molar excess of 4-vinylpyridine in the dark for 1 hour at room temperature (RT) to cap any potential free cysteine residues. Alkylated Env samples were subsequently deglycosylated with 500 U of PNGase F in 100 μl of 50 mM ammonium citrate buffer (pH 6.5) for 1 week at 37°C . The fully deglycosylated and alkylated samples were digested overnight with trypsin (protein/enzyme ratio of 30:1) at 37°C . For glycosylation analysis, samples containing 25 μg of the CH848 10.17DT SOSIP Envs were incubated with Endo H (2.5 μl , 5 units ml^{-1}) for 48 hours at 37°C in 50 mM ammonium

acetate buffer, pH 5.5. Following Endo-H treatment, Env samples were digested as described below.

Proteolytic digestion of CH848 10.17DT SOSIP Envs for glycosylation analysis

CH848 10.17DT SOSIP Envs (25 µg) were denatured with 7 M urea in 100 mM Tris buffer (pH 8.0), reduced at RT for 1 hour with TCEP (5 mM), and alkylated with 20 mM IAM at RT for another hour in the dark. The reduced and alkylated samples were buffer-exchanged using a 50-kDa MWCO filter (Millipore) prior to protease digestion. Digestion was performed using trypsin alone and a combination of trypsin and chymotrypsin at a 30:1 protein/enzyme ratio. Samples were incubated overnight at 37°C. The resulting Env digest was either directly analyzed or stored at -20°C until further analysis. To ensure reproducibility of the method, digestion was performed at least three times on different days with samples obtained from the same batch and analyzed with the same experimental procedure.

Chromatography and mass spectrometry

High-resolution LC/MS experiments were performed using an LTQ-Orbitrap Velos Pro (Thermo Scientific) mass spectrometer equipped with ETD coupled to an Acquity UPLC M-Class system (Waters). Mobile phases consisted of solvent A (99.9% deionized H₂O + 0.1% formic acid) and solvent B (99.9% CH₃CN + 0.1% formic acid). A 5-µl aliquot of the sample (~1.5 µM) was injected onto C18 PepMap 300 column (300 µm i.d. × 15 cm, 300 Å, Thermo Fisher Scientific) at a flow rate of 4 µl/min. The following CH₃CN/H₂O multistep gradient was used: 3% B for 5 min, followed a linear increase to 40% B in 50 min, then a linear increase to 90% B in 15 min. The column was held at 97% B for 10 min before reequilibration. All mass spectrometric analysis was performed in the positive ion mode using data-dependent acquisition mode: The five most intense ions in the survey scan in the mass range 400 to 2000 *m/z* were selected for alternating CID and ETD in the LTQ linear ion trap using a normalized collision energy of 30% for CID and an ion-ion reaction time of 100 to 150 ms for ETD. Full MS scans were measured at a resolution (*R*) of 30,000 at *m/z* 400. Under these conditions, the measured *R* (FWHM) in the orbitrap mass analyzer is 20,000 at *m/z* 1000 and 17,000 at *m/z* 1500.

Glycopeptide Identification and disulfide bond analysis

Details of the glycopeptide compositional analysis have been described (63–65). Briefly, compositional analysis of glycopeptides was carried out by first identifying the peptide portion from tandem MS data. Once the peptide portion was determined, plausible glycopeptide compositions were obtained using the high-resolution MS data and GlycoPep DB. The putative glycopeptide composition was confirmed manually from CID and ETD data.

Disulfide bond patterns of CH848 10.17DT SOSIP Envs were determined by mapping the disulfide-linked peptides. Data analysis was performed using Mascot (v 2.5.1) search engine for peptides containing free cysteine residues and disulfide bond patterns were analyzed manually as described (66, 67).

In vitro HIV-1 neutralization

Antibody-mediated HIV-1 neutralization was measured using Tat-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus replication in TZM-bl cells as described (68). TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by J. Kappes and X. Wu. The monoclonal antibody or serum was pre-incubated with virus (~150,000 relative light unit equivalents) for 1 hour at 37°C, and TZM-bl cells were subsequently added. After 48 hours, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the antibody concentration in µg/ml or serum reciprocal dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells (IC₅₀ and ID₅₀, respectively).

Surface plasmon resonance (SPR)

SPR experiments were performed on a BIAcore T200. To determine apparent affinities, approximately 300 RU (range 310 to 321 RU) of each antibody was captured on an anti-human IgGc immobilized Series S CM5 sensor chip (GE Healthcare). Serial dilutions of SOSIP Env was flowed over immobilized antibody in HEPES-buffered saline. To determine binding affinity, biotinylated SOSIP gp140 was immobilized on streptavidin-coated sensor chips. Serial dilutions of antibody Fab were flowed over the Env. Each concentration of Env was flowed over each immobilized antibody for 120 s and dissociation was measured for 600 s. In between injections of each Env concentration, the surface was regenerated by injecting glycine pH 2 for 30 s. Binding rate constants (k_a , k_d) were measured after global curve fitting to a Langmuir model. Curve fitting analysis was performed with BiaEvaluation software (GE Healthcare) using a 1:1 Langmuir model, or a heterogeneous binding model when appropriate to derive rate (k_a , k_d) and apparent or true equilibrium dissociation constants (K_d).

Serum IgG ELISA binding titers

Avi-tag SOSIP capture or PGT151 SOSIP capture ELISAs were performed as described (69). Protein (2 µg ml⁻¹) in sodium bicarbonate buffer was incubated in sealed Nunc-absorb (ThermoFisher) plates overnight at 4°C. Unbound protein was washed away and the plates were blocked with SuperBlock for 1 hour. Serial dilution of serum or monoclonal antibodies were added to the plate for 90 min. Binding antibodies were detected with HRP-labeled anti-IgG Fc antibody. HRP was detected with 3,3',5,5'-tetramethylbenzidine. Binding titers were analyzed as area-under-curve of the log-transformed concentrations.

Serum IgG and monoclonal antibody competition ELISAs

Serum IgG or monoclonal antibody competition assays were performed as described (61, 70). In brief, Nunc-sorp plates were coated with HIV-1 envelope, washed and blocked as stated above for direct ELISAs. After blocking was complete, mouse serum was diluted in SuperBlock at a 1:50 dilution and incubated in triplicate wells for 90 min. For monoclonal antibody competitors, a dilution series starting at 100 µg ml⁻¹ was incubated in triplicate wells for 90 min. Non-biotinylated monoclonal antibodies were incubated with the Env in

triplicate as positive controls for blocking. To determine relative binding, no plasma or no antibody was added to a group of wells scattered throughout the plate. After 90 min, the non-biotinylated antibody or plasma was washed away, and biotinylated monoclonal antibodies were incubated in the wells for 1 hour at sub-saturating concentrations. Each well was washed, and binding of biotinylated monoclonal antibodies was determined with a 1:8000 dilution of horseradish peroxidase (HRP)–conjugated streptavidin. HRP was detected with tetramethylbenzidine and stopped with 1% HCl. The absorbance at 450 nm of each well was read with a Spectramax plate reader (Molecular Devices). Binding of the biotinylated monoclonal antibody to HIV-1 envelope in the absence of plasma was compared to in the presence of plasma to calculate percent inhibition of binding. Based on historical negative controls, assays were considered valid if the positive control antibodies blocked greater than 20% of the biotinylated antibody binding.

Sortase A conjugation of HIV-1 envelope trimers to ferritin nanoparticles

CH848 10.17DT SOSIP gp140 was expressed with amino acids LPSTGG encoded at its C terminus. The CH848 10.17DT SOSIP was expressed in Freestyle293 cells and purified by affinity chromatography with broadly neutralizing trimer-specific antibody PGT151 as stated above. Trimeric gp140 was isolated by size-exclusion chromatography using a Superose6 16_60 column. Ferritin particles were expressed with a (GGGGG)₃ repeat sequence encoded at the N terminus of each subunit. For affinity purification of ferritin particles, 6×His tags were appended to the C terminus. CH848 SOSIP with a C-terminal sortase A tag and ferritin particles with a sortase A N-terminal tag were buffer-exchanged into 50 mM Tris, 150 mM NaCl, 5 mM CaCl₂, pH 7.5. 120 μM SOSIP gp140 was mixed with 120 μM ferritin subunits and incubated with 100 μM sortase A overnight at room temperature. After incubation, conjugated particles were isolated from free ferritin or free SOSIP gp140 by size-exclusion chromatography using a Superose6 16_60 column.

Antibody binding to HIV-1 envelope by biolayer interferometry (BLI)

Biolayer interferometry was performed as described (71). Antibodies were immobilized to anti-Fc sensor tips and incubated with 50 μg ml⁻¹ of Env SOSIP gp140-nanoparticle or SOSIP trimer solutions for 400 s. To compare binding profiles between Env SOSIP gp140 and Env SOSIP gp140 nanoparticles, BLI binding values were normalized to PGT151 binding response by dividing each antibody binding response value by the PGT151 binding response value.

Antigen-specific fluorescence-activated single B cell sorting

To sort Env-specific memory B cells, splenocytes were incubated with the following fluorochrome-antibody conjugates (all from BD Biosciences): FITC anti-mouse IgG1 (A85–1), FITC anti-mouse IgG2a/2b (R2–40), FITC anti-mouse IgG3 (R40–82), PE anti-mouse GL7 (GL7), PE-Cy7 anti-mouse IgM (R6–60.2), AlexaFluor700 anti-mouse CD19 (1D3), BV510 anti-mouse IgD (11–26C.2a), and BV650 anti-mouse B220 (RA3–6B2). Cells were also labeled with BV421 and AF647-conjugated SOSIP trimers (CH848 10.17) to identify Env-specific memory B cells. Env-specific memory B cells were identified as viable B220⁺CD19⁺IgM[–]IgD[–]GL7[–]IgG^{1/2/3}⁺ cells that bound both BV421- and AF647-conjugated SOSIP trimers. In some cases, the BV421-SOSIP contained the N332A mutation; in these

samples Env-specific memory B cells were identified as those binding the AF647-CH848 10.17 wild-type SOSIP but not the BV421-N332A SOSIP mutant. Single cells were sorted on a BD FACS Aria II into wells of 96-well PCR plates containing lysis buffer. Plates were immediately frozen and stored at -80°C .

Antibody cloning by PCR

Ig genes were amplified as described, with some modifications (72, 73). Ig genes from a single B cell were reverse-transcribed with Superscript III (ThermoFisher) using random hexamer oligonucleotides as primers. The complementary DNA was used to perform nested PCR for heavy and light chain genes using AmpliTaq gold (ThermoFisher). In parallel, PCR reactions were done with mouse Ig-specific primers and DH270 variable region-specific primers. In one reaction, mouse variable region and mouse constant region primers were used. In a separate reaction, human variable region and mouse constant region primers were used. Positive PCR amplification of Ig genes was identified by gel electrophoresis. Positive PCR reactions were purified using the PCR clean-up kit (Qiagen). The purified PCR amplicon was sequenced with 4 μM of forward and reverse primers. Contigs of the PCR amplicon sequence were made, and genes were inferred with the human library and the mouse library in Cloanlyst (74). Antibody genes were categorized as human or mouse according to which species had the closest sequence identity. A second aliquot of the purified PCR amplicon was used for overlapping PCR to generate a linear expression cassette. The expression cassette was transfected with Effectene (Qiagen) into 293T cells. The supernatant containing recombinant antibodies was cleared of cells by centrifugation and used for binding assays. The genes of selected heavy chains were synthesized as IgG1 (GenScript). Kappa and lambda chains were synthesized similarly. Plasmids were prepared for transient transfection using the Megaprep plasmid plus kit (Qiagen).

B cell receptor–dependent calcium flux in Ramos B cells

Ramos B cell calcium flux was measured as described (75). Protein tetramers were formed at a 4:1 molar ratio of protein to streptavidin (Invitrogen). Ramos cell lines stably expressing DH270 UCA, DH270.1, or CH65 IgM (76) were passaged (1:10) 4 days before calcium flux experiments. On the day of the experiment, cells with $>95\%$ viability were resuspended at 10^6 cells ml^{-1} in 2:1 ratio of RPMI media (GIBCO) + FLIPR Calcium 6 dye (Molecular Devices). Cells were plated in a U-bottom 96-well tissue culture plate (Costar) and incubated at 37°C $5\% \text{CO}_2$ for 2 hours. In a black clear-bottom 96-well plate (Costar) containing 50 μl of RPMI media (GIBCO) + FLIPR Calcium 6 dye (Molecular Devices) (2:1 ratio) either 0.1 nmol of proteins or 50 $\mu\text{g ml}^{-1}$ of anti-human IgM F(ab')₂ (Jackson Immuno) were added (based on a 100- μl volume). Using a FlexStation 3 multimode microplate reader (Molecular Devices), 50 μl of supernatant containing cells were transferred into the 50 μl of media containing protein or anti-human IgM F(ab')₂ (Jackson Immuno) and continuously read for 5 min. Relative fluorescent value units were background-subtracted and the data expressed as percentage of the IgM maximum signal (% IgM max).

B cell receptor–dependent calcium flux in mouse B cells

Calcium flux in murine B cells was evaluated as described (56). For experiments evaluating total splenic B cells, WT BL/6 and DH270 UCA double V_HDJ_h homozygous and V_LJ_L homozygous KI splenocytes were collected, and total B cells were enriched using a mouse Pan-B cell isolation kit (Stemcell) according to manufacturer's instructions. Enriched Pan-B cells were stained by LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific) for 30 min. For experiments evaluating splenic B cell subsets, single-cell suspensions were directly stained with cell surface marker combinations for spleen subfractionation into transitional-B and mature-B subsets including $0.5 \mu\text{g ml}^{-1}$ of anti-B220-BV786 (catalog #563894), anti-CD19 APCR700 (catalog #565473), and anti-CD93 BV650 (catalog #563807). For both sets of experiments, prestained B cells were loaded with Fluo-4 via thorough washes in HBSS, followed by mixing with equal volumes of $2\times$ Fluo-4 Direct calcium reagent loading solution (Fluo-4 Direct Calcium Assay Kits, ThermoFisher Scientific). After sequential 30-min incubations at 37°C and room temperature, cells were washed and incubated with LIVE/DEAD staining buffer for 30 min and resuspended in calcium-containing HBSS and incubated at room temperature for 5 min, before being activated by $25 \mu\text{g ml}^{-1}$ anti-IgM F(ab')₂ (Southern Biotech). Fluo-4 data for B cells were acquired on a BD LSR II flow cytometer and analyzed by FlowJo software.

T cell and B cell phenotyping of murine splenocytes

To analyze B cell phenotypes in the DH270 UCA and CH235 UCA $V_H + V_L$ KI mice, spleens were dissected from the mice and dissociated by grinding into cell suspension by mechanical disruption with a syringe plunger on a $70\text{-}\mu\text{m}$ cell strainer. Single-cell suspensions of mouse spleens were prepared then treated with ammonium chloride lysis solution or with red blood cell lysing buffer (Sigma R7757) to lyse red blood cells. Cells were counted on a Countess (ThermoFisher). For enumeration of germinal center B cells, cells were incubated with optimal concentrations of the following fluorochrome-antibody conjugates (all from BD Biosciences): PE anti-mouse GL7 (GL7), PE-CF594 anti-mouse CD93 (AA4.1), AlexaFluor700 anti-mouse CD19 (1D3), BV605 anti-mouse CD95 (Jo2), BV650 anti-mouse B220 (RA3–6B2), and BV711 anti-mouse CD138 (281–2). Cells were subsequently labeled with Live/Dead Fixable Near-IR Dead Cell Stain (ThermoFisher) to allow exclusion of dead cells from analysis. Germinal center B cells were identified as viable $\text{CD138}^-\text{B220}^+\text{CD19}^+\text{CD93}^-\text{GL7}^+\text{CD95}^+$ cells. To enumerate Tfh cells, splenocytes were stained with the following antibody conjugates (all from BD Biosciences or Biolegend): FITC anti-mouse CD4 (RM4–5), PE anti-mouse CD25 (7D4), PE-CF594 anti-mouse PD1 (J43), PE-Cy5 anti-mouse TER119 (TER119), PE-Cy7 anti-mouse CD62L, Biotin anti-mouse CXCR5 (2G8), AlexaFluor700 anti-mouse CD8 (53–6.7), BV421 anti-mouse CD127 (SB/199), BV510 anti-mouse CD3 (145–2C11), BV570 anti-mouse CD11b (M1/70), BV650 anti-mouse NK1.1 (PK136), BV711 anti-mouse CD44 (IM7), and BV786 anti-mouse B220 (RA3–6B2). Tfh cells were identified as viable $\text{TER119}^-\text{B220}^-\text{NK1.1}^-\text{CD3}^+\text{CD4}^+\text{CD8}^-\text{CD62L}^-\text{CD44}^+\text{CD25}^-\text{PD1}^+\text{CXCR5}^+$ cells. After cell labeling, cells were fixed in 2% formaldehyde. Cells were acquired on a BD LSR II cytometer and analyzed with FlowJo version 10 software. To generate the data shown in figs. S7 and S23, splenocytes were stained with the following antibodies: APC antiB220 (eBioscience 17–0452-83), PE anti-Thy1.2 (PharMingen 553006), PE anti-IgM (eBioscience

12–5790-83), PE anti-IgM^a (PharMingen 553517), PE anti-Ig λ (Biolegend 407308), FITC anti-IgD (BD PharMingen 553439), FITC anti-IgM^b (PharMingen 553520), FITC anti-Ig κ (1050–02) and viability dye, Sytox blue (Life Technologies S34857). The stained cells were analyzed on Attune NxT flow cytometer from Invitrogen and the data were analyzed with FlowJo10 software. During the analysis, a lymphocyte gate was drawn on FSC and SSC plot. Within this population, Sytox blue–negative live cells were gated. Single cells were gated on the next FSC-H and FSC-A plot. The staining pattern of these single cells are shown in figs. S7A and S23A B220⁺ B cells were further gated on single cells, and the staining patterns of B220⁺ B cells are shown in figs. S7, B to D, and S23, B to D.

Cryo-EM data collection and processing

To prepare Env complexes, CH848 10.17DT. SOSIP trimer at a final concentration of 1 mg ml^{−1} was incubated with 4- to 6-fold molar excess of the DH270 Fab fragments for 30 to 60 min. To prevent aggregation during vitrification, the sample was incubated in 0.085 mM dodecyl-maltoside (DDM). The specimen was vitrified by applying 2.5 μ l of sample to freshly plasma-cleaned Quantifoil R 1.2/13 300-mesh Cu holey carbon grids, allowing the sample to adsorb to the grid for 60 s, followed by blotting with filter paper and plunge-freezing into liquid ethane using the Leica EM GP cryo-plunger (Leica Microsystems) (20°C, >90% relative humidity). For the DH270 UCA and DH270.6 complexes, data were acquired using the EPU automated data-acquisition program. Images were collected on a Titan Krios (Thermo Fisher) operating at 300 keV equipped with a Falcon III direct electron detector operating in counting mode. For the DH270 UCA and DH270.6 complexes, 3383 and 2844 movies, respectively, were collected at a magnification of 75,000 $\times$ with a physical pixel size of 1.08 Å per pixel using a nominal defocus range of S1.25 to −3 μ m. Each movie (30 frames) was acquired using a dose rate of $\sim$ 0.8 e[−]/pixel/s and a total exposure of 42 e[−]/Å².

For the DH270.mu1 complex, data were acquired using the Gatan Latitude data collection software installed on a Titan Krios electron microscope operating at 300 kV and fitted with a Gatan K3 direct detection device operating in counting mode. We collected 3009 movies at a nominal magnification of 22,500 $\times$ with a physical pixel size of 1.07 Å per pixel using a nominal defocus range of −1.25 to −3 μ m. Each movie (60 frames) was acquired using a dose rate of $\sim$ 1.01 e[−]/pixel/s and a total exposure of 60.6 e[−]/Å².

For the DH270 UCA and DH270.6 complexes, motion correction and dose weighting were performed using MotionCor2 (77). For the DH270.mu1 complex, motion correction and dose weighting were performed using Unblur (78). CTF was estimated using CTFFIND4 (79). Particles were picked using the Laplacian-of-Gaussian function in RELION-3 (80). These particles were imported into cryoSparc v2 (81), 2D classification was performed, and selected 2D classes representing different views of the complex were used for template-based particle picking in cryoSparc. Following further 2D classifications to remove junk, ab initio reconstruction and classification was performed using C1 symmetry. A 3D class was identified with three antibody Fabs bound symmetrically to the HIV-1 Env trimer. This initial model was refined using C3 symmetry against the clean stack of particles. Overall map resolution was reported according to the FSC_{0.143} gold-standard criterion (82).

Cryo-EM model fitting

Fits of HIV-1 trimer and Fab to the cryo-EM reconstructed maps were performed using Chimera (www.rbvi.ucsf.edu/chimera) (83). BG505 SOSIP trimer structure (PDB ID 5YFL) was used for the trimer fits and the coordinates of DH270 UCA3 (PDB ID 5U15) and DH270.6 (PDB ID 5TQA) were used for fitting the Fab in the DH270 UCA and DH270.6 complex structures, respectively. The sequences were replaced with those of the CH848 10.17DT SOSIP trimer using Coot (84). The coordinates were further fit to the electron density first using Rosetta (85), followed by an iterative process of manual fitting using Coot and realspace refinement within Phenix (86). Molprobit (87) and EMRinger (88) were used to check geometry and evaluate structures at each iteration step. Figures were generated in UCSF Chimera and PyMOL (PyMOL Molecular Graphics System, Version 2.0; Schrödinger LLC). Map-fitting cross correlations were calculated using Fit-in-Map feature in UCSF Chimera. Local resolution of cryo-EM maps was determined using RELION.

Negative-stain electron microscopy of HIV-1 envelope

Electron microscopy was performed as described (62).

Differential scanning calorimetry

Envelope thermal denaturation profiles were determined as described (89). Envelope profiles were generated in HEPES-buffered saline (HBS; 10 mM HEPES, 150 mM NaCl pH 7.4) at concentrations ranging from 0.2 to 0.4 mg ml⁻¹ using the NanoDSC platform (TA instruments, New Castle, DE). The observed, irreversible denaturation profiles were buffer-subtracted, converted to molar heat capacity, baseline-corrected with a sixth-order polynomial, and fit with three Gaussian transition models using the NanoAnalyze software (TA Instruments). The primary transition temperature (T_m) is reported as the temperature at the maximum observed heat capacity.

High-throughput heavy chain variable region sequencing

RNA was extracted from total splenocytes post-sixth immunization using the Qiagen RNeasy Mini isolation kit (Qiagen) and used for reverse transcription with random hexamer primers for cDNA synthesis. After cDNA synthesis, the DH270 or CH235 UCA KI Ig genes were amplified by PCR using a forward primer that anneals in the IGHV1 leader sequence and two reverse primers that anneal in the mouse IgG1/2 and IgG3 constant region sequences to amplify all human encoding IGHV1 IgG sequences. All primers have leading sequences that match Illumina adapter sequences for Nextera amplification. A second PCR step was performed to add Nextera index sequencing adapters (Illumina) and libraries were purified and size-selected by AMPpure bead cleanup. Libraries were quantified by quantitative PCR using the KAPA SYBR FAST qPCR kit (KAPA Biosystems) and sequenced using the Illumina Miseq V2 2× 300-bp kit.

Antibody sequence analysis

NGS reads from immunized mice were assembled using FLASH (90), quality filtered using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and deduplicated and aligned to their respective UCA sequence using in-house-developed bioinformatics programs. NGS

reads were immunogenetically annotated using Cloanalyzer (91) and sequences that were deemed nonfunctional (out-of-frame, missing invariant residues or CDRs) were discarded from analysis. Probability of mutations was estimated using the ARMADiLLO (Antigen Receptor Mutation Analyzer for Detecting Low Likelihood Occurrences) program (10). Briefly, given a UCA sequence and the number of mutations observed in the antibody sequence of interest, ARMADiLLO simulates somatic hypermutation based on a model of AID targeting and base substitution (92) and uses these simulations to estimate the probability of an amino acid at a specific position.

Mathematical modeling of improbable mutation acquisition with vaccination

We model the acquisition of improbable mutations as a Poisson process with rate parameter, λ , in which the average number of improbable mutations is 1 in a time interval T of 12 weeks based on the number of improbable mutations observed for the mice immunized biweekly in this study. For simplicity, here we assume improbable mutations are acquired at a constant rate in the absence of targeted selection during vaccination, although evolutionary rates of B cell lineages are known to fluctuate during infection (49). The probability of k or fewer Poisson-distributed events occurring within a time interval is a function of the cumulative density of the Poisson distribution:

$$P(X \leq k) = \exp(-\lambda) \sum_{i=0}^k \frac{\lambda^i}{i!}$$

The Poisson cumulative distribution function can be expressed using the regularized incomplete gamma function Q as

$$P(X \leq k) = Q[(k+1), \lambda] = \frac{\Gamma[(k+1), \lambda]}{\Gamma(k+1)}$$

The probability of k or more events occurring in the time interval is then

$$P(X \geq k) = 1 - Q(k, \lambda)$$

which can be solved for λ numerically using the inverse incomplete regularized gamma function,

$$\lambda = Q^{-1}[1 - P(X \geq k), k]$$

The total time to acquire k or more mutations is then λT .

The probability of acquiring at least $k = 4$ improbable mutations given a rate parameter of 1 improbable mutation every 12 weeks is plotted as a function of time in fig. S19C. For $P(X \geq 4) = 0.99$, the number of 12-week intervals is 10.05 to acquire at least four improbable mutations. Thus, mice would need to be immunized biweekly for ~120 weeks in order to achieve 99% probability of acquiring at least four improbable mutations.

Quantification and statistical analysis

The statistical analyses for this paper were performed in SAS 9.4 to calculate exact Wilcoxon tests for group comparisons. Due to the exploratory nature of this research and the small sample size, we are using an alpha level of 0.05 as a descriptive level for significance and have not made any adjustments to control for multiple testing. For group sizes less than 5, no paired-sample comparisons were performed due to the small sample size; only descriptive statistics are provided in these instances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Structural characterization and development of an HIV-1 Env antigen capable of binding to the DH270 unmutated common ancestor (UCA).

(A) Antibody neutralization of TZM-bl cell infection was compared for HIV-1 pseudoviruses encoding CH848 10.17 gp160 Env or the same Env with glycosylation sites at Asn¹³³ and Asn¹³⁸ (N133 and N138) removed by mutating them to Asp¹³³ and Thr¹³⁸ (10.17DT). Neutralization titers are shown as IC₅₀ values. (B) The binding affinities of DH270 UCA and the first intermediate antibody in the DH270 lineage (IA4) for CH848 10.17DT-stabilized Env trimers was determined by surface plasmon resonance. The somatic

mutations acquired in the heavy chain variable region (V_H) and light chain variable region (V_L) of DH270 IA4 are shown above and below the arrow, respectively. Improbable and probable somatic mutations were modeled on the DH270 UCA Fab crystal structure (PDB ID 5U0R) as red and green residues, respectively. **(C)** CH848 10.17DT calcium flux induction in DH270 UCA (green), DH270.1 (orange), and negative control (black) IgG-expressing Ramos B cells. **(D)** Cryo-EM reconstruction (4.2 Å resolution) of DH270 UCA (green) bound to CH848 10.17DT-stabilized Env trimers (gray), segmented by components. **(E)** Zoomed-in view of the interactive region between DH270 UCA and CH848 10.17DT. HIV-1 Env gp120 is shown in gray, with the V3 loop in orange and the V1 loop in red. Glycans are shown in stick representation and colored by elements, with oxygen atoms in red and nitrogen atoms in blue. The heavy chain of DH270 UCA is shown in green and light chain in yellow. Color code for complementarity-determining regions (CDRs) of the antibody: wheat, CDR H1; blue, CDR H2; purple, CDR H3; brown, CDR L1; pink, CDR L2; teal, CDR L3. DH270 UCA contacted the V1 loop, the V3 loop, and the surrounding glycans, with contacts made by both the heavy and light chains of the antibody. **(F to H)** Zoomed-in views of the structure showing details of the contacts DH270 UCA makes with glycan 332, shown in stick representation overlaid with dots and colored by element, with the DH270 UCA shown in surface representation (F), the V3 loop (G), and the V1 loop (H).

Fig. 2. CH848 10.17DT nanoparticle immunization of DH270 UCA knock-in mice induces serum neutralizing antibody responses and the improbable G57R mutation.

(A) Negative-stain electron microscopy of CH848 10.17DT-stabilized Env trimers conjugated to ferritin nanoparticles. A magnified image of the Env nanoparticles (top) and 2D class average (bottom) of the Env nanoparticles are shown at the right. (B) Antigenic profile of CH848 10.17DT-stabilized Env trimers conjugated to ferritin nanoparticles or unconjugated. Binding was determined by biolayer interferometry and normalized to PGT151 binding values. The normalized binding is shown in the heat map, with a value of 1 being equal to PGT151 binding. OD glycan, outer domain glycans in the high-mannose

patch. **(C)** CH848 10.17DT Env trimer nanoparticle (red) induction of calcium flux in homozygous ($V_H^{+/+}$, $V_L^{+/+}$) DH270 UCA double knock-in (double KI) mouse B cells. **(D)** Heterozygous ($V_H^{+/-}$, $V_L^{+/-}$) DH270 UCA double KI mouse immunization regimen with CH848 10.17DT SOSIP trimer nanoparticles. **(E)** Quantification of the frequency of splenic germinal center B cells (top) and follicular helper T cells (bottom) in vaccinated DH270 UCA mice. Unvaccinated, wild-type C57BL/6 mice (BL6) served as baseline controls. Means $\pm$ SEM are shown by black bars. * $P < 0.05$ (Wilcoxon exact test). **(F)** Serum antibody neutralization of autologous tier 2 CH848 10.17DT (top) and CH848 10.17 (bottom) virus infection of TZM-bl cells. Group geometric mean ID_{50} titers are shown (nanoparticle, $n = 5$; adjuvant only, $n = 6$). Arrows indicate immunization time points. **(G)** Top: Enumeration by V_H next-generation sequencing of the frequency of unique DH270 sequences encoding the G57R amino acid change. Frequencies of G57R were determined in total splenocytes 1 week after the sixth immunization. Each mouse is shown by an individual symbol; horizontal bars indicate the group mean. Bottom: Relative (fold) increase in G57R frequency in heterozygous DH270 UCA KI mice immunized with CH848 10.17DT Env nanoparticle and adjuvant compared to adjuvant-only control mice. Box-and-whisker plots indicate the minimum, maximum, median, and interquartile range fold increase for each group. Each symbol represents an individual mouse. * $P < 0.05$ (Wilcoxon exact test).

Fig. 3. CH848 10.17DT Env trimer nanoparticle immunization elicits neutralizing antibody DH270.mu1 that encodes both S27Y and G57R improbable amino acid changes.

(A) The percentage of CH848 10.17-specific B cells sorted from CH848 10.17DT Env trimer nanoparticle-immunized mice that encode DH270 V_H and V_L regions. CH848 10.17-specific B cells encoding one or more mouse Ig chains were classified as other antibodies. Total number of antibodies isolated is shown in the center of the pie chart. (B) The percentage of DH270-expressing B cells that have acquired one or more somatic mutations. Total number of DH270 antibodies isolated [based on data in (A)] is shown in the center of the pie chart. (C) Amino acid mutation frequency of the DH270 V_H and V_L regions cloned from the sorted CH848 10.17-specific B cells shown in (B). Box-and-whisker plots show

the range, median, and interquartile range. **(D)** Env nanoparticle vaccine–induced monoclonal antibody DH270.mu1 possessed both the G57R and S27Y amino acid changes in its HCDR2 and LCDR1 loops, respectively. Shown is an amino acid alignment of select DH270 clonal lineage antibodies and DH270.mu1. The two critical improbable mutations are highlighted in yellow. **(E)** DH270.mu1 neutralizes autologous and heterologous viruses with potency nearly identical to that of DH270 IA4. Neutralization titers are shown as IC₅₀ for each individual virus. Left, vaccine-induced antibody; right, DH270 intermediate antibody inferred from the clonal lineage elicited during chronic HIV-1 infections. **(F)** DH270.mu1 possesses neutralization breadth (16/23 or 70% of viruses) similar to that of DH270 IA4. Neutralization IC₅₀ titers are shown in a heat map. **(G)** The somatic mutations shared between the vaccine-elicited antibodies and the bnAb DH270.1. The DH270.1 somatic mutations observed after vaccination with 10.17DT trimer nanoparticle are indicated with blue squares; unobserved mutations are shown by white squares. **(H)** The frequency of the total (**n** = 134) mouse mAbs isolated from mice immunized with the 10.17DT SOSIP nanoparticle that had the indicated numbers of shared mutations with DH270.1. Amino acid abbreviations: A, Ala; D, Asp; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Fig. 4. Vaccine-induced antibody DH270.mu1 with improbable mutations binds to the HIV-1 Env SOSIP trimer with the same binding mode as DH270 bnAbs.

(A) Cryo-EM reconstruction (4.3 Å resolution) of DH270.6 (cyan) bound to CH848 10.17DT-stabilized Env trimers (gray) segmented by components. (B) Same as (A) but for the DH270.mu1 complex (3.5 Å resolution) with DH270.mu1 shown in pink. (C) Zoomed-in view of the interactive region between DH270.6 and CH848 10.17DT. HIV-1 Env gp120 is shown in gray with the V3 loop in orange and the V1 loop in red. Glycans are shown in stick representation and colored by elements, with oxygen atoms in red and nitrogen atoms in

blue. The heavy chain of DH270.6 is shown in cyan and light chain in yellow. Color code for CDRs: wheat, CDR H1; blue, CDR H2; purple, CDR H3; brown, CDR L1; pink, CDR L2; teal, CDR L3. **(D)** Same as (C) but for the DH270.mu1 complex, with DH270.mu1 heavy chain in pink. **(E)** Overlay of the DH270-bound V1 (green), DH270.6-bound V1 (blue), and DH270.mu1-bound V1 (magenta). **(F to H)** Zoomed-in views showing details of the contacts made by heavy chain residue 57. Black dashed lines indicate hydrogen bonds. **(I to K)** Zoomed-in views showing details of the contacts made by light chain residue 27.

Fig. 5. Env trimer immunization elicits neutralizing antibodies with the critical, improbable K19T amino acid change in the CD4 binding site bnAb lineage CH235.

(A) CH235 UCA neutralization of autologous CH505 virus infection of TZM-bl cells is enhanced by the acquisition of the K19T somatic mutation in the V_H. Neutralization titers are shown as IC₅₀ values. A mixture of CH01 and CH31 was used as a positive control. (B) CH235 UCA (het/het) KI mouse (n = 5) immunization comparing Man₅GlcNAc₂-enriched versus heterogeneously glycosylated CH505 M5.G458Y SOSIP gp140 trimer. (C) Comparable titers of serum antibody neutralization of CH505 M5.G458Y virus infection of TZM-bl cells were elicited by Man₅GlcNAc₂-enriched versus heterogeneously glycosylated CH505 M5.G458Y SOSIP gp140 trimer. Neutralization activity was sensitive to a N280D amino acid change in the CD4 binding site. Neutralization titers are shown as ID₅₀ with each symbol representing one mouse serum sample collected 1 week after the fourth immunization. Group geometric means are shown by horizontal bars. Murine leukemia virus was negative at each time point. (D) Enumeration by V_H next-generation sequencing of the frequency of unique CH235 sequences encoding the K19T amino acid change in mice

immunized with M5 gp120 or Man₅GlcNAc₂-enriched M5.G458Y trimer. Frequencies of K19T were determined in total splenocytes 1 week after the final immunization. Each mouse is shown by an individual symbol; horizontal bars indicate the group mean. ***P** < 0.05 (Wilcoxon exact test); ns, not significant. **(E)** Fold increase in K19T frequency in heterozygous CH235 UCA KI mice immunized with Man₅GlcNAc₂-enriched M5. G458Y trimer and M5 gp120. Symbols and bars are the same as in (D). ***P** < 0.05 (Wilcoxon exact test). **(F)** M5.G458Y gp120-reactive single splenic B cells from a Man₅GlcNAc₂-enriched CH505 M5.G458Y Env trimer-immunized mouse were sorted by fluorescence-activated cell sorting (FACS) 1 week after the final immunization. All of the 57 recovered antibody sequences originated from the CH235 KI variable regions. The pie chart shows the percentage of CH235-expressing B cells that have acquired one or more somatic mutations. **(G)** Amino acid alignment of V_H sequences from vaccine-induced and infection-induced CH235 antibodies shows the occurrence of the K19T mutation. **(H)** Vaccine-induced CH235 antibodies encoding K19T neutralize autologous CH505 virus infection of TZM-bl cells more potently than does the CH235 UCA. Viruses were grown under conditions that result in Man₅GlcNAc₂ enrichment (left) or heterogeneous glycosylation (right). The heat maps depict IC₅₀ neutralization titers for each individual virus.

Fig. 6. CH505 M5.G458Y SOSIP nanoparticle immunization of rhesus macaques elicits serum autologous neutralizing antibodies against the CD4 binding site.

(A) Two-dimensional class averages of negative-stain electron microscopy images of GnTI^{-/-} cell-expressed M5.G458Y-stabilized SOSIP gp140 ferritin nanoparticles. (B) Rhesus macaque immunization with M5.G458Y-stabilized SOSIP gp140 ferritin nanoparticles enriched for Man₅GlcNAc₂ glycans. (C) Plasma IgG binding over time to GnTI^{-/-} cell-expressed M5.G458Y-stabilized SOSIP gp140. (D) Serum neutralization kinetics against GnTI^{-/-} cell-produced HIV-1 CH505 M5.G458Y virus. Each curve represents an individual macaque. (E) Macaque serum after three immunizations neutralizes Man₅GlcNAc₂-enriched and heterogeneously glycosylated CH505 M5.G458Y virus. Horizontal bars are the geometric mean for the group (n = 4). SVA is a negative control murine leukemia virus. (F)

Macaque serum after three immunizations neutralizes Man₅GlcNAc₂-enriched CH505 M5.G458Y, M5, and TF viruses. Horizontal bars are the geometric mean for the group (**n** = 4). (**G**) Geometric mean of the neutralization titers of all four animals against CH505 M5.G458Y wild-type and CD4bs CH235 bnAb knockout (N280D) viruses over time. (**H**) Individual macaque neutralization titers after three immunizations against M5.G458Y wild-type and CD4bs CH235 bnAb knockout (N280D) viruses. Horizontal bars are the geometric mean for the group (**n** = 4). (**I**) Fold decrease in week 10 neutralization ID₅₀ titers shown in (H) for each macaque upon mutation of the CD4 binding site with a N280D mutation.