Initiation of HIV neutralizing B cell lineages with sequential envelope immunizations

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A strategy for HIV-1 vaccine development is to define envelope (Env) evolution of broadly neutralizing antibodies (bnAbs) in infection and to recreate those events by vaccination. Here, we report host tolerance mechanisms that limit the development of CD4-binding site (CD4bs), HCDR3-binder bnAbs via sequential HIV-1 Env vaccination. Vaccine-induced macaque CD4bs antibodies neutralize 7% of HIV-1 strains, recognize open Env trimers, and accumulate relatively modest somatic mutations. In naive CD4bs, unmutated common ancestor knock-in mice Env⁺B cell clones develop anergy and partial deletion at the transitional to mature B cell stage, but become Env⁻ upon receptor editing. In comparison with repetitive Env immunizations, sequential Env administration rescue anergic Env⁺ (non-edited) precursor B cells. Thus, stepwise immunization initiates CD4bs-bnAb responses, but immune tolerance mechanisms restrict their development, suggesting that sequential immunogen-based vaccine regimens will likely need to incorporate strategies to expand bnAb precursor pools.

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The HIV-1 envelope (Env) is the target of neutralizing antibodies (nAb)\(^1\). However, Env-immunogens including stabilized trimers have thus far been ineffective for inducing broadly neutralizing antibodies (bnAbs) in humans or wild-type animals\(^2\)–\(^4\). Antibody-virus co-evolution studies from the time of HIV-1 transmission through bnAb development have shown that bnAbs arise after extensive Env diversification; and when bnAbs develop, they are subdominant with respect to other Env lineages\(^5\)–\(^8\).

BnAb knock-in (KI) mice have proved useful for bnAb development and regulation studies. Several reports with such models have demonstrated that portions of bnAb maturation pathways can be completed by immunization regimens, including: (1) initiation or partial completion of bnAb-like responses with immunogens that target B cell repertoires generated from knocked-in unarranged bnAb germ line segments\(^9\) or B cell-bearing partially reverted (V\(_{H}\) germ line/mature HCDR3 hybrid) knocked-in rearrangements\(^10\)–\(^13\) and (2) induction of bnAb responses with immunogens that can engage B cells expressing either near-mature or fully affinity matured bnAb V(D)J rearrangements\(^12\)–\(^15\). However, several mouse models of bnAb development have also demonstrated that bnAb maturation of membrane proximal external region (MPER)-targeting or CD4-mimicking bnAbs\(^16\)–\(^18\) is likely to be limited at some point in development, either by central or peripheral tolerance controls. We have previously shown that both mature and UCA gp41 MPER bnAb heavy- (HC) and light-chain (LC) gene-rearranged (VHDJH/L) KI mice have severe bone marrow (BM) deletion, and the few remaining B cells in the periphery are anergic, resulting in massive reduction in BM precursor frequency of MPER bnAbs\(^16\).

Similarly, immunization of rhesus macaques with Env immunogens has initiated bnAb-like lineages that have been controlled either by deletion or affinity reversion (maturation off-target) due to selection of non-bnAb HCDR3 regions\(^19\). In contrast, the precursor frequency of CD4-mimicking type of CD4-binding site bnAbs (VRC01-class) has been found to be normal in UCA KI mice in one study\(^9\), but abnormal with BM deletion, receptor editing, and peripheral anergy in another\(^17\).

In contrast to the VRC01-class of CD4-binding site bnAbs, the CD4-binding site HCDR3-binder class of bnAbs make contacts with gp120 via their CDR3 loops. CH103, a prototype of the HCDR3-binder class of CD4-binding site bnAbs, is one of the only two bnAb lineages whose complete virus-Ab co-evolution pathway has been comprehensively characterized\(^6\), and whose co-evolved Env maturation pathway, from which sequential immunogens have been derived for this study, can now also be investigated in SHIV CH505-infected non-human primates\(^20\). No studies have yet been done, however, to characterize the HCDR3-binder-class responses to immunization, nor have any bona fide unmutated common ancestors (UCAs) from full, patient-derived bnAb lineages been studied in the setting of a bnAb KI model. Moreover, the in vivo host controls have yet to be systematically examined in a physiologically relevant setting -- that is, in which all such controls (including LC receptor editing) are available for the immune system to utilize.

We report here the immunogenicity in rhesus macaques and CH103 CD4-binding site bnAb UCA KI mice of sequential Env immunogens derived from the CH505 HIV-1-infected individual who made the CH103 bnAb lineage. In macaques, vaccine-induced nAbs had epitopes overlapping that of CH103, bound only open trimers, and neutralized rare tier 2 viruses. While the V\(_{H}\) genes encoding vaccine-induced antibodies in macaques were similar to the V\(_{H}\) gene of CH103, the V\(_{L}\) genes were not, raising the possibility of receptor editing. In CH103 bnAb V\(_{H}\) + V\(_{L}\) UCA mice, we found that ~70% of BM UCA B cells were deleted at the transitional to mature B cell development stage, with most of the remaining B cells edited with alternative V\(_{L}\). Sequential gp120 Env immunizations could, however, select for B cells bearing paired CH103 V\(_{H}\) and V\(_{L}\), thereby enlarging the pool of bnAb B cell precursors for further lineage maturation.

**Results**

**Immunizations with CH505 4-valent gp120 Env monomers.** We have previously reported isolation from an individual of a CD4-binding site bnAb B cell lineage, CH103, and CH505 Env monomers that evolved sequentially during the time of bnAb development\(^6\). From this group of Envs, we generated 113 autologous recombinant Envs and tested them by ELISA for binding to members of the CH103 bnAb lineage\(^20\). We selected four Envs based on binding to stages of the CH103 bnAb lineage: the CH505 transmitted founder (TF) and three natural CH505 variants (week 53, 78, and 100)\(^20\). To determine if CH505 sequential vaccine regimens could initiate CH103-like B cell lineages in non-human primates, we vaccinated macaques with CH505 gp120 monomers in the observed temporal order, either sequentially or cumulatively, with the CH505 TF gp120 at all timepoints as control.

CH505 gp120 Envs in AS01E (NHP88, N = 16) or GLA-SE (NHP79, N = 24) adjuvants and administered either repeatedly as TF Env immunogen alone or as sequential Envs immunogens induced statistically similar plasma-neutralizing antibody profiles in macaques after six immunizations (Fig. 1a–c). However, sequential gp120 Env-immunized macaques showed a trend of qualitatively better serum plasma-neutralizing antibody responses relative to those immunized repeatedly with TF Env gp120, both in terms of potency and breadth (Fig. 1a, b). Furthermore, sequentially-immunized macaques had a trend for enhanced autologous tier 1 neutralization titers relative to TF Env-immunized animals (Fig. 1c), with macaques administered with CH505 TF Env gp120 in GLA-SE (N = 4) inducing mean autologous tier 1 (CH505.w4.3)-neutralizing antibody ID50-titers of 614 ± 597 (mean ± SD), vs. those administered with CH505 sequential gp120 Envs in GLA-SE (N = 4) inducing mean autologous tier 1 (CH505.w4.3)-neutralizing antibody ID50-titers of 933 ± 712. This trend reached near significance (P = 0.07, exact Wilcoxon test) in groups administered with CH505 sequential gp120 Envs when they were formulated in AS01E (N = 8), with mean autologous tier 1 (CH505.w4.3)-neutralizing antibody ID50-titers of 2264 ± 2932, relative to those administered with CH505 TF Env gp120 alone formulated in AS01E (N = 8), which by comparison, induced mean autologous tier 1 (CH505.w4.3)-neutralizing antibody ID50-titers of 630 ± 383.

The enhanced neutralizing antibody responses in sequentially-immunized groups would be anticipated to be only revealed as a trend in a complete polyclonal system, such as macaques, since any potential enhancements in plasma-neutralizing antibody responses are most likely to be subdominant in nature. Such results therefore predict that general CD4-binding site binding responses will be predominantly non-specific, and thus similar between all immunization groups and adjuvants tested. To examine this, we measured plasma binding in macaques, and as expected, observed no statistical differences or obvious trends in levels of plasma-binding titers to TF Env gp120 (Fig. 1d, e). Specifically, after sixth immunizations, CH505 TF Env gp120 in AS01E (N = 8) or GLA-SE (N = 4) induced autologous (CH505 TF gp120)-binding Ab Log AUC-titers of 9.3 ± 1.0 and 8.9 ± 1.6 (P = 0.8, exact Wilcoxon test), respectively, (Fig. 1d) and CH505 sequential Env gp120s in AS01E (N = 8) and GLA-SE (N = 4) induced autologous Env-binding Ab Log AUC-titers of 10.2 ± 0.9 and 10.0 ± 0.8 (P = 0.7, exact Wilcoxon test), respectively, (Fig. 1e).

**Vaccine-induced blood memory B cell repertoires in macaques.** To further examine the quality of vaccine-induced antibodies...
targeting the CD4-binding site, we compared the binding characteristics of plasma with those of CD4-binding site CH103 early-lineage members. We previously reported that the CH103 UCA bound CH505 TF gp120 Envs, but not Env variants with a deletion of isoleucine at amino acid position 371 (Δ371I), which disrupts the CD4-binding site. We termed antibodies with this profile “CH505 differential binders.” We found that plasma from macaques immunized with TF or sequential CH505 Envs bound wild-type CH505 TF gp120 and equally well to the CD4-disrupted mutant protein. However, plasma from immunized macaques blocked binding of a CD4-binding site CH103 lineage member (CH106) and of sCD4 to CH505 TF gp120 Envs

### Table A

| Vaccine group | Antigen | C | B | A | D |
|---------------|---------|---|---|---|---|
| Group 7       | CH505   | 100 | 93 | 87 | 80 |
|               | CH106   | 95  | 85 | 75 | 60 |
|               | CH108   | 90  | 75 | 65 | 50 |

### Table B

| Vaccine group (VVG) | Antigen | C | B | A | D |
|---------------------|---------|---|---|---|---|
| Group 1             | CH505   | 100 | 95 | 90 | 85 |
|                     | CH106   | 95  | 90 | 85 | 80 |
|                     | CH108   | 90  | 85 | 80 | 75 |

### Figure A

#### Figure A (AS01E adjuvant)

| Vaccine group (VVG) | Antigen | C | B | A | D |
|---------------------|---------|---|---|---|---|
| Group 1             | CH505   | 100 | 95 | 90 | 85 |
|                     | CH106   | 95  | 90 | 85 | 80 |
|                     | CH108   | 90  | 85 | 80 | 75 |

### Figure B

#### Figure B (GLA-SE adjuvant)

| Vaccine group (VVG) | Antigen | C | B | A | D |
|---------------------|---------|---|---|---|---|
| Group 1             | CH505   | 100 | 95 | 90 | 85 |
|                     | CH106   | 95  | 90 | 85 | 80 |
|                     | CH108   | 90  | 85 | 80 | 75 |

### Figure C

#### Figure C (AS01E or GLA-SE adjuvants)

| Vaccine group (VVG) | Antigen | C | B | A | D |
|---------------------|---------|---|---|---|---|
| Group 1             | CH505   | 100 | 95 | 90 | 85 |
|                     | CH106   | 95  | 90 | 85 | 80 |
|                     | CH108   | 90  | 85 | 80 | 75 |

### Figure D

#### Figure D (AS01E or GLA-SE adjuvants)

| Vaccine group (VVG) | Antigen | C | B | A | D |
|---------------------|---------|---|---|---|---|
| Group 1             | CH505   | 100 | 95 | 90 | 85 |
|                     | CH106   | 95  | 90 | 85 | 80 |
|                     | CH108   | 90  | 85 | 80 | 75 |
These results further indicate that while there were high levels of CD4-binding site-like antibodies induced by CH505 Env s in macaques in plasma, this response did not have a predominance of differential-binding bnAb-like activity.

We extended our analysis of differential responses in CH505 Env-immunized macaques by enumerating the frequency of CH505 differential-binding memory (IgD−, CD27 all) B cells induced at various time points (Fig. 2a; Supplementary Fig. 1), and found that macaques immunized with sequential CH505 gp120 Env s appeared to have an increased number of CH505 differential-binding memory B cells over background, compared to macaques immunized with CH505 TF gp120 Env alone. Furthermore, the number of responders for CH505 differential-binding memory B cells, and frequency of CH505 differential-binding memory B cells, appeared to go down after the third immunization, suggesting an off-target effect on CH505 differential-binding memory B cell lineages initiated by early CH505 Env s in the sequential Env regimens. To further examine CH505 differential-binding responses induced by TF-repetitive Env immunizations or sequential Env regimens in macaques, we recovered CH505 Env-reactive recombinant antibodies from sorted memory B cells isolated from animals immunized with TF Env in GLA-SE or AS01E, and compared their binding characteristics with those from macaques immunized with sequential combinations of Env s in GLA-SE or AS01E. One hundred and twenty antibodies were isolated using the TF Env gp120 as a fluorophore-labeled memory B cell hook from macaques immunized with TF Env (macaque N = 5), and 232 antibodies were isolated from macaques immunized with sequential combinations of Env s (macaque N = 11) (Fig. 2b, Table 1). Among macaques immunized with TF gp120 Env, 11% of the CH505 Env-reactive antibodies were CH505 differential binders; however, among those immunized with sequential combinations of CH505 gp120 Env s, 16% were CH505 differential binders (Fig. 2b). Thus, consistent with the observed trend of enhanced subdominant neutralizing antibody responses induced in the plasma by sequential immunization (Fig. 1a–c), we observed a trend for animals immunized with sequential CH505 gp120 Env s to make more CH505 differential-binding antibodies, albeit not statistically significant.

While the observed trends of enhanced plasma-neutralizing antibody responses, as well as higher frequencies of differential memory B cells and recombinant antibodies were seen in sequentially immunized macaques relative to those vaccinated with TF Env, no differences in the immunogenetics of CH505 differential-binding antibodies were observed between Ab groups isolated from macaques immunized with TF alone in AS01E or GLA-SE, and sequential CH505 Env s in AS01E or GLA-SE (Fig. 2c, d). Strikingly however, among all CH505 Env-reactive antibodies in CH505 Env-immunized macaques (regardless of adjuvant), the heavy-chain genes of CH505 differential binders (macaque N = 12) were less mutated (P = 0.008, exact Wilcoxon test with false discovery rate (FDR) correction) and had longer HCDR3 lengths (P = 0.008, exact Wilcoxon test with false discovery rate (FDR) correction) than non-differential binder antibodies (macaque N = 14) (Fig. 2e, f). Non-HIV-1 antibodies (macaque N = 13) had similar mean heavy-chain gene-mutation frequencies, but shorter HCDR3 lengths than CH505 differential-binding antibodies. These data suggest that CH505 differential-binding antibodies arose from a pool of precursor B cells with longer HCDR3s and more limited affinity maturation capacity, features normally associated with B cells under clonal deletion/anergy host controls16, and/or that are excluded from mature B2 subsets, and thus are predisposed to generating either suboptimal T-dependent responses or using T-independent pathways.

Vaccine-induced macaque CD4-binding site-nAbs. We selected 29 differential-binding recombinant antibodies based upon degree of antibody differential binding to wild-type and mutant CH505 Δ371I Env s, and autologous CH505.w4.3 virus neutralization or V1-V4 usage (human CH103 used V1-V4−59) (Supplementary Fig. 2a, b). CH505 differential-binding antibodies isolated from macaques immunized with TF or sequential combinations of CH505 Env s had similar autologous Env-binding and neutralization patterns (Supplementary Fig. 2c, d). While CH505 differential-binding monoclonal antibodies (mAbs) neutralized autologous tier 1 CH505.w4.3 (Supplementary Fig. 2d), none neutralized the autologous tier 2 CH505 TF isolate. The antibody clonal lineage most similar to CH103 we identified was an IGHV4-4, CH505 differential-binding clonal lineage (DH522) with autologous tier 1, and heterologous tier 1 and weak tier 2 neutralization activities derived from memory B cells of RM-5556 immunized with additive CH505 Env s (Supplementary Fig. 2e). DH522 lineage antibodies, DH522.1 and DH522.2, were isolated from blood memory B cells after four and six immunizations, respectively (Fig. 3a). DH522 lineage mAbs did not neutralize tier 2 CH505 TF (Fig. 3b), neutralized 7% of a panel of 199 HIV-1 primary isolates (Supplementary Fig. 2f), and blocked the binding of CH103 lineage Ab (CH106) to CH505 TF gp120 (Fig. 3c). Chimeric Abs of heavy- and light-chain genes of DH522 and
CH103 mAbs improved neither binding nor neutralization breadth of DH522 (Supplementary Fig. 2f), suggesting that compensatory mutations in CH103 heavy-and light-chain genes are necessary for mAb binding and neutralization.

It was previously reported that glycans occluded access to the CD4-binding site on SOSIP trimers by CD4-binding site bnAbs, including CH103 \(^{21, 22}\), and glycan-deleted trimers generally induced high titer autologous-nAbs in rhesus macaques or mice models\(^9, 22\). In the context of gp120 monomers, we found that the CH103 lineage antibodies bound equally well to natively glycosylated Envs and Envs with a deletion of glycans in the vicinity of the CD4-binding site\(^{22}\) (Supplementary Fig. 3a).

**CH505 differential binder rAbs**

|                  | CH505-D | CH505-ND |
|------------------|---------|----------|
| CH505 TF Env     | 14%     | 86%      |
| CH505 sequential + additive Envs | 11% | 89%      |
| CH505 additive Envs | 16% | 84%      |
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| CH505 sequential + additive Envs | 11% | 89%      |
| CH505 additive Envs | 16% | 84%      |

**IGHV mutation frequency**

- **CH505 TF Env**
  - +GLA-SE: 20%
  - +AS01E: 20%
- **CH505 sequential Envs**
  - +GLA-SE: 20%
  - +AS01E: 20%
- **CH505 additive Envs**
  - +GLA-SE: 20%

**IGHV CDR3 length**

- **CH505 TF Env**
  - +GLA-SE: 30
  - +AS01E: 30
- **CH505 sequential Envs**
  - +GLA-SE: 30
  - +AS01E: 30
- **CH505 additive Envs**
  - +GLA-SE: 30
However, CH103 antibody demonstrated improved neutralization titers against HIV-1 strains bearing Envs with a deletion of glycans proximal to the CD4-binding site compared to the viruses bearing natively glycosylated Envs (Supplementary Fig. 3b), suggesting that glycans in the vicinity of the CD4-binding site may impact the neutralization capacity of CH103 lineage antibodies. We also found that neither the UCA nor mature CH103 antibodies bound free N-glycans (Supplementary Fig. 3c). For comparison, we found that the DH522 lineage antibodies bound natively glycosylated and glycans-deleted CH505 TF gp120 Envs equally well, but did not neutralize HIV-1 pseudoviruses bearing these Envs nor bound free N-glycans (Supplementary Fig. 3). However, we also found DH522 lineage antibodies to be polyreactive with host antigens (Fig. 3d) similar to previously reported for CH103 bnAb.

We determined the crystal structure of the DH522.2 Fab in complex with a deglycosylated chimeric B.YU2 gp120 core to 2.8 Å resolution (Supplementary Figs. 4 and 5 and Supplementary Table 1). We found that the DH522.2 HCDR3 was in an extended conformation, with its hydrophobic tip inserted into a pocket on the surface of gp120 near the junction of the inner domain, outer domain, and bridging sheet (Fig. 3e). This pocket was part of a broad hydrophobic surface underlaid by loop B and adjacent to the well-characterized Phe43 cavity. A shift in the bridging sheet represented a departure from the CD4-bound state, exposing the hydrophobic patch rather than preserving the full depth and shape of the Phe43 cavity. The loop B hydrophobic pocket, the core epitope for DH522.2, is also the binding site of NBD-556 and related CD4 mimetic compounds (Fig. 3e, g). The HCDR3 hydrophobic tips of both DH522.2 and the poorly neutralizing CD4-binding site antibody F105 reach into the loop B pocket, but the overall orientations of the two Fabs with respect to the gp120 core are quite distinct (Fig. 3e). Although the DH522.2 HCDR3 probed the CD4-binding site Phe43 cavity, superposition of the DH522.2 Fab-gp120 complex onto the BG505 Env SOSIP.664 trimer indicated that DH522.2 Fab would clash with a nearby gp120 subunit (Fig. 3f). A 3D reconstruction of DH522.2-bound CH505 SOSIP.664 fully glycosylated trimer by negative stain electron microscopy confirmed that DH522.2-bound Env trimers in an open conformation (Fig. 3h–i), a mode of binding consistent with an Env conformation between the CD4- and b12-bound states (Fig. 3h). A broader comparison of CD4-binding site antibodies showed that other bnAbs included loop D and V5 in their epitopes rather than loop B (Fig. 3g); those bnAbs also had more favorable orientations of binding trimeric Envs with much less steric occlusion than found with DH522.2. Thus, DH522.2 interacts poorly with a closed Env trimer, explaining the limited neutralization breadth of antibodies in its lineage.

CH103 bnAb maturation in CH103 UCA V81DJH4 + VJL4 KI mice. While the rhesus IGHV4-1 is similar to the human IGHV4–59 that encoded the CH103 bnAb heavy chain, an IGLV2 encoded the DH522 light chain, rather than an IGLV3 gene as in CH103. One hypothesis for this difference is that receptor editing may have limited IGLV3 usage. A second hypothesis is that the macaque does not have a sufficiently similar IGLV3 that can pair with IGHV4-1 and yield CH103 bnAb-like activity. Because the macaque Ig loci show high inter-individual diversity, we used next-generation sequencing (NGS) using IGLV3-specific primers to further probe macaque 5556 for germ line gene segments closer to IGLV3-1. The closest plausible germ line candidate is again 85% identical. Thus, these data suggested that insufficient macaque and human gene identity was a plausible reason for inability of macaques to make CH103-like bnAbs.

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To study directly the immune mechanisms controlling development of HCDR3-binder type bnAbs and to more robustly reveal potential immunogenicity differences between vaccination groups in a less stringent (i.e., less competitive polyclonal system), we turned instead to a humanized immunoglobulin mouse model. We generated a double KI mouse, designed to express the rearranged CH103 UCA heavy- and light-chains (double KI; V91DJH4+VJL4) and (V51DSK6+VJL5+). Naive B cells from naïve CH103 UCA double KI mice were not clonally deleted at the first (central) tolerance checkpoint, that is, at the pre-B to immature B cell transition in BM (Fig. 4a) as observed in naive 2F5 germ line/UA double KI mice (Supplementary Fig. 6a). Instead, they exhibited a blockade at the BM transitional to mature B cell stage (70% mean reduction relative to WT) (Fig. 4a, b), and a subpopulation of residual mature B cells with lowered B cell receptor (BCR) densities (Fig. 4a). In line with specific negative selection of mature BM B cells, CH103 UCA double KI mice also had lower numbers of total peripheral B cells (Fig. 4c, d), a larger fraction of which accumulated in the splenic transitional compartment (Fig. 4c, e). Thus, these data are consistent with the processes of both anergy and deletion of CH103 UCA double KI-expressing positive B cells at the second tolerance checkpoint.

Light-chain editing in CH103 UCA KI mice. Since self-reactivity of bnAbs often correlate with their HIV-1 Env reactivity, we determined the frequency of CH505 differential-binding (CH505 TF gp120+, CH505 TF gp120 Δ371I–) B cells in the mature
It is required for differential binding. This suggested that the KI UCA binding, combined with previous findings, that the KI LC allele with a specific targeting strategy, in which we knocked in the human CH103 VJ rearrangement at mouse Ig κ locus. (rather than Igκ; Supplementary Fig. 7) and retained downstream Jκ4/5 mini-gene segments. This approach allowed CH103 UCA het double KI mice to undergo multiple secondary LC rearrangements both in cis and in trans, and generated a more diverse repertoire of VJ rearrangements than possible at the a tissue-specific mouse Ig κ locus.

As predicted by their potential for both alternative κLC and HC rearrangement, B cell subsets from naive CH103 UCA heterozygous double KI spleen had significantly lower CH505 differential reactivity than did those from the CH103 UCA double KI model, including a fourfold reduction in differential binders found in their transitional B cell subset, and proportionally even lower differential reactivity in the mature B cell subset (more than sevenfold reduction in differential binders). The result was a near complete lack of Env reactivity, that is, >98% of mature B cells. As expected, loss of Env reactivity in CH103 UCA heterozygous double KI mice was also associated with rescue of mature B cells development, which resembled the normal developmental phenotype of homozygous CH103 UCA VDJκ/λ KI mice (Supplementary Fig. 6a–d).

We also isolated by RT-PCR VH4JH4 and VκJκ Ig gene pairs from single splenic mature follicular B cells in naive CH103 het double KI mice. Half (23/46) of the sorted mature B cells used the KI VJ rearrangement, and only 1/23 (4.3%) of the KI VH4JH4-expressing clones used the original CH103 UCA VJκ rearrangement (Fig. 5f), instead expressing Vκ5-39 almost exclusively. In addition, single-sorted clones were skewed toward Jκ4/5 usage (Fig. 5f), further indicating selection for κ-edited clones and suggesting that the CH103 UCA VκJκ rearrangement had been replaced by secondary rearrangement events on the KI allele. Moreover, we did not detect by flow staining higher Jκ LC usage in mature B cells from CH103 het double KI mice than in similar cells from wild-type (WT) B6 mice (Supplementary Fig. 6e), nor did we detect by single-cell repertoire analysis any single-cell clones bearing VκJκ rearrangements (Fig. 5f).

### Table 1 Frequency of antibodies isolated from CH505 envelope-vaccinated macaques

| CH505 vaccine group | Animal ID | Total antibody lineages isolated | CH505 differentials | Unique clonal lineages  
|---------------------|-----------|-----------------------------------|---------------------|------------------------
|                     |           | All antibody lineages isolated | CH505 Env+ | CH505 differentials | CH505 Env+ | % Of total Abs | Count | % Of total Abs | Count |
| CH505 TF alone      | 5346      | 15 2 13 4 | 27% | 13 2 11 2 | 15% | 2 1 1 0 | 0% |
|                     | 5356      | 2 1 1 0 | 0% | 2 1 1 0 | 0% | 0 0 0 0 | 0% |
|                     | 5360      | 11 2 9 0 | 0% | 10 2 8 0 | 0% | 4 1 3 0 | 0% |
|                     | 5261      | 78 4 74 9 | 12% | 68 4 64 7 | 10% | 25 4 21 0 | 0% |
|                     | 5670      | 27 4 23 0 | 0% | 2 0 2 1 | 50% | 0 0 0 0 | 0% |
| CH505 sequential    | 5362      | 2 0 2 1 | 50% | 2 0 2 1 | 50% | 14 0 14 1 | 7% |
|                     | 5363      | 14 0 14 1 | 7% | 11 0 10 1 | 9% | 34 11 23 3 | 9% |
|                     | 5551      | 35 1 11 24 3 | 9% | 34 11 23 3 | 9% | 34 11 23 3 | 9% |
|                     | 5692      | 40 1 35 4 4 | 10% | 39 5 34 4 | 10% | 39 5 34 4 | 10% |
|                     | 5694      | 34 1 12 22 6 4 | 18% | 33 12 21 6 | 18% | 33 12 21 6 | 18% |
|                     | 5699      | 35 1 1 35 4 | 11% | 29 1 28 4 | 14% | 29 1 28 4 | 14% |
| CH505 additive      | 5554      | 23 4 19 3 | 13% | 21 4 17 3 | 14% | 21 4 17 3 | 14% |
| sequential          | combination) | 5556      | 49 10 39 9 | 18% | 43 10 33 7 | 16% | 43 10 33 7 | 16% |
|                     | 5558      | 28 5 23 4 | 14% | 25 4 21 4 | 16% | 25 4 21 4 | 16% |
|                     | 5560      | 10 1 9 1 | 10% | 10 1 9 1 | 10% | 10 1 9 1 | 10% |
| Summary (total/%)   |           | 415 63 352 50 | 12% (50/415) | 379 61 317 44 | 12% (44/379) |
To examine if the CH103 UCA had poly and/or autoreactivity in vitro, and if LC editing of the KI LC using the editor Vκ5-39 could eliminate or mitigate it, we performed the >9600 host proto-array screen on the CH103 UCA mAb and a representative recombinant mAb, M5808-4D1, consisting of the natural V<sub>H</sub>D<sub>11</sub>/V<sub>J<sub>λ<sub>1<sub> pair from a single mature B cells (sorted from a CH103 UCA het double KI mouse) expressing an unmutated CH103 UCA HC, paired with a LC bearing the editor Vκ5-39-Jκ5 rearrangement. The CH103 UCA mAb had minimal polyreactivity but had moderate affinity for several host protein and high...
affinity for two, BAG5 and MCCC1 (Fig. 5g; left). The V_{L}-edited MS808-4D1 Ab did not bind these two candidate autoantigens, and it also lacked the moderate reactivity with various host proteins characteristic of the CH103 UCA (Fig. 5g). Thus, most naive mature B cells with Vk5-39 had low autoreactivity but also no binding to CH505 Env.

**BCR responsiveness in naive CH103 UCA double KI mice.** To study Env-reactive and Env-non-reactive mature follicular subpopulations, we used CH103 UCA double KI (homozygous) mice with sufficiently high frequencies of CH505 differentially binding double KI-positive (“Env^+”) clones (~10%; Fig. 5b, e) to allow their detection for comparison with LC-edited (“Env^-”) clones. Since one of the hallmarks of anergic B cells is downmodulation of surface IgM31, we used flow cytometry to measure IgM mean fluorescence intensities (MFIs) in both transitional and mature B cell subsets, further subfractionated based on Env reactivities. Both Env^- and Env^- transitional B cell subsets had IgM MFIs comparable to those in WT B6 mice. In contrast, Env^- mature B cells from naive CH103 UCA double KI mice had lower surface IgM densities than did Env^- mature B cells (Fig. 6a, b), which had IgM MFIs comparable to those of WT B6 transitional B cells (Fig. 6b), suggesting that the Env^- cells had become anergic after bypassing the second checkpoint. The majority of mature B cells that had undergone LC editing (and lost Env reactivity) were not only rescued from clonal deletion but also rescued from anergy.

Consistent with the above IgM MFI data, both Env^- and Env^- transitional B cells from CH103 UCA double KI mice had Ca++ responses to saturating concentrations of anti-Ig similar to those of transitional B cells from WT (B6) mice (Fig. 6c, d). At sub-saturating concentrations, however, Env^- splenic transitional cells had attenuated Ca++ responses (Fig. 6d), suggesting they were partially anergic. In contrast, Env^- mature B cells from CH103 UCA double KI mice had modestly attenuated Ca++ responses to saturating concentrations of anti-Ig, but more attenuated Ca++ signaling at limiting amounts of anti-Ig (Fig. 6c, d). This gradient of anergy, in which the most accentuated decreases in BCR signaling capacity were in mature Env^- B cell subsets, either present in mature follicles or recirculating in BM, rather than in the prototypical T3 transitional compartment32, is analogous to auto-Ab KI models in which anergic clones have also been found in various mature B cell subsets32, 33.

**Env activation of CH103 double KI-positive B cells.** TF Env gp120 binds the CH103 UCA IgG antibody in vitro more tightly (K_D in the high nM range5) than activation thresholds estimated for triggering naive B cells in vivo35. Given the anergic status of naive CH103 UCA double KI-positive mature B cells (Fig. 6a–d), however, such clones could require stronger BCR and/or T cell signals, the amounts of which can vary depending on stage and degree of silencing32.

To test whether TF Env could activate the naive B cell repertoire in CH103 UCA double KI mice, we examined Ca++ responses of their transitional and mature B cells in vivo, using TF Env concentrations that saturated their BCRs (Fig. 6c). Since we could not directly compare responses in Env^- and Env^- subsets (due to cross-blocking issues associated with using TF Env both as a detection and BCR-stimulating reagent), we evaluated total transitional and mature subsets that are enriched for Env^- and Env^- clones, respectively. TF Env gp120 tetramers induced Ca++ flux near levels induced by the anti-Ig control in transitional B cells from CH103 UCA double KI mice, but not in WT B6 mice (Fig. 6e; bottom left), showing that double KI clones could be specifically activated by this CH103 UCA-targeting immunogen. The effect was not seen with TF gp120 monomers (Supplementary Fig. 8a), indicating a requirement for multimeric presentation of antigens to mediate the Ca++ flux in anergic double KI-positive clones. Similar results were also seen in peripheral (splenic) transitional and mature B cells (Supplementary Fig. 8b).

TF Env gp120 tetramers induced low levels of Ca++ mobilization (10% of those induced by the anti-Ig control) in mature B cell subsets of CH103 UCA double KI mice (Fig. 6e; bottom right), but did not induce Ca++ flux in mature B cells of WT B6 mice (Fig. 6e; bottom row). While the low, but detectable Ca++ induction may have been expected, given the anergic status of the double KI mature B cell subset, the level induced was similar to the percentage of clones in the subset that retained Env reactivity (10%). Thus, despite their anergic status, these mature CH103 UCA expressing B cell clones can respond to TF Env gp120, provided that the latter is present at high enough concentration and in multimeric form.

**Vaccine-induced responses in CH103 UCA double KI mice.** We next studied B cell responses from CH103 het double KI mice immunized either twice with TF Env in the TLR4 agonist adjuvant, GLA-SE, or after the first two steps of the 4-valent sequential vaccine regimen, that is, TF and week 53 CH505 Env^6 (both in GLA-SE), to evaluate whether immunization could initiate the CH103 bnAb B cell lineage (Fig. 7a, b, Supplementary Fig. 8).

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**Fig. 3** Characteristics of macaque DH522 lineage antibodies. a Immunogenetics of clonally related DH522 lineage antibodies inferred by the Clonolyst software program. DH522.1 and DH522.2 antibodies were isolated after 4 (21 weeks) and 6 (59 weeks) immunizations, respectively. b Neutralization profile of DH522.1 and DH522.2 monoclonal antibodies (mAbs) for viruses previously tested by plasma from CH505 Env-vaccinated macaques. Neutralization was performed in TZM-bl cells and titers reported as μg/mL IC50. c DH522.1 and DH522.2 competition for binding epitopes targeted by CD4-binding site bnAbs and soluble CD4. Palivizumab was used as a negative control. d Binding profile of DH522 lineage antibodies to a panel of >9600 autoantigens via lucern as previously described44. Shaded gray region; autoantigen binding to each mAb, and a polyreactivity profile for each mAb. e Footprints of DH522.2 and two related antibodies, on the surface of deglycosylated gp120 Env monomeric cores. Surface of deglycosylated gp120 in gray and antibody footprint in blue; the surface of deglycosylated HIV-1 YU2 gp120 core in the DH522.2 complex structure is underlain by a trace of the deglycosylated gp120 core in the compound-probed structure. (DH522.2) Structure of DH522.2—hydrophobic side chains of the Val-Leu-Phe motif at the tip of DH522.2 HCDR3 interact with a pocket on the surface of gp120 lined by loop B and adjacent to the CD4-binding site; (F105) the Val-Phe-Tyr hydrophobic tip of the F105 HCDR3 interacts with the same hydrophobic pocket on gp120 (PDB: 3HI5); (CH103) the CH103 footprint rests heavily on the CD4-binding loop and V5 (PDB: 4JAN). Crystal structure of the CH103 complex had only the gp120 outer domain; the inner domain (lighter gray) was modeled from the DH522.2-bound structure. f Superposition onto the BG505 SOSIP.664 gp140 trimer model of the deglycosylated gp120 cores in structures of complexes with DH522.2, F105, and CH103; view is along the gp140 threefold axis. g Footprints of CD4-mimic, CD4-binding site bnAbs (VRC01, CH235), and CD4 attachment inhibitor N-(4-bromophenyl)-(N^+)(2,6,6-tetramethylylperidin-4-yl)ethanediamide (NBD-557). h Negative stain electron microscopy analysis of DH522.2 in complex with the fully glycosylated CH505 transmitted-founder (TF) SOSIP.664 trimmer. h Electron microscopy (EM) reconstruction of a DH522.2-SOSIP.664 complex; b12-bound gp120 was docked into the EM map (PDB: 2NY7). i 2D class averages of the DH522.2-SOSIP.664 complex. Fabs indicated by the red arrows. j The Fourier shell correlation curve is shown along with the resolution and is determined using FSC = 0.5.
Fig. 4 Immune tolerance in CH103 germ line knock-in mice. a-c Bone Marrow B cell development in naive CH103 unmutated common ancestor (UCA) double knock-in (dKI) mice, compared to wild type (B6), showing anergic phenotype and reduced frequencies of mature B cells associated with the second tolerance checkpoint. a Flow histograms indicating percentages in progenitor/precursor (Pro/pre), Immature (Imm), Transitional (Trans), and Mature (Mat) subsets. Data gated on live, total (CD19+/B220+) B cells. b Graphical summary of data shown in (a) for several mice. c-e Peripheral B cell development in naive CH103 UCA dKI mice, showing reduced total B cell numbers and frequencies of follicular mature B cells. e Representative flow histograms indicating percentages of total, live gated splenic B cells in transitional (Trans), marginal zone (MZ), and mature follicular (Mat) subsets. Graphical representation of total splenic B cell numbers (d) or mature to transitional ratios (e), as a measure of developmental arrest (transitional B cell accumulation) in periphery. Circles represent individual mice, and means are denoted by black bars. *P < 0.05; **P < 0.005, two-tailed Student’s t-test. n.s. not significant.
Fig. 5 Analysis of receptor editing in CH103 germ line knock-in mice. a–c Env reactivity in mature B cell compartments of CH103 unmutated common ancestor (UCA) double knock-in (dKI) mice. a Representative pseudocolored dot plots showing typical patterns of reduced TF Env+ mature (follicular) B cells in naive CH103 UCA dKI mice. Shown as controls for background TF Env staining are WT (B6) mice. b Graphical summaries of percentages of differential binding in transitional and mature compartments in naive CH103 UCA dKI mice. Each dot represents an individual animal. ***P < 0.001, two-tailed Student’s t test. c Regression analysis of developmental blockade severity and residual mature B cell fraction retaining Env specificity. d Loss of CH103 KI light chain (LC) rescues splenic mature B cell development and reverses anergic phenotype in CH103 UCA V_{H4-59}^{dKI}+/+ (“heavy-chain (HC)-only”) KI mice. **P < 0.005, two-tailed Student’s t test. e Progressively decreased differential binding of splenic transitional and mature B cells (means ± SEM) in CH103 UCA dKI (n = 6), heterozygous (het) dKI (n = 5), and V_{H4-59}^{dKI}+/+ KI (n = 4) mice. CH01 het dKI mice (n = 2), a model that undergoes no negative selection, and WT B6 mice (n = 4) are shown as positive and negative controls for Env binding, respectively. f, g Extensive and highly-restricted receptor editing of the CH103 J3-20 LC by “master editor” Vx5-39, as revealed in CH103 UCA het dKI mice. f Receptor editing in CH103 UCA het dKI mice. Shown are pie charts of HC/LC usage and breakdown of Vx family and Jx usage, among LCs paired to the KI V_{H4-59}^{dKI}+/+ HC. Individual mature B cells from naive CH103 het dKI mice were obtained by single-cell sorting on the total (unselected for TF Env binding) mature follicular B cell repertoire, and V_{H4-59}^{dKI}/V_{Jx} Ig gene pairs from single cells were recovered by RT-PCR for sequencing and immunogenetic analysis. g Polyreactivity/autoreactivity profiles of the CH103 UCA and Vx5-39 editor MS808 monoclonal antibodies (mAbs) on human proteoarrays. The >500-fold binding compared to control mAb, that is, the official autoreactivity “cutoff,” is indicated by dashed lines. Also shown are candidate autoantigens bound by the CH103 UCA mAb (circled in blue, in the red shaded box) and to which binding by MS808 is eliminated (green-shaded box).
precursors in follicles of CH103 UCA heterozygous double KI mice can be initially induced to expand, switch, secrete serum Abs, and form antigen-specific memory in vivo, in response to sequential CH103 lineage-targeting Env immunogens.

We sorted single, differential-binding IgG+ memory splenic B cells after the second immunizations and recovered HC/LC pairs for sequence analysis. As expected, differentially sorted IgG+ memory B cells from immunized mice had higher frequencies of KI HC-expressing clones that paired with KI LCs than did total IgG+ memory B cells from control mice immunized with saline or GLA-SE alone (Fig. 7d, e). This enriched, immunization-induced, IgG+ differentially sorted population (32% overall, compared to 4% in IgG+B cells from saline-immunized animals), confirms that some of the differentially binding memory population in
sequentially immunized animals were bona fide anergic double KI-positive clones that had been re-activated to switch and form memory. Of these double KI IgG memory clones, somatic hypermutation (SHM) rates were low but detectable, with six out of nine having amino acid changes in their KI HC and/or LCs (Fig. 7f), but the relatively modest changes (Supplementary Fig. 10) suggest incomplete (partial) activation, and are analogous to the differential-binding population with relatively longer HCDR3s observed in macaques (Fig. 2f, g). In contrast to the high fraction of differentially binding double KI-positive clones bearing somatic mutations, none of the differentially binding clones that retained only KI HCs and just 18% of those that retained only KI LCs bore somatic mutations (Fig. 7f). This difference suggests that immunization might break anergy in rare double KI-positive clones, initiating SHM as well as driving switch and Ab secretion, while LC (or HC)-edited (non Env cross-reactive) clones (which are fully competent to signal (Fig. 6a–d)), yet are not engaged by TF Env ex vivo (Fig. 6e), are also not primed by TF Env in vivo. An alternative, non-mutually exclusive possibility is that SHM might be initiated in double KI-positive clones, to drive specificity away from self (“affinity reversion”), which would not be required in clones that have already expressed their KI HC or LCs.

### Discussion

We have demonstrated in this study that sequential immunization of both macaques and CD4-binding site HCDR3-binder nAb VH + Vλ KI mice can initiate neutralizing antibody lineages. In macaques, the sequential immunogen induced antibodies with limited neutralization breadth, likely due both to incomplete activation of self-reactive, lineage-specific bnAb clones as well as the unavailability of a suitable IGLV to pair with IGHV4-J for bnAb activity. In CH103 KI mice, we observed that a large proportion of antibodies used a similar VHb, but different Vλ than did the CH103 UCA, showing that receptor editing was one host tolerance mechanism limiting development of CD4-binding site HCDR3-binder bnAbs. A second major tolerance mechanism in CH103 UCA KI mice was anergy in non-receptor-edited mature B cells. These data suggest that in addition to designing sequential Env antigens to select for the desired bnAb lineage SHMs, it will be necessary to formulate sequential vaccines with adjuvants and/or drugs to transiently circumvent immune checkpoints and overcome anergy.

We have previously reported that the CH103 UCA was not polyreactive using 10 autoantibody assays, whereas mature CH103 lineage antibodies acquired polyreactivity coincident with the onset of bnAb activity. In a more stringent analysis of polyreactivity with a >9600 human protein chip assay, we found that indeed, the CH103 UCA was autoreactive. Thus, like the 3BNC60 UCA VHλ KI model, expressing naive B cell precursors bearing germ line-reverted V(D)J rearrangements of the CD4-mimicking bnAb 3BNC60,17, the HCDR3-binder CD4-binding site CH103 bnAb UCA double KI clones are also deleted in mature B cell development prior to immunization and subjected to extensive LC receptor editing. The mechanism by which editing occurred in the two models was different (lambda vs. restricted kappa usage), which could be explained either by the way the models were designed and/or by differences in stringency of editing demanded by the degree of tolerizing self-reactivity these two bnAb specificities imparted in vivo.

The CH103 UCA heterozygous double KI (het; VHDIH1λ/ -/VJκ1,-/2,3) model we used here differs in two ways from other recently reported CD4-binding site “germ line-reverted” models13, influencing our ability to study the full bnAb lineage maturation pathway that sequential immunization will likely need to recreate. The first distinction is expression of a fully unmutated VHDIH1λ rearrangement from the bona fide time-of-infection CH103 UCA, in contrast to the germ line-reverted KI models using hybrid rearrangements with germ line VHλ and matured bnAb HCDR3s10,12. The second distinction is that the specific LC targeting strategy in our model16,18 accounts for more physiologically relevant tolerance mechanisms of LC receptor editing than do those of other bnAb double KI (HC + LC) KI models38,39. This feature, when in combination with use of this model as a heterozygous knocked-in version at both the HC and LC loci, allowed us to evaluate physiological tolerance effects on the pre-immune repertoire. Thus, the CH103 KI mouse model represents a high bar for testing clonal competition, because a true germ line (UCA) precursor is present in a semi-polyclonal system that is most analogous to an adoptive transfer landscape.

Recent evidence for the mechanism of preferential induction of competing “off-target” CD4-binding site positive responses, at the expense of CD4-binding site positive bnAb responses has been recently reported in competition studies between precursors of CD4-binding site positive bnAb and non-bnAbs using transformed B cell lines in vitro.40 Our ex vivo Ca2+–signaling data in this study extend these findings and provide a direct mechanism for incomplete maturation in vivo: exclusion of anergic bnAb lineage precursors from participating in T-dependent responses at the expense of both edited and off-target clones. A model based on this mechanism predicts that bnAb clones will be at a survival disadvantage and will be outcompeted by two populations. Initially (prior to Env immunization), such anergic bnAb lineage clones will be excluded by those predominating the mature repertoire that are non Env-reactive (due to editing their LCs in response to autoantigen or environmental antigen). Such LC-edited clones, whether they completely eliminate or maintain partial self-reactivity would thus be predicted to outcompete anergic bnAb clones, since analogous clones with low-degree or “acceptable” self-reactivity, have been found to spontaneously populate the germinal centres (GC) niche in certain autoimmune mouse strains.41,42 or autoimmune conditions.13 Then later, in an immunization setting involving Env, a highly complex, multi-epitope immunogen (that even in trimeric form has a high

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**Fig. 6** Signaling responsiveness of transitional and mature B cell subsets from CH103 germ line knock-in mice. **a** Representative flow histograms showing selectively reduced IgM mean fluorescence intensity (MFI), in differential-binding transmitted-founder (TF) Envelope (Env+) mature B cell fractions in CH103 unmutated common ancestor (UCA) double knock-in (dki) mice, relative to Env− (light-chain (LC)-edited) fractions. Data show gating for Env-specific bone marrow (BM) and splenic Env+ and Env−trans or mat B cell fractions, and is gated on live total (B220+) B cells or live total splenocytes, respectively. **b** Graphical summary (means + SEM) of data shown in (a) performed on eight mice per strain. **c** Ex vivo proximal signaling responses to BCR cross-linking in naive B cells from CH103 UCA dki and control B6 mice, based on Ca2+ levels (Fluo-4 MFI) before/after anti-IgM stimulation. Shown are representative data from a single CH103 UCA dki mouse of Ca2+ responses, to varying concentrations of anti-BCR, in mature B cells, either recirculating in BM (upper row) or in spleen follicles (lower row). Also shown for comparison is proximal signaling of mature B cells from control WT B6 mice. **d** Graphical representation of Ca2+–signaling responses (means ± SD) in both transitional and mature (splenic or BM B cells). Data are cumulative from four mice and two independently performed experiments. **e** CH103 UCA dki transitional and mature B cell subset responses to priming immunogen TF Env. Data shown are from experiments performed in BM B cell fractions, using saturating amounts (100 nM) of tetramerized TF Env gp120 or anti-BCR (as a positive control for 100% of B cells signaling). *P < 0.05; **P < 0.005; ***P < 0.001; two-tailed Student’s t-test. n.s. not significant.
capacity for triggering off-target clones), anergic bnAb B cell clones may be further excluded from GCs by non-anergic, off-target (that is, non-differential Env+) clones induced by Env vaccination. We predict that such clones will begin arising at high frequencies in memory IgG populations after multiple immunizations and after numerous rounds of preferential selection by immunization. Their presence will make it difficult for bnAb lineage clones to compete for the GC niche and form long-lived memory, thus requiring much stronger local CD4 helper and/or BCR signals to overcome this hurdle.

![Diagram](image-url)

**Figure a**
- **Controls**
  - Saline only
  - GLA-SE only
- **Immunizations**
  - TF Env (×2) In GLA-SE
  - Sequential (TF+w53 Envs) In GLA-SE

**Figure b**
- Differential IgG+ memory B-cells / spleen
- Saline, GLA-SE, TF (×2), Seq. (TF+w53)

**Figure c**
- Env+ serum IgG (Log AUC)
- Post-immunization #
- Saline, GLA-SE

**Figure d**
- Sort:
- Total IgG+ memory
- Differential IgG+ memory
- V_H usage
- V_L usage (paired to KI V_K)

**Figure e**
- All immunized
- Repeated TF
- Sequential

**Figure f**
- Saline and GLA-SE
- All immunized
- Mut
- Unmut
- All KI+
- dKI (HC+LC)
- HC KI
- LC KI

V_K usage in individual mice
A key question is why TF followed by week 53 Env led to more differential Env binding (lineage-positive/undefined) B cells, that is, enriched for double KI clones, expressing the original UCA V_{H IDH/V_{L J1}} pair. Although we do not have direct evidence for the mechanism behind this, we do not think week 53 Env directly rescued such differential Env-binding B cells, since we do not think week 53 Env has high enough affinity for the UCA to activate anergic B cells expressing the UCA. However, week 53 Env may indirectly help “break” anergy in double KI clones by: (i) diverting the non-lineage positive B cell response, thus reducing competition with anergic clones; this would specifically occur via week 53 Env potentially cross-reacting more strongly than TF with either pre-immune KI light-chain-edited (TF Env+) clones and/or vaccine-induced KI heavy-chain-edited (TF Env+ but non-differential) clones that start appearing after TF priming; or (ii) activating/expanding the rare double KI-positive clones initially activated by TF to switch and expand, because, as shown in this study, such clones exhibit more SFM (although limited amounts) than heavy- or light-chain-only KI clones, which could produce a relatively high fraction of clones with affinity for week 53 Env.

In conclusion, future studies should focus on overcoming the factors that limit induction of CH103 bnAbs, by testing new CH505 Env vaccine regimens in CH103 KI mice and in macaques. These regimens should increase the precursor pool, by selection for non-LC-edited clones, and during immunization, they should enhance the strength of in vivo Th and/or BCR signals to the lineage-specific anergic clones, in order to provide a survival advantage to bnAb B cell lineages over “off-target” B cell lineages. It will also be critical to test sequential CH505 Env Clade C gp120 immunogens in humans, since macaques do not have orthologs of the human genes that encoded the CH103 bnAbs induced by CH505 Env.

Methods

Study design. We previously identified a transmitted-founder (TF) clade C (CH505 TF) HIV-1 envelope (Env) that induced CD4-binding site binding site bnAbs CH103 and CH235 in an HIV-1-infected African individual (CH5055). Ab-virus co-evolution studies in the CH505 HIV-1-infected individual revealed that bnAbs arose after extensive Env diversification. Using this roadmap of Ab-viral co-evolution, we selected a 4-valent CH505 Env regimen and produced them as gp120 candidate Env immunogens for vaccination in adult non-human primates (NHPs) and CH103 UCA KI mice. We hypothesized that the CH505 Env would induce CD4-binding site, CH103-, and/or CH235-like nAbs. Thus, to test the immunogenicity of the CH505 Env immunogens, we immunized HIV-1 uninfected rhesus macaques. Furthermore, to test the ex vivo and in vivo immunogenicity and specificity of CH505 Env Us in macaques, and to define the host mechanisms impacting the development of HC033-binding, CD4- binding site antibodies both prior to, and in response to immunization, we generated CH103 UCA heavy-chain and/or light-chain-expressing KI mice and evaluated the immune response to CH505 Env regimens used in macaques.

Ethics. Indian-origin rhesus monkeys used in the immunization studies (NHP79 and NHP88) were housed and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution in accordance with the principles of the National Institute of Health. All studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in BIOQUAL (Rockville, MD). BIOQUAL is fully accredited by AAALAC and through OLAW, Assurance Number A-3086. The animal protocol used in this study was approved by the BIOQUAL IACUC. All physical procedures associated with this work were done under anesthesia to minimize pain and distress in accordance with the recommendations of the Weatherall report, “The use of non-human primates in research.” Teklad 5038 Primate Diet was provided, once daily by animal size and weight. The diet was supplemented with fresh fruit and vegetables. Fresh water was provided ad libitum.

Recombinant Env gp120 expression. One mg of plasmid DNA per 1 liter of cells was diluted in DMEM and mixed with PEI. PEI DNA mixtures were added to 293F cells (ThermoFisher, catalog #R79007) for 4h. 293F cells were subsequently washed and diluted to 1.25 million cells per ml in Freestyle293 media (ThermoFisher). The cells were cultured for 5 days, and on the fifth day, the cell culture media was cleared of cells by centrifugation and filtration with a 0.8 μm cellulose membrane (Nalgene). The cell culture media was concentrated with a vial-fallow 50 with a 10 kD molecular weight cutoff. The concentrated cell culture supernatant was rotated with lectin beads (Vistart Labs) in MES pH 7.0 buffer overnight at 4°C. The beads were pelleted by centrifugation the next day and resuspended in MES pH 7.0 wash buffer. The beads were washed twice and the glycoblotted HIV-1 gp120 was eluted with 0.5 M methyl-β-pyranoside. The protein was buffer-exchanged into phosphate-buffered saline and stored at –80°C. Monomeric recombinant gp120 was purified by size exclusion chromatography (SEC) in phosphate-buffered saline using a Superdex200 10/300 gel filtration column (GE Healthcare) and stored at –80°C. The glycans on recombinant gp120 Env have been shown to be more complex residues compared to glycans on virus associated gp160 Env57.

HIV-1 CH505 Env immunizations. Previous studies have shown the advantages, synthetic monophosphoryl lipid A in a stable emulsion (GLA-SE)47, and liposome-based formulation with MPL and QS21 (AS01E)49, used in this study to be effective for enhancing humoral immune responses to a number of infectious agent products (eg, CH505 TF gp120 Env) were selected as Env immunogens based on affinity for intermediate (IA) and mature antibodies in the CH103 lineage20. In addition, we used surface plasmon resonance (SPR) analysis to determine the affinity of CH103 lineage antibodies to the 4 CH505 Env gp120 Env selected for our immunization regimen. Each CH103 lineage mAb binding to CH505 Env Us (TF, week 53, week 78, week 100) was measured by injecting Env proteins at varying concentrations (0.5–25 μg/mL) over each mAb captured on anti-rhesus IgG immobilized Ab (Millipore) on a CM5 sensor surface8 with the T200 SPR platform. We found that CH103UCA bound only autologous CH505 TF gp120 (321 kDa), while...
CH103 intermediate and mature Abs bound with higher binding affinity to CH505 TF and natural variants. CH505 TF (CH103 IA3, 23 nM, CH103 IA2, 12.4 nM; CH505 TF H6627 week 53 (CH103 IA3, 13.5 nM; CH103 IA2, 50.2 nM; CH106, 30.2 nM); CH505 week 78 (CH103 IA3, 96.3 nM; CH103 IA2, 30.9 nM; CH106, 12.7 nM); and CH505 week 100 (CH103 IA3, 91.2 nM; CH103 IA2, 15 nM; CH106, 12.3 nM).

We immunized a total of 44 macaques in eight groups with CH505-transmitted-founder (TF) or natural variants of the TF (week 53, week 78, week 100) envelope (Env) alone, or sequential combinations of TF and TF-virants in AS01E (NHP88 study) or GLA-SE (NHP79 study) adjuvants. Of 44 macaques aged 3–4 years, 4 (5628, 5644, 5714, and 5724) were female and the remaining 40 were males. Immunizations were administered intramuscular in the leg of each animal with 100 µg of total CH505 gp120 proteins for six vaccinations. For macaques immunized with CH505 gp120 Env in AS01E (NHP88), animals were immunized on a 6-week interval for the first 4 immunizations, followed by a 7.5-month interval until boost #5 and a 3-month interval from boost #5 to #6. Vaccine group 1 animals received sequential CH505 gp120 Env regimens (+AS01E) as follows: IMM #1–#6, IMM #2–#4, #3–#5, and #5–#6–#10. In contrast, vaccine group 2 animals received CH505 TF gp120 Env alone (+AS01E) at all immunizations (IMM #1–#6–#TF). For macaques immunized with CH505 gp120 Envs in GLA-SE (NHP79), animals were immunized on a 6-week interval for the first 5 immunizations, followed by a 8.2-month interval until boost #6. Vaccine group 3–6 animals received only one CH505 gp120 Env (+GLA-SE) for all immunizations (IMM #1–#6); group 3 (TF), group 4 (week 53), group 5 (week 78), and group 6 (week 100). In contrast, vaccine groups 7–8 animals received sequential combinations of CH505 gp120 Envs (+GLA-SE): group 7, IMM #1–#TF, IMM #2–#5, IMM #3–#7, IMM #4–#9, and IMM #5–#9–#10, and group 8, IMM #1–#TF, IMM #2–#7 + week 53, IMM #3–#7 + week 53 + week 78, IMM #4–#7 + week 53 + week 78 + week 100, IMM #5–#10 + week 3 + week 100, and IMM #6–#10 – week 100.

For all immunizations with CH103 KI Env models, a minimum of 4 mice per immunization group that were distributed equally were used, all were 8–12 weeks old at the start of the immunization study. Mice with all Duke University Institutional Animal Care and Use Committee-approved animal protocols. Mice were immunized up to 8 times with saline (control groups), 5 µg of the TLR4 agonist CpG-Oligo-2006. Immunization protocols and treatment with 0.02% sodium azide, and for samples stained with LIVE/DEAD staining buffer (LifeTech), spun down, and then stained in FACS buffer containing 1 × PBS (pH 7.2), 3% FBS (HyClone), 0.01% sodium azide, and premixed combinations of fluorochrome-labeled mAbs at titration-determined optimal concentrations. Total B cell lymphocytes. Primary labeled mAbs (all from BD Biosciences) were as follows: 0.5 µg/mL of anti-B220 BV650 (catalog #563893), anti-D19 APcR700 (catalog #565473), anti-igG BV310 (catalog #563110), anti-D19 FITC (catalog #553443), anti-igG2a/b FITC (catalog #553399), anti-D19 IgG (catalog #553403); all Abs were obtained from BD Biosciences. IgG2a memory B cells were visualized by direct staining with anti-D19 FITC (catalog #553403) followed by exclusion of LIVE/DEAD™-near stained cells (to discriminate live from dead cells), subsequent gating for CD19+ B220+ (total B cells), and finally, gating for CD38+ IgG+ IgD+ (class-switched, memory B cells). CH103 lineage specificity of the IgG2a memory B cell subset was detected by using the combination of tetramers, Alexa 488, and Brilliant Violet 421-transmitted HIV Env gp120 wild-type or mutant CH505 transmitted-founder virus-derived proteins (TF-BV421 and A371 TF-AF647, respectively, with differential binding to the former, but not the latter, indicating CH103 ba/Ab lineage-specific binding to the Env CD4-binding site, as previously described29. Differential binding (TF Env, A371 TF), switch tetramers (Δ371+CD38+IgG+IgD+) B cells induced in Env-immunized mice, and for comparison, non-HIV Env binding (WT TF, A371 TF) switched B cells from naive, control (saline or "adjuvant alone")-immunized mice, were sorted using a FACSAria II (BD Biosciences) into BioExpress 96-well plates (T-3085-1) containing 20 µl of SuperScript™ III reverse transcriptase buffer (LifeTech) as previously described29. Sorted single-cell suspensions from naive and immunized mice that were stained with fluorochrome-labeled wild-type and mutant (Δ371) CH505 TF Env gp120 tetramers, also as previously described29.

Mouse B cell phenotypic analysis by flow cytometry. Flow cytometric analysis of B cell development and responses was performed as described16, 18, 19. Briefly, single-cell suspensions from spleen, draining lymph nodes (dLNs), and BM were isolated from 8–2-week-old naive C57/10CAU vaccine or adjuvant alone mice (as controls), or mice immunized with CH103UCA het double KI mice. A total of 106 naive or immunized cells were stained with LIVE/DEAD staining buffer (LifeTech), spun down, and then stained in FACS buffer containing 1 × PBS (pH 7.2), 3% FBS (HyClone), 0.01% sodium azide, and premixed combinations of fluorochrome-labeled mAbs at titration-determined optimal concentrations. Total B cell lymphocytes. Primary labeled mAbs (all from BD Biosciences) were as follows: 0.5 µg/mL of anti-B220 BV650 (catalog #563893), anti-D19 APcR700 (catalog #565473), anti-igG BV310 (catalog #563110), anti-D19 FITC (catalog #553443), anti-igG2a/b FITC (catalog #553399), anti-D19 IgG (catalog #553403), anti-D19 PE-Cy7 (catalog #553867), anti-D19 BV214 (catalog #562750), anti-D19 FITC (catalog #553318), and anti-D19 PE-Cy5 (catalog #563805); and 0.2 µg/mL of anti-mouse T and B cell activation antigen PE (catalog #561530) and anti-Fas PE-Cy7 (CD95, catalog #557653). Flow cytometric analysis of subset B cell reactivities for CH103 lineage-specific CD4-binding site specificity was performed similarly using single-cell splenocyte, dLN, or BM suspensions from naive and immunized mice that were stained with fluorochrome-labeled wild-type and mutant (Δ371) CH505 TF Env gp120 tetramers, also as previously described29.

Isolation of macaque antibody genes and evaluation of immunogenetics. Heavy-(IGHV) and light (IGLV, IGLJ)-chain genes were isolated via single-cell PCR52, 53. The gene sequences were then computationally analyzed and evaluated for immunogenetics (gene family and segment Ds, mutation frequency, CDR3 length) determined using extended VDJ amalgams for either the common J558 or CH103 lineage. We studied using the 2015 Clostralid rhesus gene library. Antibody clone lineages were inferred as sequences that had the same IGHV VDJ rearrangement and CDR3 length, and paired with the same light chain (lg V segments). The automated inference was followed up by visual inspection of the DNA sequence alignments for conformational accuracy. Heavy and light chain gene sequences for the inferred UCA and IAs were produced commercially and used to generate purified recombinant mAbs.

Isolation of mouse antibody genes. Mouse Heavy (Hc) and Light (Lc) genes were isolated via single-cell PCR5, 12. The gene sequences were then computationally analyzed and evaluated for immunogenetics (gene family and segment Ds, mutation frequency, CDR3 length) determined using extended VDJ amalgams for either the common J558 or CH103 lineage. We studied using the 2015 Clostralid rhesus gene library. Antibody clone lineages were inferred as sequences that had the same IGHV VDJ rearrangement and CDR3 length, and paired with the same light chain (lg V segments). The automated inference was followed up by visual inspection of the DNA sequence alignments for conformational accuracy. Heavy and light chain gene sequences for the inferred UCA and IAs were produced commercially and used to generate purified recombinant mAbs.
Usage was determined by querying amplified sequences to both the original CH103 UCA rearrangements and relative to C57BL/6 Ig germ line sequences in the IMG-EMB/High/VQD/QV2 database software. CH103 was sequenced in Kl Vδ1D6 and Vj1. Kl rearrangements sequenced in both directions were analyzed for SHMs using DNASTAR MegAlign Pro multiple sequence alignment software.

Expression of macaque antibody genes as IgG1 recombinant mAbs. Plasmids encoding the IGHV, IGKV, and IGJL genes were generated and used for recombinant expression in embryonic kidney epithelial (HEK) cell lines 293T (ATCC, Manassas, VA; catalog #CRL3211) in small-scale transfection and in suspension Exp2 293F cells (Invitrogen; catalog #A14527) for expression of large quantities of purified mAbs. Purified recombinant mAbs were analyzed by PBS, analyzed, and stored at 4°C.

Antibody binding. Plasma antibody reactivity with CH505 Env was determined via standard enzyme-linked immunosorbent assays (ELISA) and binding titers reported as log area under the curve (AUC)70. Recombinant mAbs and plasma antibodies were screened for reactivity with HIV-1 Env and corresponding mutant proteins with a disruption of the CD4-binding site in ELISA66; these proteins included CH505 TF gp120, CH505 TF gp120 Δ371I, YU2 gp120, YU2 D368R gp120, RSC3, RSC3 Δ371I, and RSC3 P363NI371I gp120 proteins66. Abs that bound CH505 TF gp120, but not CH505 TF gp120 Δ371I, are candidate CD4-binding site antibodies referred to as CH505 differentials; CH505 non-differentials bind equally well to both proteins.

Antibody-complex inhibition. Plasma and purified recombinant mAbs were evaluated for blocking well-characterized antibodies as previously described66. For CD4 (binding site) blocking assays, we used sCD4 (Progenics Pharm Inc.) or CH106 nAb66. Competitive inhibition was measured as the ratio of binding in the presence and absence of inhibitory molecules, CH106 or sCD4.

Neutralization assays. Plasma or purified mAbs were screened for neutralization using the well-established TZM-bl assay as described65. Plasma post surgery/immunization neutralized autologous tier 1 (CH505.w4.3) and heterologous tier 1 (M9W65, SF162, 6644, D263) and two (57128) viruses. Heterologous tier 2 viruses tested for which we did not observe plasma neutralization are as follows: CON-5, 45, 01DF, JRFL, YU2, ZM173SF.P042, Q66, 1Q68, and BG1668. DH522 lineage mAbs were tested for neutralization breadth in a panel of 199 geographically diverse HIV-1 Envs used in in vitro and in vivo neutralization assays. These included CH505 TF gp120, CH505 TF gp120 Δ371I, YU2 gp120, YU2 D368R gp120, RSC3, RSC3 Δ371I, and RSC3 P363N-I371I gp120 proteins66. Abs that bound CH505 TF gp120, but not CH505 TF gp120 Δ371I, are candidate CD4-binding site antibodies referred to as CH505 differentials; CH505 non-differentials bind equally well to both proteins.

Serum ELISA assays for CH103 UCA double kl mIg. Tilters of class-switched serum antibodies reactive for the wild-type CH505 TF Env protein, or a mutant version with a disruption of the CD4-binding site (TF Δ371I), were determined by ELISA, based on described methods66. Brieﬂy, mouse sera, collected 10 days after immunization neutralized autologous tier 1 (CH505.w4.3) and heterologous tier 1 (M9W65, SF162, 6644, D263) and two (57128) viruses. Heterologous tier 2 viruses tested for which we did not observe plasma neutralization are as follows: CON-5, 45, 01DF, JRFL, YU2, ZM173SF.P042, Q66, 1Q68, and BG1668. DH522 lineage mAbs were tested for neutralization breadth in a panel of 199 geographically diverse HIV-1 Envs used in in vitro and in vivo neutralization assays. These included CH505 TF gp120, CH505 TF gp120 Δ371I, YU2 gp120, YU2 D368R gp120, RSC3, RSC3 Δ371I, and RSC3 P363N-I371I gp120 proteins66. Abs that bound CH505 TF gp120, but not CH505 TF gp120 Δ371I, are candidate CD4-binding site antibodies referred to as CH505 differentials; CH505 non-differentials bind equally well to both proteins.

Calcium flux analysis in CH103 UCA double kl mIg. For experiments evaluating specific cell lines, B WT/6 and CH103 UCA double kl 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 with LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific). Intracellular proteins evaluations were stained and cell suspensions were directly stained with various combinations of cell surface markers for BM fractionation into pre-B, immature-B, transitional B, and mature B subsets included 0.5 µg/mL of anti-B200 B786 (catalog #563894), anti-CD19 APCR700 (catalog #565473), anti-CD43 BV605 (catalog #563205), anti-IGM PE-Cy®5 (catalog #552867), and anti-IGD BV605 (catalog #563003). Likewise, cell surface marker combinations used for spleen subfractionation into transitional-B and mature-B subsets included 0.5 µg/mL of anti-B200 B786 (catalog #563894), anti-CD19 APCR700 (catalog #565473), and anti-CD3 BV605 (catalog #563807). All subsets were also further subdivided for CD4-binding site82 CH103 lineage reactivity by including BRD-21 fluorescent dye-conjugated antibodies in the pre-stain mix. For both sets of experiments, pre-stained B cells were loaded with Fluo-4 via thorough washes in HBSS, followed by mixing with equal volumes of 2x 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 µg/mL anti-IgM F(ab)2 (Southern Biotech). Fluo-4 MFI data for transitional (B200/CD93+) B cells was acquired on a BD LSR II flow cytometer and analyzed by Flowjo software.

Electron microscopy. To generate the autologous HIV-1 CH505 SOSIP.664 expression construct, we followed established SOSIP design parameters83. Briefly, the CH505 SOSIP.664 trimer was engineered with a disulfide linkage between gp120 and gp41 by introducing A501C and T605C mutations (HxB2 numbering system) that covalently links the two subunits of the heterodimer. The gp41 mutation was included in the heptad repeat region 1 (HR1) of gp41 for trimer stabilization, and a deletion of part of the hydrophobic MPER, in this case residues 664–681 of the Env ectodomain. The virion cleavage site between gp120 and gp41 (S08RRKS31) was altered to S08RRKR31 to enhance cleavage. The resulting, glycosylated protein was used to produce two, recombinant gp120 Env protein-containing fractions were concentrated to 15.1 mg/mL in the SEC buffer. The deglycosylated protein was then run through SEC as described above. The complex of CH505 TF Env with deglycosylated chimeric B.VU2 gp120 was loaded by binding gp120 with Fab in a 1:2.1 molar ratio, and then run through SEC as described above to eliminate Fab from the 1:1 complex. Peak protein-containing fractions were concentrated to 15.1 mg/mL in the SEC buffer. All protein samples were tested against commercially available screens (Qasgen, Millipore) for endotoxins and contaminating viruses as described64; DH522 mAb neutralized viruses 6095.V1.C10, DJ263.8, 6095.V1.C12, and DJ263.12; CH505 SOSIP.664 was transfected together with a 1 µg/mL of anti-B200 B786 (catalog #563894), anti-CD19 APCR700 (catalog #565473), and anti-CD3 BV605 (catalog #563807). Likewise, cell surface marker combinations used for spleen subfractionation into transitional-B and mature-B subsets included 0.5 µg/mL of anti-B200 B786 (catalog #563894), anti-CD19 APCR700 (catalog #565473), and anti-CD3 BV605 (catalog #563807). All subsets were also further subdivided for CD4-binding site CH103 lineage reactivity by including BRD-21 fluorescent dye-conjugated antibodies in the pre-stain mix. For both sets of experiments, pre-stained B cells were loaded with Fluo-4 via thorough washes in HBSS, followed by mixing with equal volumes of 2x 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 µg/mL anti-IgM F(ab)2 (Southern Biotech). Fluo-4 MFI data for transitional (B200/CD93+) B cells was acquired on a BD LSR II flow cytometer and analyzed by Flowjo software.
with 1x PBS supplemented with 0.5 M NaCl and proteins were eluted with 1 M methyl-α-D-mannopyranoside dissolved in 1x PBS. The eluate was concentrated and loaded onto a Superdex 200 10/300 GL column (GE Life Sciences) pre-
quilibrated in a buffer of 5 mM HEPS, pH 7.5, 150 mM NaCl, and 0.02% sodium azide for analysis by electron microscopy (EM). Purified CH505 SOSIP.664 trimmer was incubated with a five molar excess of DH522.2 Fab at 4°C for 1 h. A 3 µl aliquot containing ~0.01 mg/ml of the Fab–CH505 SOSIP.664 complex was applied for 30 s onto a carbon-coated 400 Cu grid mesh that had been glow discharged at 20 mA for 30 s, followed by negative staining with 2% uranyl formate for 20 s. Samples were imaged using a FEI Tecnai T12 microscope operating at 120 kV, at a magnification of 52,000x that resulted in a pixel size of 2.13 Å at the specimen plane. Images were acquired with a 2 K CCD camera using a nominal defocus of 1500 nm at 10° tilt increments, up to 50°. The tilt provided additional particle orientations to improve the image reconstructions. Particles were picked semi-automatically using EMAN2 and put into a particle stack. Initial, reference-free, two-dimensional class averages were calculated and particles corresponding to complexes (with one, two, or three Fab bounds) were selected into a substack for determination of an initial model. The initial model was cal-
culated in EMAN2 using threefold symmetry and EMAN2 was used for subsequent refinement using threefold symmetry. In total, 15,269 particles were included in the final reconstruction for the three-dimensional average of CH505 SOSIP.664 trimmer complex with DH522.2. The resolution of the final model was determined using a Fourier shell correlation (FSC) cutoff of 0.5. The cryo-electron tomography structure of b12-bound gp120 trimer (PDB ID: 3DNL) was used for subsequent reconstruction for the three-dimensional average of CH505 SOSIP.664 complex with DH522.2. The resolution of the final model was determined using a Fourier shell correlation (FSC) cutoff of 0.5. The cryo-electron tomography structure of b12-bound gp120 trimer (PDB ID: 3DNL) was used for subsequent refinement using threefold symmetry. In total, 15,269 particles were included in the final reconstruction for the three-dimensional average of CH505 SOSIP.664 trimmer.
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Author contributions
B.F.H. conceived and designed the study, evaluated all data, and wrote the paper; L.V. conceived and designed the mouse studies, analyzed the data, co-wrote, and edited the paper; W.B.W. designed and performed experiments for NHP studies, analyzed data, co-wrote, and edited the paper; F.G., J.Z. and C.J. designed and/or performed experiments for mice studies and analyzed data; N.I.N. performed X-ray crystallography studies; D.F. and S.C.H. performed negative stain electron microscopy studies; M.A.M., K.L., D.I.M. and J.F.W. performed flow cytometry sorts and/or isolated antibodies; T.B.K., A.R., K.W. and J.A.H. analyzed antibody gene sequences; T.B. performed NGS; N.V. performed statistical analyses; S.M.A., A.F. and R.P. characterized antibody-binding specificities; H.-X.L. and K.O.S. provided recombinant Envs and mAbs; D.C.M., M.B., M.L., J.R.M., S.-M.X. and A.E. performed neutralization assays and data analysis; S.S., R.S., L.S. and C.B. performed NHP immunizations and care; A.N. and H.B.-V. performed mice immunizations and care; H.B., X.N. and G.K. characterized antibody polyreactivity; S.G.R., C.B.F., and K.C. provided adjuvants; and M.K. and D.F. provided adjuvants and contributed to the development of the NHP study protocol.

Additional information
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Competing interests: S.G.R., C.B.F. and K.C. are employees of Infectious Disease Research Institute (Seattle, WA). M.K. and D.F. are employees of the GSK group of companies. The remaining authors declare no competing financial interests.

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