Boosting with AIDSVAX B/E enhances Env constant region 1 and 2 antibody-dependent cellular cytotoxicity breadth and potency

By

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Abstract

Induction of protective antibodies is a critical goal of HIV-1 vaccine development. One strategy is to induce non-neutralizing antibodies (NNAbs) that kill virus-infected cells as these antibody specificities have been implicated in slowing HIV-1 disease progression and in protection. HIV-1 Env constant region 1 and 2 (C1C2) monoclonal antibodies (mAbs) frequently mediate potent antibody dependent cellular cytotoxicity (ADCC)
making them an important vaccine target. Here we explore the effect of delayed and repetitive boosting of RV144 vaccine-recipients with AIDSVAX B/E on the C1C2-specific mAb repertoire. It was found that boosting increased clonal lineage specific ADCC breadth and potency. A ligand crystal structure of a vaccine-induced broad and potent ADCC-mediating C1C2-specific mAb showed that it bound a highly conserved Env gp120 epitope. Thus, boosting to affinity mature these type of IgG C1C2-specific antibody responses may be one method by which to make an improved HIV vaccine with higher efficacy than seen in the RV144 trial.

Significance

Over one million people become infected with HIV-1 each year making the development of an efficacious HIV-1 vaccine an important unmet medical need. The RV144 human HIV-1 vaccine-regimen is the only HIV-1 clinical trial to date to demonstrate vaccine-efficacy. An area of focus has been on identifying ways by which to improve upon RV144 vaccine-efficacy. The RV305 HIV-1 vaccine-regimen was a follow-up boost of RV144 vaccine-recipients that occurred 6-8 years after the conclusion of RV144. Our study focused on the effect of delayed boosting in humans on the vaccine-induced Env constant region 1 and 2 (C1C2) – specific antibody repertoire. It was found that boosting with a HIV-1 Env vaccine increased C1C2-specific antibody dependent cellular cytotoxicity potency and breadth.

INTRODUCTION

CD4-inducible (CD4i) epitopes within HIV-1 envelope (Env) constant regions 1 and 2 (C1C2) are targets for antibodies that mediate antibody dependent cellular
cytotoxicity (ADCC) (1). C1C2-specific antibody epitopes have been termed Cluster A (1) and defined by two Env-targeted monoclonal antibodies (mAbs), A32 (2) and C11 (1). Structural analyses of antigen complexes formed by A32, A32-like (3-5) and C11-like mAbs (6) indicate that these mAbs bind distinct Env epitopes. The A32 epitope involves a discontinuous sequence within Env layers 1 and 2 of the inner domain (4, 5) while the C11 epitope maps to the inner domain eight-stranded β sandwich (6). Importantly, both mAbs are non-neutralizing for tier 2 HIV strains, but are capable of broad and potent ADCC (1, 2).

The secondary analysis of HIV-1 infection risk in RV144 (NCT00223080) indicated that ADCC in the presence of low anti-Env IgA responses correlated with decreased HIV-1 acquisition (7). While antibodies representative of the Env variable region 2 (V2) response inversely correlated with HIV-1 acquisition (7), we previously demonstrated that synergy between A32-blockable C1C2-specific mAbs and V2-specific mAbs increased ADCC potency of the V2 mAbs induced in the RV144 trial (8).

Here we studied the effect of late boosting of RV144 vaccinees in the RV305 HIV-1 vaccine trial (NCT01435135) focusing specifically on C1C2-specific mAb affinity maturation, ADCC potency and ADCC breadth. We found that the RV144 ALVAC/AIDSVAX B/E immunization regimen induced durable C1C2-specific memory B cells and that boosting with AIDSVAX B/E could increase C1C2-specific mAb variable heavy and variable light (V_H + V_L) chain gene mutation frequency along with increasing ADCC breadth and potency.

RESULTS
AIDSVAX B/E N-terminal deletion alters C1C2-specific antibody responses.

The AIDSVAX B/E protein used in the RV144 and RV305 HIV-1 vaccine trials had an eleven amino acid N-terminal deletion (9) that removed a majority of the C11-like mAb epitope (6), whereas the CRF_01 AE gp140 Env 92TH023 encoded in ALVAC (vCP1521) retained the gp120 N-terminal 11 amino acids (10). To determine if C11 could bind to gp120 proteins with an 11 amino acid N-terminal deletion, we assayed A32 and C11 mAbs for binding to full length AE.A244gp120 or to AE.A244gp120Δ11 (N-terminal 11 aa deleted). A32 bound to full length AE.A244gp120 and binding was enhanced on AE.A244gp120Δ11 (Fig. 1A) (9). In contrast, C11 only bound to the full length AE.A244gp120 (Fig 1A). From these data we concluded that C11-like antibody responses were unlikely to be boosted by AIDSVAX B/E.

PBMCs collected from four vaccine-recipients two weeks after the second RV305 boost with AIDSVAX B/E (RV305 Group II) were used for AE.A244gp120-specific single B cell sorting and antibody variable region RT-PCR. A total of 19 RV305-derived NNAbs were identified that blocked the C1C2 mAb A32 binding to AE.A244gp120Δ11 (Fig 1B and Table 1). Compared to previously published RV144 C1C2-specific mAbs (11), the RV305 C1C2-specific mAbs had significantly more V_H and V_L chain gene mutations (Wilcoxon rank sum test P < 0.0001) (Fig 1C), suggesting that RV305 boosting induced additional somatic mutations in C1C2-specific antibodies.

To determine if the RV305 boosted A32 blockable mAbs contained a binding epitope similar to A32, we used the A32 ligand crystal structure (5) to identify critical A32 antibody contact residues, and then designed an AE.A244gp120Δ11 mutant protein (AE.A244gp120Δ11 F53S, H72L, V75A, E106K, D107H, S110A, Q114L) to eliminate
A32 mAb binding (Fig 1A). In ELISA, the RV305 antibody, DH838, was the only mAb with binding eliminated by mutating the A32 epitope (Fig 1D). Likewise, DH838 was the only mAb that used a VH3 family gene while all other ALVAC/AIDSVAX B/E–induced C1C2-specific mAbs used VH1 genes (Table 1). Thus, as in RV144, AIDSVAX B/E boosting preferentially expanded C1C2-specific antibodies that used VH1 family genes (11). A majority of the mAbs assayed in this study bound epitopes distinct from A32 but in close enough proximity to be sterically cross-blocked by A32 (Fig 1B).

Boosting increased C1C2-specific ADCC breadth and potency. RV305 C1C2-specific mAbs and a subset of RV144 C1C2-specific mAbs were next assessed for ADCC against CEM.NKRCCR5 cells infected with one of seven HIV-1 infectious molecular clones (IMCs) representing three different clades (HIV-1 AE.CM235, B.WITO, C.TV-1. C.MW965, C.1086C, C.DU151 and C.DU422) (Table 2). These IMCs were chosen because they represent clusters of IMCs with different sensitivity to ADCC (unpublished data). To ascertain ADCC breadth and potency mAbs were ranked using an ADCC score (see methods) analogous to calculating mAb neutralization breadth. Apart from the RV144-derived A32 blockable mAb CH38, which was naturally an IgA mAb but tested here as a recombinant IgG1 mAb, ADCC scores for 16/19 RV305 mAbs ranked higher than the RV144 mAbs (Table 3 and 4).

Boosting of RV144 vaccine-recipients with AIDSVAX B/E in the RV305 trial increased ADCC breadth and potency of the RV144 derived C1C2-specific, DH677 clonal lineage. Next the C1C2-specific DH677 memory B cell clonal lineage was used to study affinity maturation and ontogeny of AIDSVAX B/E-induced ADCC responses. B cell clonal lineage member DH677.1 was isolated from PBMCs collected from a
vaccine-recipient two weeks after the last boost in the original RV144 trial (ALVAC + AIDSVAX B/E). DH677.2, DH677.3 and DH677.4 clonal lineage members were isolated from PBMCs collected from the same vaccine-recipient two weeks after the second boost with AIDSVAX B/E alone given in the RV305 clinical trial (RV305 Group II). Thus, this B cell clonal lineage belongs to a long-lived memory B cell pool started by the RV144 vaccine-regimen and boosted many years later with the RV305 vaccine-regimen (Fig 2). DH677.1, DH677.2, DH677.3 and DH677.4 mAb sequences were used to infer with Cloanalyst (12) three intermediate ancestors (IA) – IA1, IA2, IA3 – and an unmutated common ancestor (UCA) for the DH677 clonal lineage. The DH677 clonal lineage was then assayed by surface plasmon resonance for binding to the AIDSVAX B/E proteins - AE.A244gp120Δ11 and B.MNgp120Δ11 – as well as full length AE.A244gp120. The DH677 UCA did not bind to B.MNgp120Δ11, had minimal binding to the full length AE.A244gp120 and this binding was enhanced with AE.A244gp120Δ11 (Fig 2). The RV305 boosts more than doubled the V\textsubscript{H} chain gene mutation frequency from 1.04% (DH677.1; RV144) up to 4.51% (DH677.4; RV305) which resulted in 100-fold increase in apparent affinity for the AIDSVAX B/E proteins (DH677.1 AE.A244gp120Δ11 \(K_d= 45.2\) nM & B.MNgp120Δ11 \(K_d=219\) nM to DH677.4 AE.A244gp120Δ11 \(K_d= 0.49\) nM & B.MNgp120Δ11 \(K_d=2.86\) nM) and also improved binding to full length AE.A244gp120 (DH677.1 AE.A244gp120 Full length \(K_d= 152\) nM to DH677.4 \(K_d=2.29\) nM) (Fig 2).

The ontogeny of vaccine-induced ADCC was studied by assaying the DH677 clonal lineage against a panel of IMC infected CEM.NKR\textsubscript{CCR5} cells (AE.CM235, B.WITO, C.TV-1, C.MW965, C.1086C, C.DU151 and C.DU422) (Table 2). The RV144 prime-
boost immunization regimen minimally increased ADCC breadth and potency (DH677 UCA ADCC Score = -2.32; DH677.1 ADCC Score = -2.20 (see methods)). Conversely, RV305 boosting substantially increased ADCC breadth and potency (DH677.3 ADCC Score = 4.56) (Fig 2). These data indicate that the RV144 prime-boost regimen was insufficient to fully affinity mature this C1C2-specific B cell clonal lineage. Rather RV305 trial boosting of this particular RV144 vaccine-recipient profoundly enhanced DH677 lineage ADCC breadth and potency.

Crystal structure of the potent ADCC-mediating mAb DH677.3. We next determined the crystal structure of the antigen binding fragment (Fab) of the highest ranking RV305 ADCC mAb DH677.3 (Table 4) - alone and in complex with clade AE gp12093TH057 core, plus the CD4-mimetic M48-U1 (Fig 3 and Table 5). DH677.3 Fab-gp12093TH057 core, M48U1 complex (Fig 4) showed that, similar to other Cluster A mAbs, DH677.3 approaches gp120 at the face that is buried in the native Env trimer (3-5) and binds the C1C2 region exclusively within the gp120 inner domain. The gp120 residues involved in DH677.3 binding map to the base of the 7-stranded β-sandwich (residues 82, 84, 86-87, 222-224, 244-246, and 491-492) and extend into the mobile layers 1 (residues 53, 60, 70-80) and 2 (residues 218-221). By docking at the layer 1/2/β-sandwich junction the Fab buried surface area (BSA) utilizes 248 Å² of the β-sandwich, 542 Å² of layer 1 and 135 Å² of layer 2 (Fig 4 and Table 5). The majority of contacts providing specificity involve a network of hydrogen bonds and a salt bridge (Fig 4, inset) contributed by the antibody heavy chain and gp120 side chain atoms of layer 1 (α turn connecting the β1-β0 strands, D78 and N80) and the 7-stranded-β-sandwich (strand β7, Q246). The contacts provided by the light chain are less specific
and consist of hydrogen bonds to the gp120 main chain atoms and hydrophobic contacts within a hydrophobic cleft formed at the layer 1/2/β-sandwich junction (Fig 4B and C). Overall DH677.3 utilizes all six of its complementary determining regions (CDRs), and relies approximately equally on both heavy chain and light chain with a total BSA of $973 \text{ Å}^2$: $498 \text{ Å}^2$ for the light chain and $475 \text{ Å}^2$ for the heavy chain (Fig 5 and Table 5). Interestingly, 25 of 29 gp120 contact residues are conserved in $>$80% of sequences in the HIV Sequence Database Compendium (https://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/compendium.html) with 15 of 29 being effectively invariant ($>$99% conserved) (Fig 4B).

Comparison of the DH677.3 mode of binding and epitope footprint to Cluster A prototype mAbs. Antigen complex structures of A32 and N12-i3 (C11-like) (3, 6), mAb isolated from HIV-1-infected individuals, confirm that DH677.3 recognized a unique epitope between the A32 and C11 antibody-binding sites involving Env epitope elements of both (Fig 6). While the A32 mAb epitope consists exclusively of gp120 mobile layers 1 and 2 (76% and 24% of gp120 BSA, respectively; (Fig 6 and Table 5)), DH677.3 relies less on layers 1 and 2 (53% and 14% of gp120 BSA, respectively) and effectively utilizes the gp120 7-stranded β-sandwich (24% of gp120 BSA) (Fig 6 and Table 5). The ability to recognize the 7-stranded β-sandwich renders DH677.3 similar to the C11-like antibody N12-i3, which almost exclusively depends on the β-sandwich for binding (94% of its total gp120 BSA; (Fig 6 and Table 5)). Interestingly, N12-i3 and other C11-like mAbs require the N-terminus of gp120 for binding and recognize a unique gp120 conformation formed by docking of the gp120 N-terminus as an 8th strand to the β-sandwich to form an 8-stranded-β-sandwich structure (6). The DH677.3
complex crystals were obtained with gp12093TH057 core, which lacks the N-terminus (Δ11 aa deletion) and therefore the direct judgment, based on structure, whether or not the 8th strand is involved in binding was not possible. However, we were able to model the N/C-termini-gp12093TH057 core, from the N12-i3 Fab complex structure (PDB code: 5W4L) to the DH677.3 Fab-gp12093TH057 core-M48U1 complex without any steric clashes (Fig 6A, inset). Both the conformation and orientation of CDR H1 and 2 of DH677.3 allowed easy access to the 8-stranded-β-sandwich structure and enabled contacts to the 8th strand. These data indicated that DH677.3 is capable of accommodating both the 7 and 8-stranded-β-sandwich conformations of gp120 with effective contacts to the 8th strand. Thus, the vaccine-induced C1C2-specific mAb DH677.3 has a unique binding angle to the C1C2 region compared to the infection-induced C1C2-specific mAbs C11 and A32.

DH677 lineage mAbs mediate ADCC against CD4 downmodulated HIV-1 infected cells. During natural infection the HIV-1 accessory protein Nef downregulates CD4 expression on the surface of virus infected cells (13, 14). Cell surface expressed CD4 facilitates the exposure of CD4i Env epitopes – like C1C2 - by binding to co-expressed cell surface Env (15). The analyses of ADCC breadth was performed using target cells infected with IMCs containing the Renilla luciferase (LucR) reporter gene, which restricts Nef expression leading to incomplete CD4 downregulation (16). Nevertheless, Vpu expression can compensate for Nef function and induce CD4 downregulation during the 72 hour incubation of the target cells before assays were performed. To exclude any possible impact of this technical aspect of IMCs with LucR on our ADCC results, full length IMCs (n=7) that do not contain a report gene were used to evaluate
ADCC of the affinity matured RV305 C1C2-specific mAbs DH677.3 and DH677.4 (Fig 2) and A32 (2). Since clade CRF01_AE possess a histidine at Env HXB2 position 375 that influences sensitivity to CD4i antibody binding and ADCC (17, 18) only clade B and clade C HIV-1 IMCs were used (Table 2).

As these full-length IMCs did not contain a reporter gene, we used an infected cell elimination assay, which measures the reduction of live p24+, p24+CD4+ and/or p24+CD4- cell populations in the presence of effector cells (Fig 7). When evaluating elimination of total p24+ cells no significant difference (Wilcoxon rank sum test; p > 0.05) in specific killing was noted among the three mAbs (Fig 8A). However, when infected cells were separated into p24+CD4+ (Fig 8B) and p24+CD4- (Fig 8C) it was found that the RV305-boosted DH677.3 mAb mediated ADCC against 4 out of seven HIV-1 IMCs whereas DH677.4 and A32 mAb mediated ADCC against two or none of these IMCs, respectively. Although DH677.3 % of specific killing against these IMCs was low (mean 6%, range 0-24%), it was significantly higher (Wilcoxon rank sum test p = 0.03) at mediating ADCC against p24+ CD4- infected cells (Fig 8C) compared to A32.

This is likely related to the unique DH677 clonal lineage epitope which may be more frequently exposed on Env conformations on the surface of IMC infected cells even in the context of CD4 downmodulation.

DISCUSSION

In this study it was found that late boosting of RV144 vaccinees increased C1C2-specific mAb V_H + V_L chain gene mutation frequency and increased clonal lineage specific ADCC breadth and potency (Fig 1, 2 and Table 4). We analyzed the somatic hypermutation frequency of mAbs obtained after boosting and did not observe a clear
correlation between somatic hypermutation frequency and ADCC breadth or potency. Boosting likely affinity matures antibody clonal lineages that are capable of acquiring broad and potent ADCC, as well as, antibody clonal lineages incapable of acquiring broad and potent ADCC. For example, DH689 clonal lineage members DH689.1 and DH689.2 were both ~10% mutated (Table 1) but ADCC breadth and potency was worse than most of the RV144 mAbs assayed (Table 4). Conversely, boosting increased somatic hypermutation along with ADCC breadth and potency within the DH677 clonal lineage (Fig 2). Thus, clonal lineages with fine epitope specificities capable of being matured to increased ADCC breadth and potency greatly benefited from the two AIDSVAX B/E boosts given in the RV305 clinical trial. It should be noted that in VAX003 and VAX004 clinical trials ADCC responses peaked at 3-4 immunizations and declined after 5-7 immunizations (19). Collectively these data indicate that while the RV144 clinical trial was under boosted, repetitive subsequent boosting beyond RV305 may not necessarily lead to continuously better functional antibody outcomes.

Improving vaccine-induced NNAb effector function will also require more detailed immunological studies on the timing and frequency of boosting. In the VAX003 (NCT00002441) and VAX004 (NCT00002441) trials, frequent protein immunizations skewed Env-specific antibody subclass usage from the highly functional IgG3 to IgG4 (20-22). The RV305 boosts that were studied here occurred several years (6-8yrs) after the final RV144 boost, unlike previous HIV-1 vaccine trials. Whether the boosting interval can be shortened without skewing antibody subclass usage is not known, but it is possible that boosting with long rest intervals (≥1-2 years) will be necessary.
The AIDSVAX B/E protein used for boosting in the RV144 and RV305 HIV-1 vaccine trial contained a N-terminal 11 amino acid deletion. Previously it was shown that this modification enhanced exposure of the C1C2 region and V2 loop (9). Here we show that this modification disrupts C11-like mAb binding (Fig 1) but does create a germline-targeting immunogen for DH677-like B cell lineages (Fig 2). Ligand crystal structure analysis found that DH677.3 recognized a unique C1C2 epitope that involves epitope footprints of Cluster A mAb A32 and N12-i3 (C11-like), as well, as new elements of the inner domain Layer 1 and the 7-stranded-β-sandwich (Fig 6). The DH677.3 epitope is positioned midway between the A32 and N12-i3 binding sites with most residues being highly conserved. Interestingly, DH677.3 binds at the edge of the gp120 inner domain 7-stranded β-sandwich and with layers 1 and 2. This binding mode allows it to bind a gp120 conformation emblematic of the late stages of HIV entry recognized by C11 and C11-like mAbs (6). Most likely this allows DH677.3 to recognize a broader range of Env targets, emerging in both early (when the A32 epitope becomes available) and late stage (when the C11 epitope becomes available) of the viral entry process.

Identification of a stage 2A of the HIV-1 Env expressed on the surface of infected cells in presence of the CD4 molecule or CD4 mimetics reiterate the importance of targeting these epitopes by vaccine induced responses as detected in our assays (23). In addition, a model of DH677.3 in complex with gp120 antigen bound to a CD4 of a target/infected cell confirms that the recognition site and angle of approach position the DH677.3 IgG for easy access for effector cell recognition and Fc-effector complex formation (Fig 6A). Interestingly, we recently characterized JR4, a mAb
isolated from a NHP that like DH677.3 recognizes an epitope that includes elements of both the A32 and C11 binding sites (5). JR4 uses its CDR H1 to contact layer 2 residues of A32 epitope region and its CDR H3 to reach the residues of 7-stranded β-
sandwich. However, in contrast to DH677.3 that largely relies on recognizing the 7-
stranded β-sandwich, access of JR4 to this region is limited and involves only few residue contacts. In this regard JR4 is more like A32 with main anchoring contacts to layer 2 that limits its reach to the 7-stranded β-sandwich. DH677.3 misses these A32 layer 2 residues and shifts its epitope footprint more toward the 7-stranded β-sandwich placing it midway between the two epitope regions.

ADCC-mediating antibodies have been shown to reduce mother-to-child HIV-1 transmission (24-26), slow virus disease progression (26-28) and in RV144 correlated with reduced risk of infection in vaccine-recipients with lower anti-Env plasma IgA responses (7). Synergy between the RV144 C1C2 and V2 mAbs suggest a role for the C1C2 plasma responses that could not be directly identify by the correlates of protection study. That DH677.3 was better than A32 at mediating ADCC against HIV-1 clade B and C CD4 down-modulated cells (Fig 8) make this mAb an attractive candidate for targeting HIV-1 infected cells in vivo in the setting of HIV-1 infection. We have previously shown that the C1C2 mAb A32 when formulated as a bi-specific antibody can potently opsonize and kill HIV-1 infected CD4+ T cells (29). Whether DH677.3-type of mAbs are superior to A32 for targeting virus-infected cells remains to be determined.

In summary, our data demonstrate that if the RV144 vaccine trial had been boosted, ADCC-mediating C1C2-specific antibodies would have undergone affinity
maturation for both ADCC potency and breadth of recognition of HIV-1-infected CD4+ T cells.

**MATERIALS AND METHODS**

**Ethics Statement.** The RV305 clinical trial (NCT01435135) was a boosting of 162 RV144 clinical trial participants (NCT00223080) six-eight years after the conclusion of RV144 (30). Donors used in this study were from groups boosted twice with either AIDSVAX B/E + ALVAC-HIV (vCP1521) (Group I) or AIDSVAX B/E alone (Group II). PBMCs were only collected two weeks after the second boost. The RV305 clinical trial (NCT01435135) received approvals from Walter Reed Army Institute of Research, Thai Ministry of Public Health, Royal Thai Army Medical Department, Faculty of Tropical Medicine, Mahidol University, Chulalongkorn University Faculty of Medicine, and Siriraj Hospital. Written informed consent was obtained from all clinical trial participants. The Duke University Health System Institutional Review Board approved all human specimen handling.

**Antigen-specific single-cell sorting.** All PBMCs used in this study were collected two weeks after the second boost in RV305. A total of 1 x 10^7 PBMC per vaccine-recipient were stained with AE.A244gp120Δ11 fluorescently labelled proteins and a human B cell flow cytometry panel. Viable antigen-specific B cells (AqVd-CD14-CD16-CD3-CD19+IgD-) were single-cell sorted with a BD FACS Aria II-SORP (BD Biosciences, Mountain View, CA) into 96 well PCR plates and stored at -80°C for RT-PCR.
single-cell reverse transcriptase PCR. Single B cell cDNA was generated with random hexamers using SSIII. The antibody variable heavy and light chain variable regions were PCR amplified using AmpliTaq360 Master Mix (Applied Biosystems). PCR products were purified (Qiagen, Valencia, CA) and sequenced by Genewiz. Gene rearrangements, clonal relatedness, unmutated common ancestors and intermediate ancestor inferences were made using Cloanalyst (12). DH677 clonal lineage tree was generated using FigTree.

Monoclonal antibody production. PCR-amplified heavy and light chain gene sequences were transiently expressed as previously described (31). Ig containing cell culture supernatants were used for ELISA binding assays. For large scale expression, \( V_H \) and \( V_L \) chain genes were synthesized (\( V_H \) chain in the IgG1 4A backbone) and transformed into DH5α cells (GeneScript, Piscataway, NJ). Plasmids were expressed in Luria Broth, purified (Qiagen, Valencia, CA) and Expi293 cells were transfected using ExpiFectamine™ (Life Technologies, Carlsbad, CA) following the manufacturers protocol. After five days of incubation at 37°C 5% CO₂ the Ig containing media was concentrated, purified with Protein A beads and the antibody buffer exchanged into PBS.

Antibody binding and blocking assays. Direct ELISAs were performed as previously described (31). Briefly, 384-well microplates were coated overnight with 30ng/well of protein. Antibodies were diluted and add for one hour. Binding was detected with an anti-IgG-HRP (Rockland) and developed with SureBlue Reserve TMB One Component (KPL). Plates were read on a plate reader (Molecular Devices) at
450nm. A32-blocking assays were performed by adding the RV305 antibodies followed by biotinylated A32 and detecting with streptavidin HRP.

**Neutralization assays.** TZM-bl neutralization assays were performed as previously described (32). No neutralization was detected for the mAbs assayed in this study.

**Infectious molecular clones (IMC).** The HIV-1 reporter viruses used were replication-competent IMC designed to encode the env genes of CM235 (subtype A/E; GenBank No. AF259954.1), WITO (subtype B; GeneBank No. JN944948), 1086.c (subtype C; GeneBank No. FJ444395), TV-1 (subtype C; GeneBank No. HM215437), MW96.5 (subtype C; GeneBank No.), DU151 (subtype C; GeneBank No. DQ411851), DU422 (subtype C; GeneBank No. DQ411854) in cis within an Nef deficient isogenic backbone that expresses the Renilla luciferase reporter gene (33). The subtype AE Env-IMC-LucR viruses used were the NL-LucR.T2A-AE.CM235-ecto (IMC<sub>CM235</sub>) (plasmid provided by Dr. Jerome Kim, US Military HIV Research Program), and clinical env IMCs from the RV144 trial that were built on the 40061-LucR virus backbone. All other IMCs were built using the original NL-LucR.T2A-ENV.ecto backbone as originally described (34). Reporter virus stocks were generated by transfecting 293T cells with proviral IMC plasmid DNA, and virus titer was determined on TZM-bl cells for quality control (34).

**Infection of CEM.NKR<sub>CCR5</sub> cell line with HIV-1 IMCs.** CEM.NKR<sub>CCR5</sub> cells were infected with HIV-1 IMCs as previously described (35). Briefly, IMCs were titrated in order to achieve maximum expression within 48-72 hours post-infection as determined by detection of Luciferase activity and intra-cellular p24 expression. IMC infections
were performed by incubation of the optimal dilution of virus with CEM.NKRCCR5 cells for 0.5 hour at 37°C and 5% CO₂ in presence of DEAE-Dextran (7.5 μg/ml). The cells were subsequently resuspended at 0.5x10⁶/ml and cultured for 48-72 hours in complete medium containing 7.5 μg/ml DEAE-Dextran. For each ADCC assay, we monitored the frequency of infected target cells by intracellular p24 staining. Assays performed using infected target cells were considered reliable if cell viability was ≥60% and the percentage of viable p24⁺ target cells on assay day was ≥20%.

**Luciferase ADCC Assay.** ADCC activity was determined by a luciferase (Luc)-based assay as previously described (8, 36). Briefly, CEM.NKRCCR5 cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Alexandra Trkola) (37) were used as targets after infection with the HIV-1 IMCs. PBMC obtained from a HIV-seronegative donor with the heterozygous 158F/V and 131H/R genotypes for FcγR3A and FcγR2A (38, 39), respectively, were used as a source of effector cells, and were used at an effector to target ratio of 30:1. Recombinant mAbs were tested across a range of concentrations using 5-fold serial dilutions starting at 50 μg/mL. The effector cells, target cells, and Ab dilutions were plated in opaque 96-well half area plates and were incubated for 6 hours at 37°C in 5% CO₂. The final read-out was the luminescence intensity (relative light units, RLU) generated by the presence of residual intact target cells that have not been lysed by the effector population in the presence of ADCC-mediating mAb (ViviRen substrate, Promega, Madison, WI). The % of specific killing was calculated using the formula: percent specific killing = ([number of RLU of target and effector well − number of RLU of test well]/number of RLU of target and effector well] ×100. In this analysis, the RLU of the target plus effector wells represents...
spontaneous lysis in absence of any source of Ab. The ADCC endpoint concentration (EC), defined as the lowest concentration of mAb capable of mediating ADCC in our in vitro assay, was calculated by interpolation of the mAb concentration that intersected the positive cutoff of 15% specific killing. The RSV-specific mAb Palivizumab was used as a negative control.

**ADCC Score.** Antibodies were tested across a range of concentrations using 5-fold serial dilutions starting at 50 µg/mL. Since the dilution curves are not monotonic due to pro-zone effect of mAbs, non-parametric area under the curve (AUC) was calculated using trapezoidal rule with activity less than 15% set to 0%. In this study we have used Principal Component Analysis (PCA) to compute an ADCC score which explains both potency and breadth of the mAbs. This method of using PCA to calculate breadth score for neutralizing antibodies has been used in a previous study [42]. Similarly, in 2011 a study published in Atherosclerosis aimed to study the effect of various risk factors on increase in carotid and femoral intima-media thickness used PCA to calculate a cumulative risk score (40).

PCA is the most commonly used method to reduce the dimensionality of the data set (41). It uses Eigen vector decomposition of the correlation matrix of the variables, where each variable is represented by a HIV-1 IMCs in our study. Most of the shared variance of the correlations of ADCC AUC of HIV-1 IMCs is explained by first principal component (PC1) (42). Ideally, one would want to explain 70% of the variance but should not be at the expense of adding principal components with an Eigenvalue less than 1 (43). Eigenvalue is a measure of variance in the data along that Principal Component (PC) and a larger value would mean that corresponding PC explains a
larger amount of variance in the data. The rationale behind this methodology of calculating ADCC score is that mAbs will not target all the seven HIV-1 IMCs equally, hence calculation of breadth score for mAbs needs to account for this variation.

In this study a panel of 7 HIV-1 IMCs were tested which implies that our data set has seven dimensions. ADCC activity was measured as AUC. In our analysis PC1 and PC2 have Eigen values above 1 and together account for 80.57% variance (Table 6). Scores obtained from the first Principal Component can be interpreted as weighted average of the 7 HIV-1 IMCs that would account for both potency as well as breadth of the mAbs (43). Higher PC1 score would mean that mAb has higher breadth as well as potency for ADCC activity. To calculate the ADCC score, the standardized AUC value for each mAb is first calculated for each HIV-1 IMCs and then multiplied by factor loading of the corresponding HIV-1 IMCs. Lastly these products are added together. Factor loadings are the correlation coefficients between PCs and HIV-1 IMCs (40).

ADCC score obtained from PC1 is a weighted average of the standardized AUCs where factor loadings obtained from PC1 are used as weights to calculate the weighted average. Standardized AUC values imply zero mean and unit standard deviation. The AUC values below the value of mean AUC will result in negative PC1 scores.

**Infection of primary cells with HIV-1 IMCs.** IMCs encoding the full-length transmitted/founder sequence of seven individuals infected with either subtype B or C viruses from the CHAVI acute infection cohort (CH77, CH264, CH0470, CH042, CH185, CH162 and CH236) were constructed as previously described (44, 45) and used to infect primary CD4+ cells. To infect cells, cryopreserved PBMCs were thawed and stimulated in R20 media (RPMI media (Invitrogen) with 20% Fetal Bovine Serum...
(Gemini Bioproducts), 2mM L-glutamine (Invitrogen), 50 U/mL penicillin (Invitrogen), and 50 μg/mL Gentamicin (Invitrogen)) supplemented with IL-2 (30U/mL, Proleukin), anti-CD3 (25ng/mL clone OKT-3, Invitrogen) and anti-CD28 (25ng/mL, BD Biosciences) antibodies for 72 hours at 37°C in 5% CO₂. CD8 cells were depleted from the PBMCs using CD8 microbeads (Miltenyi Biotec, Germany) according to the Manufacturer’s instructions and 1.5 x 10⁶ cells were infected using 1 mL virus supernatant by spinoculation (1125 x g) for 2 hours at 20 °C. After spinoculation, 2 mL of R20 supplemented with IL-2 was added to each infection and infections were left for 72 hours. Infected cells were used if viability was >70% and more than 5% of cells were p24+. 

**Infected Cell Elimination Assay.** HIV-1-infected or mock-infected CD8-depleted PBMCs cells were used as targets and autologous cryo-preserved PBMCs rested overnight in R10 supplemented with 10ng/ml of IL-15 (Miltenyi Biotec) were used as a source of effector cells. Infected and uninfected target cells were labelled with a fluorescent target-cell marker (TFL4; OncoImmunin) and a viability marker (NFL1; OncoImmunin) for 15 min at 37 °C, as specified by manufacturer. The labeling of the target cells with these two markers allowed to clearly identify only the live viable cells in our gating strategy and exclude artifacts related to the presence of dead cells staining. Cells were washed in R10 and adjusted to a concentration of 0.2x10⁶ cells/mL. PBMCs were then added to target cells at an effector/target ratio of 30:1 (6 x 10⁶ cells/mL). The target/effector cell suspension was plated in V-bottom 96-well plates and co-cultured with 10 μg/mL of each mAb. Co-cultures were incubated for 6 h at 37 °C in 5% CO₂. After the incubation period, cells were washed and stained with anti-CD4-PerCP-Cy5.5.
(eBioscience, clone OKT4) at a final dilution of 1:40 in the dark for 20 min at room temperature (RT). Cells were then washed, resuspended in 100 μL/well Cytofix/Cytoperm (BD Biosciences), incubated in the dark for 20 min at 4 °C, washed in 1x Cytoperm wash solution (BD Biosciences) and co-incubated with anti-p24 antibody (clone KC57-RD1; Beckman Coulter) to a final dilution of 1:100, and incubated in the dark for 25 min at 4 °C. Cells were washed three times with Cytoperm wash solution and resuspended in 125 μL PBS-1% paraformaldehyde. The samples were acquired within 24 h using a BD Fortessa cytometer. The appropriate compensation beads were used to compensate the spill over signal for the four fluorophores. Data analysis was performed using FlowJo 9.6.6 software (TreeStar). Mock-infected cells were used to appropriately position live cell p24+/− and CD4+/− gates. Specific killing was determined by the reduction in % of viable p24+ cells in the presence of mAbs after taking into consideration non-specific killing, and was calculated as:
\[
p24\% (\text{target} + \text{effector cells}) - p24\% (\text{targets} + \text{effectors} + \text{mAb/plasma})
\]
\[
p24\% (\text{target} + \text{effector cells})
\]
CH65 (an anti-influenza monoclonal antibody, kindly provided by Dr. Moody) was used as negative control. To remove background signal, the highest value of percent specific killing induced by CH65 was subtracted from the calculated reduction in % of p24+ cells and then negative values were rounded to 0%. As the data was background subtracted, no positivity criteria were applied to the data.

**Surface plasmon resonance.** The binding and kinetic rates measurement of gp120 proteins against RV305 antibodies were obtained by surface plasmon resonance (SPR) using the Biacore 3000 instrument (GE Healthcare). SPR measurements were
performed using a CM5 sensor chip with anti-human IgG Fc antibody directly immobilized to a level of 9000-11000RU (response unit). Antibodies were then captured at 5ul/min for 60s to a level of 100-300RU. For binding analyses, the gp120 proteins were diluted to approximately 1000nM in PBS and injected over the captured antibodies for 3 minutes at 30ul/min. For kinetics measurements, the gp120 proteins were diluted from 5-750 nM and injected using a high performance kinetics injection for 5 minutes at 50ul/min. This was followed by a dissociation period of 600s and surface regeneration with Glycine pH2.0 for 20s. Results were analyzed using the Biacore BiaEvaluation Software (GE Healthcare). Negative control antibody (Ab82) and blank buffer binding were used for double reference subtraction to account for non-specific protein binding and signal drift. Subsequent curve fitting analysis was performed using a 1:1 Langmuir model with a local Rmax and the reported rate constants are representative of two measurements.

Protein preparation and complex crystallization. DH677.3 Fab alone was grown and crystallized at concentration ~10 mg/ml. The structure was solved by molecular replacement with PDB ID 3QEG in space group P2_1 to a resolution of 2.6 Å. Clade A/E 93TH057 gp120 core_e (gp120_{93TH057} core_e, residues 42-492 (Hxbc2 numbering)), lacking the V1, V2 and V3 variable loops and containing a H375S mutation to allow binding of the CD4 mimetic M48U1 (46) was used to obtain crystals of DH677.3 Fab-antigen complex. gp120_{93TH057} core_e was prepared and purified as described in (3). Deglycosylated gp120_{93TH057} core_e was first mixed with CD4 mimetic peptide M48U1 at a molar ratio of 1:1.5 and purified through gel filtration chromatography using a Superdex 200 16/60 column (GE Healthcare, Piscataway, NJ). After concentration, the
gp120<sub>93TH057</sub> core<sub>e</sub>-M48U1 complex was mixed with a 20% molar excess of DH677.3 Fab and passed again through the gel filtration column equilibrated with 5 mM Tris-HCl buffer pH 7.2 and 100 mM ammonium acetate. The purified complex was concentrated to ~10 mg/ml for crystallization experiments. The structure was solved by molecular replacement using the DH677.3 Fab and PDB ID 3TGT as searching models in space group P1 to a resolution 3.0 Å. The final $R_{\text{factor}}/R_{\text{free}}$ (%) for the Fab structure is 19.9/26.1 and the final $R_{\text{factor}}/R_{\text{free}}$ for the complex is 21.4/27.4 (Table 7). The PDB IDs for the deposited structures are 6MFJ and 6MFP respectively. In each case the asymmetric unit of the crystal contained two almost identical copies of Fab or the Fab-gp120<sub>93TH057</sub> core<sub>e</sub> complex (Fig 3).

**Crystallization and data collection.** Initial crystal screens were done in vapor-diffusion hanging drop trials using commercially available sparse matrix crystallization screens from Hampton Research (Index), Emerald BioSystems (Precipitant Wizard Screen) and Molecular Dimensions (Proplex and Macrosol Screens). The screens were monitored periodically for protein crystals. Conditions that produced crystals were then further optimized to produce crystals suitable for data collection. DH677.3 Fab crystals were grown from 20% PEG 3000, 100 mM HEPES pH 7.5, and 200 mM sodium chloride. DH677.3 complex crystals were grown from 25% PEG 4000 and 100 mM MES pH 5.5. Crystals were briefly soaked in crystallization solution plus 20% MPD before being flash frozen in liquid nitrogen prior to data collection.

**Data collection and structure solution.** Diffraction data were collected at the Stanford Synchrotron Radiation Light Source (SSRL) at beam line BL12-2 equipped with a Dectris Pilatus area detector. All data were processed and reduced with
HKL2000 (47). Structures were solved by molecular replacement with Phaser (48) from the CCP4 suite (49). The DH677.3 Fab structure was solved based on the coordinates of the N12-i2 Fab (PDB: 3QEG), and the DH677.3 complex was then solved with coordinates from the DH677.3 Fab model, gp120 (PDB: 3TGT), and M48U1 (PDB: 4JZW). Refinement was carried out with Refmac (50) and/or Phenix (51). Refinement was coupled with manual refitting and rebuilding with COOT (52). Data collection and refinement statistics are shown in Table 7.

**Structure validation and analysis.** The quality of the final refined models was monitored using the program MolProbity (53). Structural alignments were performed using the program lsqkab from the CCP4 suite (49). The PISA (54) webserver was used to determine contact surfaces and residues. All illustrations were prepared with the PyMol Molecular Graphic suite (http://pymol.org) (DeLano Scientific, San Carlos, CA, USA). Conservation of the DH677.3 epitope was calculated using the HIV Sequence Database Compendium (https://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/compendium.html) comparing gp120 residues relative to clade B Hxbc2. Only unique sequences in the database having an equivalent residue at each position were included in the calculated percentage representing approximately 32,000 sequences on average.

**Statistical Methods.** For luciferase based ADCC assay background correction was performed by subtracting the highest value of percent specific killing induced by CH65 and then rounding off the negative values to zero. In Figure 5 positivity cut-off criteria was not applied but background correction was performed.
In order to assess if two groups have different response pairwise comparisons between groups was conducted using Wilcoxon rank sum test. Statistical analysis was performed using SAS software (SAS Institute Inc., Cary, N.C.).
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AUTHOR CONTRIBUTIONS

Author contributions were as follows: **Conceptualization**, David Easterhoff, Nelson L. Michael, Jean-Louis Excler, Merlin L. Robb, Supachai Rerks-Ngarm, Barton F. Haynes, Justin Pollara, Marzena Pazgier, Guido Ferrari; **Methodology**, David Easterhoff, Nelson L. Michael, Jean-Louis Excler, Merlin L. Robb, Supachai Rerks-Ngarm, Barton F. Haynes, Justin Pollara, Guido Ferrari; **Development of Antibody Sequence Analysis software**, Kevin Wiehe, Thomas B. Kepler; **Data validation**, David Easterhoff, Justin Pollara.
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**CONFLICT OF INTEREST**

B.F.H., G.F. and D.E. have patents submitted on antibodies listed in this paper.
DISCLAIMER

The views expressed are those of the authors and should not be construed to represent the positions of the Uniformed Services University, U.S. Army, Department of Defense, or the Department of Health and Human Services. The investigators have adhered to the policies for protection of human subjects as prescribed in AR-70.

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FIGURE LEGENDS

Figure 1. Identification of RV305 C1C2-specific mAbs. (A) The C1C2-specific mAbs A32 or C11 were assayed by ELISA for reactivity with full length AE.A244gp120 or AE.A244g120Δ11. An A32-like mutant protein was designed (AE.A244g120Δ11 F53S H72L V75A E106K D107H S110A Q114L) to identify A32-like mAb responses.
19B was used as a positive control and CH65 as a negative control. (B) RV305 non-neutralizing mAbs were assayed for A32-blocking by ELISA. (C) RV305 non-neutralizing A32-blockable mAb heavy and light chain gene sequence mutation frequencies were analyzed by Cloanalyst (12) and compared to previously published RV144 heavy and light chain gene sequence mutation frequencies (% nucleotide) (11). Statistical significance was determined using a Wilcoxon rank sum test. Red bar represent the mean. (D) RV305 non-neutralizing A32-blockable mAbs were assayed by ELISA for binding to AE.A244g120Δ11 and AE.A244g120Δ11 F53S H72L V75A E106K D107H S110A Q114L. Data are expressed as % binding of the mutant protein relative to wild type. Shown are the mean with standard deviation of two independent experiments.

Figure 2. RV305 boosting increased the apparent affinity and antibody dependent cellular cytotoxicity breadth and potency of the C1C2-specific RV144 derived DH677 memory B cell clonal lineage. DH677.1 was isolated by AE.A244gp120Δ11-specific single-cell sorting PBMC collected from a vaccinee two weeks after the final boost in the RV144 vaccine trial. DH677.2, DH677.3 and DH677.4 were isolated by AE.A244gp120Δ11-specific single-cell sorting PBMC collected from the same vaccinee after the second AIDSVAX B/E (RV305 Group II) boost given in RV305 (~7yrs later). The intermediate ancestor one (IA1), intermediate ancestor two (IA2), intermediate ancestor three (IA3) and unmutated common ancestor (UCA) were inferred using Cloanalyst (12). The mAbs were recombinantly expressed and assayed by surface plasmon resonance for binding to the AIDSVAX B/E proteins - AE.A244g120 full length, AE.A244g120Δ11 and B.MNg120Δ11. Shown are the antibody apparent affinity...
measurements \( (K_d) \) expressed in nM. Were indicated NB = no detectable binding. mAbs were also assayed for antibody dependent cellular cytotoxicity (ADCC) against AE.C235, B.WITO, C.TV-1, C.MW965, C.1086C, C.DU151 and C.DU422 infected CEM.NKR\(_{CCR5}\) cells. An ADCC score (see methods) was used to account for ADCC breadth and potency.

**Figure 3.** Comparison of the two copies of the DH677.3 Fab-gp120\(_{93TH057\text{core-e-M48U1}}\) complex and the two Fab copies in the apo Fab structure from the asymmetric unit of crystals. (A) The root mean square deviation (RMSD) between complex copies is 0.946 Å for main chain residues. (B) The RMSD between the Fab copies in the apo Fab structure is 0.540 Å for main chain residues. (C) Comparison of the free and bound DH677.3 Fab. The \( \alpha \)-carbon backbone diagram of superposition of the structures of DH677.3 Fab alone (dark cyan-heavy chain and light cyan-light chain) and N5-i5 Fab bound to CD4-triggered gp120 (dark brown-heavy chain and light brown-light chain). The average RMSD between free and bound Fabs is 0.818 Å for main chain residues.

**Figure 4.** Crystal structure of the DH677.3 Fab-gp120\(_{93TH057\text{core-e-M48U1}}\) complex. (A) The overall structure of the complex is shown as a ribbon diagram (left) and with the molecular surface displayed over the Fab molecule (middle), colored based on electrostatic charge- red negative and blue positive. The gp120 outer domain is gray and inner domain colored to indicate inner domain mobile layer 1 (yellow), 2 (cyan), 3 (light orange) and the 7-stranded b-sandwich (magenta). Complementary determining regions (CDRs) are colored: CDR H1 (light blue), CDR H2 (dark green), CDR H3 (black), CRL1 (light green), CDR L2 (brown) and CDRL3 (blue). A blow-up view shows
the network of hydrogen (H) bonds formed at the Fab-gp120 interface. H-bonds contributed by side chain and main chain atoms of gp120 residues are colored in magenta and blue, respectively. (B) Fab buried surface area (BSA) and gp120 residues forming DH677.3 epitope are shaded in blue according to BSA (antibody) and percent conservation of gp120 residues (Env). gp120 main chain (blue) and side chain (red) hydrogen bonds (H) and salt bridges (S) are shown above the residue. (C) The DH677.3 Fab-gp120core interface. CDRs are shown as ribbons (left) and balls-and-sticks of residues contributing the binding (right) over the gp120 core. The molecular surface of gp120 is colored as in (A) (left) and by electrostatic potential (right).

**Figure 5.** DH677.3 heavy and light chain contact residues. mAb side chain (+) and main chain (-) contact residues colored green for hydrophobic, blue for hydrophilic and black for both as determined by a 5 Å cut off value over the corresponding sequence. CDRs are colored as in Figure 1 and buried surface residues as determined by PISA are shaded.

**Figure 6.** Recognition of HIV-1 Env by DH677.3 and other Cluster A mAbs. (A) The overlay of DH677.3 and Cluster A mAbs A32 and N12-i3 (C11-like) bound to the gp120 core. Crystal structures of the gp120 antigen in complex with the Fab of DH677.3, A32 (PDB code 4YC2) and N12-i3 (PDB code 5W4L) superimposed based on gp120. The d1 and d2 domains of the target cell receptor CD4 was added to replace peptide mimetic M48U1 of the DH677.3 Fab-gp120core-M48U1 complex. Molecular surfaces are displayed over Fab molecules and colored in lighter and darker shades of brown, blue and green for the heavy and light chains of DH677.3, A32 and N12-i3,
respectively. A blow up view shows details of the DH677.3 interaction with the 8-stranded β-sandwich of the gp120 inner domain. The 8th strand (colored in blue) formed by the 11 N-terminal residues of gp120 in the N12-i3 bound conformation (PDB: 5W4L) was modeled into the DH677.3 Fab-gp120 \textsubscript{93TH057} core\textsubscript{e}-M48U1 complex. CDR H1 and H2 of DH677.3 are colored light blue and dark green, respectively. (B) and (C) Comparison of DH677.3, A32 and N12-i3 epitope footprints. In (B) the DH677.3 epitope footprint (shown in red) is plotted on the gp120 surface with layers colored as in Figure 1 with the A32 and N12-i2 epitope footprints shown in black. (C) DH677.3, A32 and N12-i3 gp120 contact residues are mapped onto the gp120 sequence. Side chain (+) and main chain (-) contact residues are colored green for hydrophobic, blue for hydrophilic and black for both as determined by a 5 Å cut off value over the corresponding sequence. Buried surface residues as determined by PISA are shaded. The DH677.3 epitope footprint overlays with the epitopes of both A32 and N12-i3.

**Figure 7.** Gating strategy for infected cell elimination assay. Cells are gated on forward scatter and side scatter followed by distinguishing viable cells using NFL-1 (a dead cell marker) and TFL-4 (target cell marker). Target cells are then gated on CD4 and p24. Mock-infected cells are used to determine positioning of the gate for p24+. The proportion of p24+, p24+CD4+ and p24+CD4- target cells in the presence of effectors only, effectors +negative control or effectors + sample was determined.

**Figure 8.** RV305 derived C1C2-specific mAb DH677.3 is significantly better than A32 at mediating antibody-dependent cellular cytotoxicity against CD4 down modulated infectious molecular clone infected cells. Cells were infected with clade B and clade C full length infectious molecular clones (IMC) that do not contain a
Surface CD4 expression was analyzed by flow cytometry and p24 expression was measured in live/viable (A) all p24+ (B) p24+ CD4+ and (C) p24+ CD4-
IMC infected cell populations. Data are shown with the mean and standard deviation.

Table 1. Immunogenetics of non-neutralizing A32-blocking RV305 C1C2-specific mAbs. PBMCs used for mAb isolation were collected two weeks after the final boost in RV144 or two weeks after the second boost in RV305. All four RV305 vaccinees studied were from Group II – boosted 2X with AIDSVAX B/E. RT-PCR amplified variable heavy and variable light chain genes were Sanger sequenced (Genewiz) and analyzed with Cloanalyst (12).

Table 2. Properties and subtype of viruses used in Luciferase and Infected cell elimination assays.

Table 3. C1C2-specific antibody dependent cellular cytotoxicity (ADCC) of infectious molecular clone infected cells. Data are expressed as ADCC endpoint concentration (µg/mL).

Table 4: Ranking C1C2-specific mAbs by ADCC breadth and potency. RV305 and RV144 C1C2-specific mAbs were assayed for antibody-dependent cellular cytotoxicity against AE.CM235, B.WITO, C.TV-1, C.MW965, C.1086C, C.DU151 and C.DU422 infectious molecular clone infected CEM.NKR_{CCR5} cells. mAbs were ranked using an ADCC Score that accounts for breadth and potency (see methods). Number of strains recognized was determined by ADCC endpoint concentration.

Table 5. Details of the DH677.3, A32, and N12-i3 interfaces based on the DH677.3-gp120_{3TH057core} -M48U1, A32 Fab-ID2_{3TH057}, and N12-i3 Fab-
gp120\textsubscript{g3TH657core+N/C-M48U1} structures as calculated by the EBI PISA server \url{http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver}. The two copies in the asymmetric unit of the DH677.3, A32, and N12-i3 complexes are averaged in the table.

Table 6. Eigenvalues and variance explained by Principle Components. Eigenvalue is a measure of variance in the data along that Principal Components. Proportion of variance explains the percentage of variability explained by every Principal Components. As seen from the table first two PCs have Eigen values more than 1 and they account for 80.57\% of variance. ADCC scores are derived from PC1 scores which has an Eigen value of 5.424 and cumulative variance of 67.81 percent.

Table 7. DH677.3 structural data collection and refinement statistics.
| Study | Visit | PTID | DH#   | Variable Segment | Joining Segment | CDR3 Length (aa) | % mut (nt) | Ig Isotype/subclass | Variable Segment | Joining Segment | CDR3 Length (aa) | % mut (nt) |
|-------|-------|------|-------|------------------|-----------------|------------------|------------|-------------------|-----------------|-----------------|------------------|------------|
| RV144 | 8     | RV144_140 | DH877.1 | 1~8 1          | 16              | 1.04             | IgG1       | x1~27 5          | 9               | 0.857           |
| RV305 | 5     | RV305_094 | DH877.3 | 1~8 1          | 16              | 3.47             | IgG1       | x1~27 5          | 9               | 2.87            |
| RV305 | 5     | RV305_082 | DH697   | 1~46 6         | 24              | 7.99             | IgG1       | A1~44 3          | 11              | 4.87            |
| RV305 | 5     | RV305_094 | DH877.2 | 1~8 1          | 16              | 2.43             | IgG1       | x1~27 5          | 9               | 2.86            |
| RV305 | 5     | RV305_082 | DH836   | 3~23 4         | 12              | 4.17             | IgG1       | A8~61 3          | 10              | 2.22            |
| RV305 | 5     | RV305_094 | DH877.4 | 1~8 1          | 16              | 4.51             | IgG1       | x1~27 5          | 9               | 2.57            |
| RV305 | 5     | RV305_082 | DH695   | 1~2 6          | 22              | 5.56             | IgG1       | x1~29 3          | 9               | 5.30            |
| RV305 | 5     | RV305_082 | DH694   | 1~46 6         | 22              | 10.42            | IgG1       | x1~29 5          | 9               | 8.71            |
| RV305 | 5     | RV305_094 | DH888.1 | 1~46 6         | 23              | 6.97             | IgG3       | x1~29 4          | 9               | 6.00            |
| RV305 | 5     | RV305_094 | DH705.5 | 1~46 6         | 23              | 5.77             | IgG3       | K3~20 2          | 10              | 4.49            |
| RV305 | 5     | RV305_094 | DH705.4 | 1~46 6         | 23              | 6.01             | IgG1       | x1~29 5          | 9               | 3.43            |
| RV305 | 5     | RV305_311 | DH886   | 1~2 4          | 12              | 8.38             | IgG3       | x1~29 4          | 9               | 4.92            |
| RV305 | 5     | RV305_031 | DH690   | 1~46 6         | 24              | 6.92             | IgG1       | A1~44 2          | 11              | 3.62            |
| RV305 | 5     | RV305_094 | DH692   | 1~46 6         | 25              | 1.66             | IgG1       | x1~29 4          | 9               | 2.57            |
| RV305 | 5     | RV305_094 | DH693   | 1~46 4         | 26              | 5.18             | IgG1       | x1~9 5           | 9               | 2.86            |
| RV305 | 5     | RV305_094 | DH836   | 1~46 6         | 16              | 5.32             | IgG1       | x1~12 4          | 9               | 2.86            |
| RV305 | 5     | RV305_094 | DH888.2 | 1~46 6         | 23              | 7.69             | IgG3       | x1~29 4          | 9               | 6.57            |
| RV305 | 5     | RV305_031 | DH889.1 | 1~46 6         | 22              | 10.17            | IgG3       | x1~29 2          | 9               | 9.71            |
| RV305 | 5     | RV305_031 | DH889.2 | 1~46 6         | 22              | 10.41            | IgG3       | x1~39 1          | 8               | 8.55            |
| RV305 | 5     | RV305_031 | DH887.2 | 1~46 4         | 23              | 5.77             | IgG1       | K3~20 3          | 10              | 1.97            |
| Virus Name | Subtype | IMC type | Assay Used                      |
|-----------|---------|----------|---------------------------------|
| 1086c     | C       | Luciferase Env-IMC | Luciferase                     |
| CM235     | AE      | Luciferase Env-IMC | Luciferase                     |
| Du151     | C       | Luciferase Env-IMC | Luciferase                     |
| Du422     | C       | Luciferase Env-IMC | Luciferase                     |
| MW96.5    | C       | Luciferase Env-IMC | Luciferase                     |
| TV-1      | C       | Luciferase Env-IMC | Luciferase                     |
| WITO      | B       | Luciferase Env-IMC | Luciferase                     |
| CH77      | B       | Full-length IMC   | Infected cell elimination assay|
| CH264     | C       | Full-length IMC   | Infected cell elimination assay|
| CH0470    | B       | Full-length IMC   | Infected cell elimination assay|
| CH042     | C       | Full-length IMC   | Infected cell elimination assay|
| CH185     | C       | Full-length IMC   | Infected cell elimination assay|
| CH162     | C       | Full-length IMC   | Infected cell elimination assay|
| CH236     | C       | Full-length IMC   | Infected cell elimination assay|
| Sample | IMC infected cell ADCC endpoint concentration (µg/mL) |
|--------|-----------------------------------------------------|
| A32    | 0.0006104  0.002097  0.0006104  0.0009766  0.0009997  0.001628  0.009766 |
| CH58   | 0.0006104  0.00675  0.001343  0.01723  0.008663  0.0333  >40 |
| CH57   | 0.008608  >40  5.368  >40  9.307  0.1427  7.142 |
| CH90   | 0.06479  23  1.281  >40  0.4033  4.474 |
| CH54   | 0.08913  1.079  0.1192  >40  1.944  0.1169  >40 |
| DH77.1 | 0.008816  1.01  >40  >40  >40  >40 |
| DH77.3 | RV305  0.0007527  0.006146  0.002376  6.151  0.01439  0.02068  0.02528 |
| DH697  | RV305  0.004967  0.03909  0.02134  0.0657  0.08982  0.1096  1.047 |
| DH77.2 | RV305  0.002912  0.02705  0.01351  >40  0.03581  >40 |
| DH638  | RV305  0.006104  0.008903  0.03242  >40  0.05042  >40 |
| DH677.4| RV305  0.00182  0.008885  0.03447  6.947  0.02838  0.3027  >40 |
| DH695  | RV305  0.005664  0.5867  0.07879  1.325  0.3076  0.3342  12.15 |
| DH694  | RV305  0.0091  0.1159  0.1249  >40  0.2651  1.854  >40 |
| DH688.1| RV305  0.03942  0.09033  0.1375  8.079  0.3554  1.268  >40 |
| DH705.5| RV305  0.0122  0.07738  0.09824  >40  0.08881  0.3366  >40 |
| DH705.4| RV305  0.03119  0.2424  0.1735  2.813  0.1999  1.8  >40 |
| DH686  | RV305  0.01321  0.1425  0.1367  >40  1.583  0.1721  >40 |
| DH690  | RV305  0.09713  0.1758  0.3679  7.525  0.6221  2.862  4.34 |
| DH692  | RV305  0.07932  0.1438  0.3851  5.228  0.1444  6.399  >40 |
| DH693  | RV305  0.01995  2.168  0.05216  0.8583  1.361  2.194  6.363 |
| DH683  | RV305  0.02868  0.6244  0.1854  >40  0.1397  12.02  >40 |
| DH688.2| RV305  0.009277  0.1436  0.1338  >40  0.5658  4.409  >40 |
| DH691  | RV305  0.03596  0.9201  0.465  8.113  0.6149  2.376  >40 |
| DH688.1| RV305  0.0352  1.629  1.69  >40  4.817  9.348  30.49 |
| DH688.2| RV305  0.3429  2.225  0.4746  >40  5.814  6.567  1.094 |
| DH687.2| RV305  >40  >40  20.61  >40  2.033  >40  >40 |
| Rank | Antibody | Study    | Score | Number of strains recognized |
|------|----------|----------|-------|------------------------------|
| 1    | A32      |          | 6.62  | 7                            |
| 2    | CH38     | RV144    | 6.28  | 6                            |
| 3    | DH677.3  | RV305    | 4.56  | 7                            |
| 4    | DH697    | RV305    | 2.70  | 7                            |
| 5    | DH677.2  | RV305    | 1.72  | 5                            |
| 6    | DH838    | RV305    | 1.62  | 4                            |
| 7    | DH677.4  | RV305    | 1.30  | 6                            |
| 8    | DH695    | RV305    | 0.86  | 7                            |
| 9    | DH688.1  | RV305    | 0.66  | 6                            |
| 10   | DH694    | RV305    | 0.58  | 5                            |
| 11   | DH705.5  | RV305    | 0.52  | 5                            |
| 12   | DH705.4  | RV305    | 0.46  | 6                            |
| 13   | DH690    | RV305    | 0.36  | 7                            |
| 14   | DH692    | RV305    | 0.08  | 6                            |
| 15   | DH693    | RV305    | 0.06  | 7                            |
| 16   | DH886    | RV305    | -0.08 | 5                            |
| 17   | DH688.2  | RV305    | -0.30 | 5                            |
| 18   | DH836    | RV305    | -0.32 | 5                            |
| 19   | CH57     | RV144    | -0.48 | 5                            |
| 20   | CH90     | RV144    | -1.06 | 5                            |
| 21   | CH54     | RV144    | -1.20 | 5                            |
| 22   | DH689.2  | RV305    | -1.42 | 6                            |
| 23   | DH689.1  | RV305    | -1.68 | 6                            |
| 24   | DH677.1  | RV144    | -2.20 | 2                            |
| 25   | DH687.2  | RV305    | -2.70 | 2                            |
| Buried Surface Area, Å² | DH677.3 Fab - gp120<sub>93TH057</sub> core<sub>e</sub>-M48U1 | A32 Fab- iD2<sub>93TH057</sub> (4YC2) | N12-i3 Fab- gp120<sub>93TH057</sub> core<sub>e</sub> +N/C- M48U1 (5W4L) |
|------------------------|-------------------------------------------------|---------------------------------|---------------------------------|
| gp120 total            | 925                                             | 850                             | 803                             |
| 7/8-stranded β-sheet   | 248                                             | 0                               | 754                             |
| Layer 1                | 542                                             | 645                             | 49                              |
| Layer 2                | 135                                             | 205                             | 0                               |
| Layer 3                | 0                                               | 0                               | 0                               |
| **Heavy chain total**  | **475**                                         | **614**                         | **711**                         |
| FWR                    | 0                                               | 17                              | 16                              |
| CDR H1                 | 16                                              | 103                             | 39                              |
| CDR H2                 | 84                                              | 83                              | 440                             |
| CDR H3                 | 375                                             | 411                             | 216                             |
| **Light chain total**  | **498**                                         | **234**                         | **142**                         |
| FWR                    | 95                                              | 0                               | 0                               |
| CDR L1                 | 238                                             | 126                             | 10                              |
| CDR L2                 | 27                                              | 0                               | 0                               |
| CDR L3                 | 138                                             | 108                             | 132                             |
| **Heavy and light chain total** | **973**                                         | **848**                         | **853**                         |
### Eigenvalues of the Correlation Matrix

| PC | Eigenvalue   | Difference  | Proportion of variance | Cumulative variance |
|----|--------------|-------------|------------------------|---------------------|
| 1  | 5.42464828   | 4.40337817  | 0.6781                 | 0.6781              |
| 2  | 1.02127012   | 0.28980217  | 0.1277                 | 0.8057              |
| 3  | 0.73146795   | 0.32157054  | 0.0914                 | 0.8972              |
| 4  | 0.40989741   | 0.19988330  | 0.0512                 | 0.9484              |
| 5  | 0.21001411   | 0.0263      | 0.9747                 |                     |
| DH677.3 Fab | DH677.3 Fab-gp12093TH057-M48U1 |
|-------------|-----------------------------|
| **Data collection** | | |
| Wavelength, Å | 0.979 | 0.979 |
| Space group | P2₁ | P1 |
| Cell parameters | | |
| a, b, c, Å | 81.7, 70.5, 84.3 | 63.5, 80.1, 88.7 |
| α, β, γ, ° | 90, 98.3, 90 | 84.8, 82.3, 82.2 |
| Complexes/a.u. | 2 | 2 |
| Resolution, (Å) | 50-2.62 (2.67-) | 50-3.0 (3.05-3.0) |
| # of reflections | 2.62 | 2.62 |
| Total Unique | 96,040 | 30,469 |
| # of reflections | 28,247 | 9.2 (44.2) |
| Complexes/a.u. | 0.99 (0.84) | 10.2 (1.2) |
| Resolution, Å | 50.0 - 2.62 | 50.0 – 3.0 |
| Protein | 140 | 12,278 |
| Water | 28 | 426 |
| Ligand/Ion | Overall B value (Å)² | 38 | 102 |
| Protein | 32 | – |
| Water | 58 | 112 |
| Ligand/Ion | RMSD¹ | 0.004 | 0.004 |
| Bond lengths, Å | 1.3 | 1.3 |
| Bond angles, ° | 96.0 | 86.7 |
| Ramachandran² favored, | 3.8 | 10.3 |
| allowed, % | 0.2 | 3.0 |
| outliers, % | 6MFJ | 6MFP |

Values in parentheses are for highest-resolution shell

¹₂₆merge = Σ | I - <I> | Σ I, where I is the observed intensity and <I> is the average intensity obtained from multiple observations of symmetry-related reflections after rejections

²R₁ = as defined in (1)

³CC¹/² = as defined by Karplus and Diederichs (2)
\[ dR = \sum |F_o| - |F_c| / \sum |F_o|, \] where \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively.

\( ^eR_{\text{free}} = \) as defined by Brünger (3)

\( ^f\text{RMSD} = \) Root mean square deviation

\( ^g\text{Calculated with MolProbity} \)