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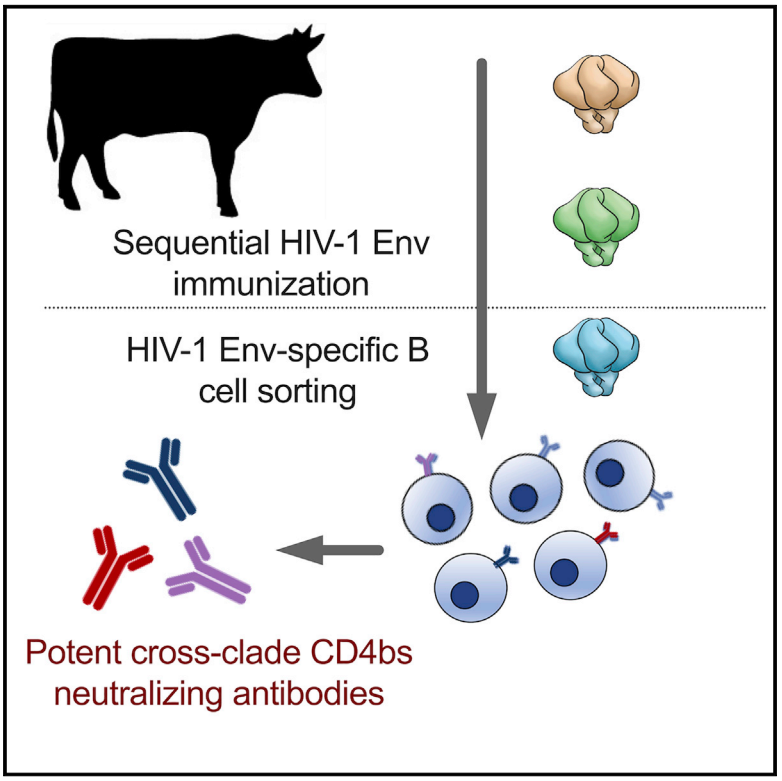
Graphical abstract



Authors

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In brief

Heydarchi et al. report that cows vaccinated with HIV-1 envelope can induce ultra-potent neutralizing antibodies targeting the key conserved sites on HIV-1 envelope. Isolated anti-HIV-1 bovine neutralizing antibodies are broader and more potent than a majority of anti-HIV-1 human broadly neutralizing antibodies.

Highlights

- Sequential vaccine with different SOSIP trimers could elicit bNAbs
- Cross-clade B-cell-sorting probe could select ultra-potent bNAbs
- Bovine CD4bs monoclonal antibody neutralizes HIV-1 isolates potently

Heydarchi et al., 2022, Cell Reports Medicine *3*, 100635
May 17, 2022
https://doi.org/10.1016/j.xcrm.2022.100635

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Broad and ultra-potent cross-clade neutralization of HIV-1 by a vaccine-induced CD4 binding site bovine antibody

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https://doi.org/10.1016/j.xcrm.2022.100635

SUMMARY

Human immunodeficiency virus type 1 (HIV-1) vaccination of cows has elicited broadly neutralizing antibodies (bNAbs). In this study, monoclonal antibodies (mAbs) are isolated from a clade A (KNH1144 and BG505) vaccinated cow using a heterologous clade B antigen (AD8). CD4 binding site (CD4bs) bNAb (MEL-1872) is more potent than a majority of CD4bs bNAbs isolated so far. MEL-1872 mAb with CDRH3 of 57 amino acids shows more potency (geometric mean half-maximal inhibitory concentration [IC_{50}]: 0.009 μg/mL; breadth: 66%) than VRC01 against clade B viruses (29-fold) and than CHO1-31 against tested clade A viruses (21-fold). It also shows more breadth and potency than NC-Cow1, the only other reported anti-HIV-1 bovine bNAb, which has 60% breadth with geometric mean IC_{50} of 0.090 μg/mL in this study. Using successive different stable-structured SOSIP trimers in bovines can elicit bNAbs focusing on epitopes ubiquitous across subtypes. Furthermore, the cross-clade selection strategy also results in ultra-potent bNAbs.

INTRODUCTION

An effective human immunodeficiency virus type 1 (HIV-1) vaccine is considered the best way to halt the ongoing HIV-1 infections that have caused a major impact on social and economic development. Producing a broadly effective vaccine against HIV-1 has proven difficult, principally due to the HIV-1 high sequence variability and the limitations of the human immunoglobulin repertoire to develop broadly neutralizing antibodies (bNAbs).[1] An effective vaccine will optimally present conserved epitopes represented[2] on many strains in a manner where B-lymphocyte recognition elicits potent bNAbs against many strains. But to achieve this during natural infection, most bNAbs require a high level of affinity maturation over several years.[3,4] Although vaccination of human[5] and animals[6,7] has yielded no or strain-specific neutralizing antibodies, cows have been able to induce bNAbs in a short period.[8–10] Bovine antibodies naturally have some unique features, such as ultra-long CDRH3[11] or presence of certain amino acids[12] that are similar to those playing roles in neutralization by human bNAbs. bNAbs prevent HIV-1 infection by targeting conserved epitopes on the envelope glycoprotein (Env),[13] and a stabilized soluble immunogen must mimic native membrane-bound pre-fusion Env. Although HIV-1 Envs truncated to remove the trans-membrane domain were initially stabilized by mutating the gp120 furin cleavage site,[14,15] resulting in uncleaved (Unc) Env gp140, this version had an open conformation and was antigenically distinct from proteolytically cleaved native pre-fusion Env on virions.[16,17] The development of a stabilized cleavable version of Env, BG505 SOSIP, the prototype of native-like Env trimer, so

far facilitated authentic presentation of the pre-fusion Env epitopes for multiple bNAbs with very few epitopes for non-neutralizing antibodies, thus avoiding immunological disruptions.[18]

Although BG505 SOSIP Env antigenically mimics HIV-1 Env, initial studies showed limited success in rabbits and non-human primates with induction of mainly autologous neutralizing antibodies with very little heterologous neutralizing activity.[6,7] Optimization of BG505 SOSIP and germ-line-targeting priming antigens have resulted in broader neutralizing antibodies against most tier 1, autologous tier 2, and some heterologous tier 2 viruses.[19] Despite the small success in eliciting tier 2 neutralizing responses in commonly used animal models, vaccination of cows with SOSIP BG505 Env[18] resulted in elicitation of potent bNAbs. Analysis of cow B-cell clones led to isolation of NC-Cow1 bNAb, which was more potent than the majority of human bNAbs isolated so far.[10] While NC-Cow1 showed the potential advantages of the bovine immune system in eliciting bNAbs against HIV-1, such advantages are not limited to SOSIP trimers. Indeed, our results showed that vaccinated cows could produce bNAbs in a much shorter time than human or other animals, with breadth achieved when cows were exposed to only non-native HIV-1 AD8 Unc gp140 HIV-1 Env vaccine without needing sequential immunogen modifications to cultivate an important germline lineage.[8–10] These immunoglobulin Gs (IgGs) were able to neutralize diverse HIV-1 viruses and inhibit Env binding of anti-CD4 binding site human bNAbs[8,9] and V3-loop-binding human bNAb.

Currently, there is a focus on therapeutic antibodies to provide immediate passive immunity to prevent or treat HIV-1 disease. Although there are numerous approved drugs against HIV-1 infection,[20] passive antibody prophylaxis and immunotherapy could hold a valuable place in both prevention and treatment due to the prospect of high stability and long bioavailability of Fc-engineered monoclonal antibodies (mAbs). Passive transfer of some human bNAbs demonstrated both pre-exposure prophylaxis and treatment in macaque,[21] humanized mouse models,[10,11] and human.[22] The result from antibody-mediated therapy to evaluate the safety, tolerability, and efficacy of the VRC01 antibody in preventing HIV-1 infection among men, transgender persons, and at-risk women showed proof of concept[23] while emphasizing the need to develop bNAb combinations to achieve optimal potency and breadth.

Surprisingly, treatment with bNAbs could also reduce cell-associated HIV-1 DNA and aid the development of HIV-1-specific T cell immunity,[25,26] which suggests a vaccine-like effect of bNAb immunotherapy.[27] Thus, in this study, we aimed to study anti-HIV-1 bovine bNAbs as an advanced approach to design HIV-1 therapeutic and prophylactic agents and to discover potential neutralizing sites on HIV-1 Env to aid rational vaccine design. Here, we immunized cows with different forms and strains of HIV-1 Env and showed that a well-ordered trimer (SOSIP gp140) elicited bovine antibodies and, using a cross-clade selection strategy, could successfully select broadly active and ultra-potent monoclonal bNAbs with ultralong CDRH3 regions.

RESULTS

Serum binding and neutralization

Female Holstein cattle were vaccinated prior to, during, and after pregnancy with different HIV Env proteins. To evaluate the antibody responses against HIV-1 vaccines, sera from different time points were collected and binding of bovine IgGs to Env immunogen was measured by direct ELISA (Figure 1B). Pre-immune sera presented low binding, while all samples at phase 1 presented endpoint titers of 100. At phase 2, re-vaccination with BG505 SOSIP in cow no. 1 increased binding to above 3,000, while changing the immunogen in cow no. 3 (from AD8 Unc gp140 on phase 1 to AD8 SOSIP on phase 2) showed a moderate increase in antibody titer (1,000). Cow no. 2, on the other hand, did not show an improved antibody titer despite changing the immunogen from AD8 Unc gp140 at phase 1 to BG505 SOSIP at phase 2. Neutralizing activity of sera from vaccinated animals was also investigated in a neutralization assay against a panel of seven pseudoviruses, including autologous Envs (Figure 1C). Cows no. 1 and no. 2 showed neutralization against JRCSF in pre-immunization phase, while neutralization decreased post-immunization. Neutralization against other tested viruses was induced only after immunization. Cow no. 1 showed the highest neutralizing activity against two pseudoviruses at phase 1 (ZM109F.PB4 and BG505 with median infectious dose [ID50] values of 26.7 and 67.2, respectively), and both potency and breadth increased at phase 2 with neutralizing activity against pseudoviruses MN, SF162, BG505, and AD8 (ID50 values of 274.3, 262.3, 1,000, and 257.5, respectively).

Although both cows no. 2 and no. 3 vaccinated with AD8 Unc gp140 were unable to induce an autologous neutralization, they induced neutralizing antibodies against multiple pseudoviruses. On phase 1, cow no. 2 induced neutralization against MN (ID50 of 25.9), and this increased in phase 2 for MN (ID50 of 127.4). Cow no. 3 showed neutralization against MN, SF162, BG505, and JRCSF at phase 1 (ID50s of 56.9, 27.6, 31.6, and 88, respectively), and the neutralizing activity enhanced against all mentioned viruses at phase no. 2 (ID50 values of 1,000, 55.9, 47.3, and 108.9, respectively).

HIV-1-specific bovine monoclonal antibodies

To isolate bovine B cells producing bNAbs, we developed a stabilized cleavable version of AD8 Env, AD8 SOSIP, to present the pre-fusion Env epitopes for multiple bNAbs with very few epitopes for non-neutralizing antibodies. The produced AD8 SOSIP was affinity purified using the 2G12 antibody followed by size exclusion chromatography (SEC) on a Superdex S200 16/600 column and characterized in reducing and non-reducing SDS gel, blue native (BN)-PAGE analysis, and capture ELISA (Figures S1C and S1D). Characterization of AD8 SOSIP confirmed that the proteins were predominantly trimeric and exposed epitopes of human bNAbs. Negative-stain electron microscopy (Figures S1G and S1H) also confirmed the trimeric structure, which appeared similar to BG505 SOSIP and closely resembled native Env spikes. AD8 SOSIP gp140 v4.1 binding to human bNAbs was confirmed in capture ELISA. HIV-1 Env-specific single B cells were sorted by fluorescence-activated cell sorting (FACS) from peripheral blood mononuclear cells

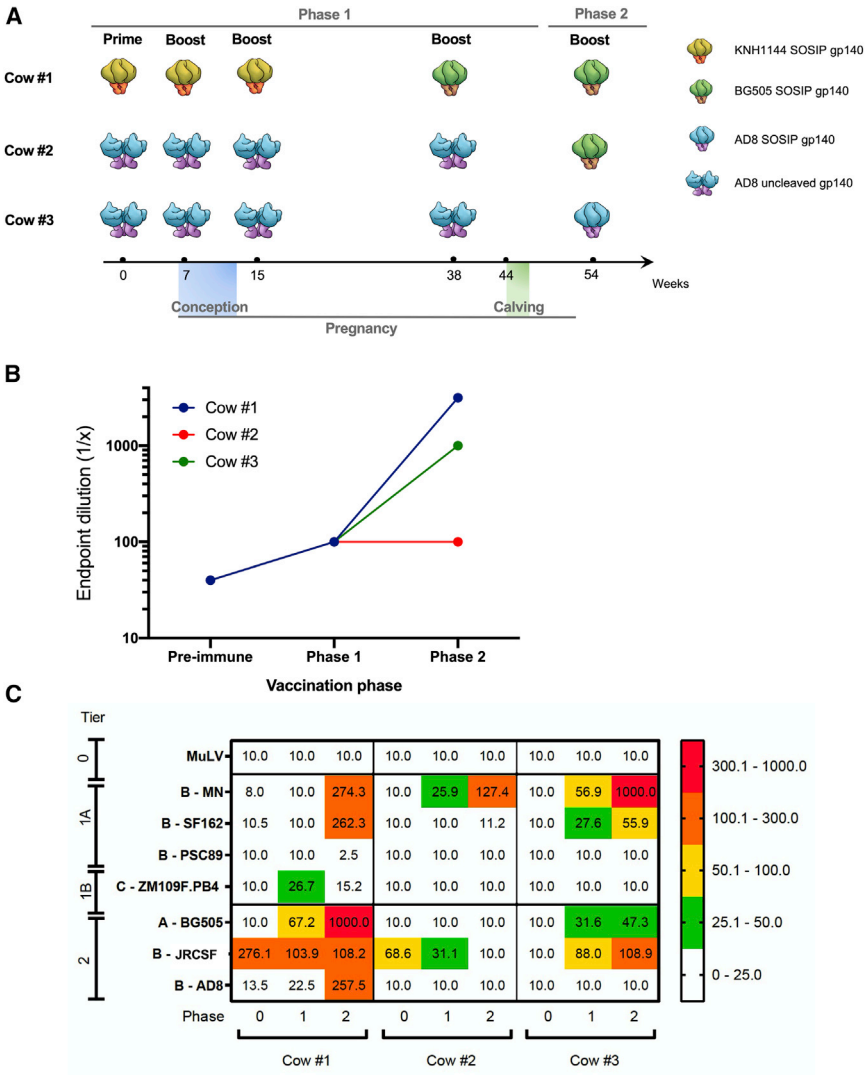


Figure 1. HIV Env binding and neutralization of serum samples collected from immunized cows

(A) Vaccination of cows during pregnancy. Cow no. 1 was vaccinated with KNH1144 SOSIP gp140 and BG505 gp140, while cows no. 2 and no. 3 were vaccinated with AD8 uncleaved gp140 followed by BG505 uncleaved gp140 and AD8 SOSIP gp140, respectively.

(B) Env binding of bovine IgGs in sera of vaccinated cows. Binding was measured against Env vaccine (cow no. 1: BG505 SOSIP; cow no. 2 and cow no. 3: AD8 SOSIP) through direct ELISA.

(C) Neutralization assays were performed against seven pseudoviruses from clades A, B, and C and tiers 1A, 1B, and 2; as negative control, MuLV pseudovirus was used. The values show ID_{50}. Heatmap scale shows no neutralization from a value of ID_{50} = 10 (white values) to the highest neutralization achieved at ID_{50} = 1,000 (red values). ELISA assays were performed in duplicate with two independent biological replicates.

(PBMCs) of cows no. 1, no. 2, and no. 3 using AD8 SOSIP. AD8 strain (clade B) is a tier 2 HIV-1 clade B virus, and we used this Env as bait to isolate B cells from AD8-vaccinated animals (cows no. 2 and no. 3). In addition, we used this bait to isolate B cells producing cross-clade anti-HIV-1 antibodies from the animal vaccinated with clade A Env (cow no. 1). HIV-1 Env-specific single B cells (IgG^+ and AD8 $SOSIP^+$) were sorted from PBMCs of vaccinated cows (Figure 2A), and after antibody variable gene amplification and further cloning of such genes into human antibody constant region expression vector, 47 chimeric mAbs were successfully produced from cow no. 1, from which 27 showed binding to AD8 SOSIP, four of which isolated from this cow showed neutralization against AD8 pseudovirus. Out of 87 chimeric mAbs constructed from cow no. 2, 46 mAbs could bind to AD8 SOSIP gp140 v4.1 trimer in capture ELISA (Figure S2). However, none of these mAbs from cow no. 2 neutralized autologous HIV-1 AD8 pseudovirus. From cow no. 3, 60 chimeric mAbs were produced, and although only 19 mAbs showed binding to SOSIP AD8, two of these antibodies showed autologous neutralization against AD8 pseudovirus in TZM-bl neutralization assay. The mAbs isolated from cow no. 1, MEL-1842 and MEL-1872, showed higher Env binding against AD8 SOSIP than VRC01 antibody. It is worth mentioning that, since the variable light gene was not rescued from MEL-1842 and MEL-1872 mAbs, these antibodies were produced using the light chain of MEL-2129 mAb. Three mAbs (MEL-1842, MEL-1872, and MEL-2129) belonged to the same antibody clonal family with ultralong CDRH3 length of 58 amino acids for MEL-2129 mAb and 57 amino acids for MEL-1842 and MEL-1872 mAbs. Sequences of the isolated antibody variable genes are deposited in GenBank and listed in Figure S3 and Table S3.

Anti-HIV-1 bovine MEL-1872 mAb is extraordinarily potent

To evaluate the neutralization properties of AD8-SOSIP-binding antibodies, TZM-bl neutralization was performed using a virus panel including HIV-1 12-virus global panel and several heterologous HIV-1 viruses. Anti-HIV-1 mAbs isolated from cow no. 2 showed a narrow breadth, but mAbs of cow no. 3 showed a moderate breadth against clade B viruses (Figure S4; Tables S5 and S6). Most of the isolated bNAbs from all three cows neutralized <50% of HIV-1 viruses with geometric mean IC_{50} of above 0.09 μg/mL (Figure S5A), while three mAbs of MEL-1842, MEL-1872, and MEL-2129 isolated from cow no. 1 showed the highest breadth (64%, 66%, and 51%, respectively). Among them, MEL-1842 and MEL-1872 mAbs demonstrated the greatest potency with geometric mean IC_{50} of 0.013 μg/mL and 0.009 μg/mL and IC_{80} of 0.045 μg/mL and 0.033 μg/mL, respectively (Tables S5

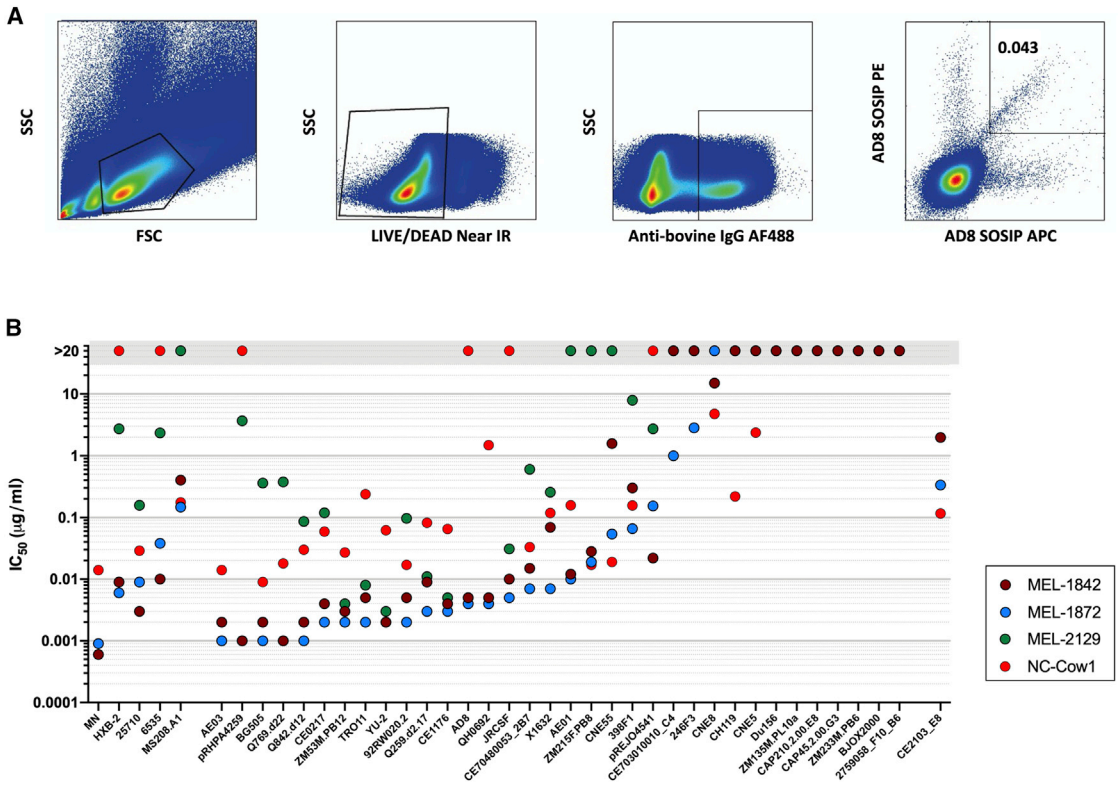


Figure 2. Isolation of bNAbs from HIV-1 vaccinated cows

(A) Cow PBMCs were sorted for IgG$^+$ cells that bound to biotinylated AD8 SOSIP-AviTag conjugated to phycoerythrin (PE) and antigen-presenting cell (APC) fluorophores. FSC, forward scatter; SSC, side scatter.

(B) Bovine bNAbs showed potent cross-clade neutralization against tier 1 and tier 2 viruses with low geometric mean IC_{50}. Neutralization assays were performed in duplicates with two independent biological replicates.

and S6). Among mAbs isolated from cow no. 1, there was a correlation between low geometric mean IC_{50} and high breadth (Figure S5B). However, for AD8 neutralizing mAbs, there was a lack of correlation between IC_{50} and half-maximal effective concentration (EC_{50}) for this strain (Figure S5C). Antibody MEL-1842, MEL-1872, and MEL-2129 all showed cross-clade neutralization against tier 1 and tier 2 viruses, with MEL-1872 neutralizing most of tier 2 viruses with IC_{50} value of 0.1 μg/mL (Figure 2B).

Antibodies MEL-1842 and MEL-1872 demonstrated broader and more potent HIV-1 neutralizing activity compared with NC-Cow1 (60% breadth with IC_{50} of 0.09 μg/mL) against tested viruses (Figures 3A and 3B). MEL-1842 and MEL-1872 showed the greatest potency against clade B viruses with geometric mean IC_{50} of 0.004 μg/mL followed by clade A viruses with geometric mean IC_{50} of 0.011 μg/mL and 0.005 μg/mL, respectively (Figures 3C and 3D). Antibody MEL-1872 was 29-fold more potent than VRC01 (geometric mean IC_{50} of 0.117 μg/mL) against tested clade B viruses (Figure S4) and 21-fold more potent than CHO1-31 (mixture of two CHO-1 [PG9-like] and CHO-31 [VRC01-like] bnAbs) against tested clade A viruses (geometric mean IC_{50} of 0.042 μg/mL).

The alignment of germline genes with the heavy-chain and light-chain gene of MEL-1872 mAbs is shown in Figure 4. Sequences were then annotated with IMGT High V-Quest (http://www.imgt.org/HighV-QUEST/; Table S4). Conventional Kabat numbering was used for antibody light-chain and for heavy-chain amino acids (H1-100b and H101), but CDRH3 residues encoded by the IGHD8-2 gene segment (between H100b and H101 and its connection to the J chain) were numbered using a "D" identifier sequentially.[28] IMGT was able to annotate both VH and JH germline genes for the bovine mAbs while failing to assign the germline genes for ultralong CDRH3. IgDiscover[29] and AbMining Toolbox[30] were used for identification of DH germline genes. As shown, there are limited hypersomatic mutations in the light gene and VH and JH of the heavy gene while, compared with germline DH (IGHD8-2*01), there are significant mutations in CDRH3 region of MEL-1872 mAb.

Bovine bNAbs bind to non-trimer-specific epitopes on HIV-1 Env

As shown in Figure 5A, bovine bNAbs displayed binding to monomeric AD8 gp120, Unc HIV-1 gp140, and AD8 SOSIP gp140 v4.1, confirming that their epitopes were present on gp120 monomers of both cleaved and Unc Env trimers. These mAbs could also bind to ConM SOSIP, which is an Env trimer based on a consensus sequence of all HIV-1 group M isolates. This trimer displays most bNAb epitopes and is made to minimize clade-specific and strain-specific antigenic determinants.[31]

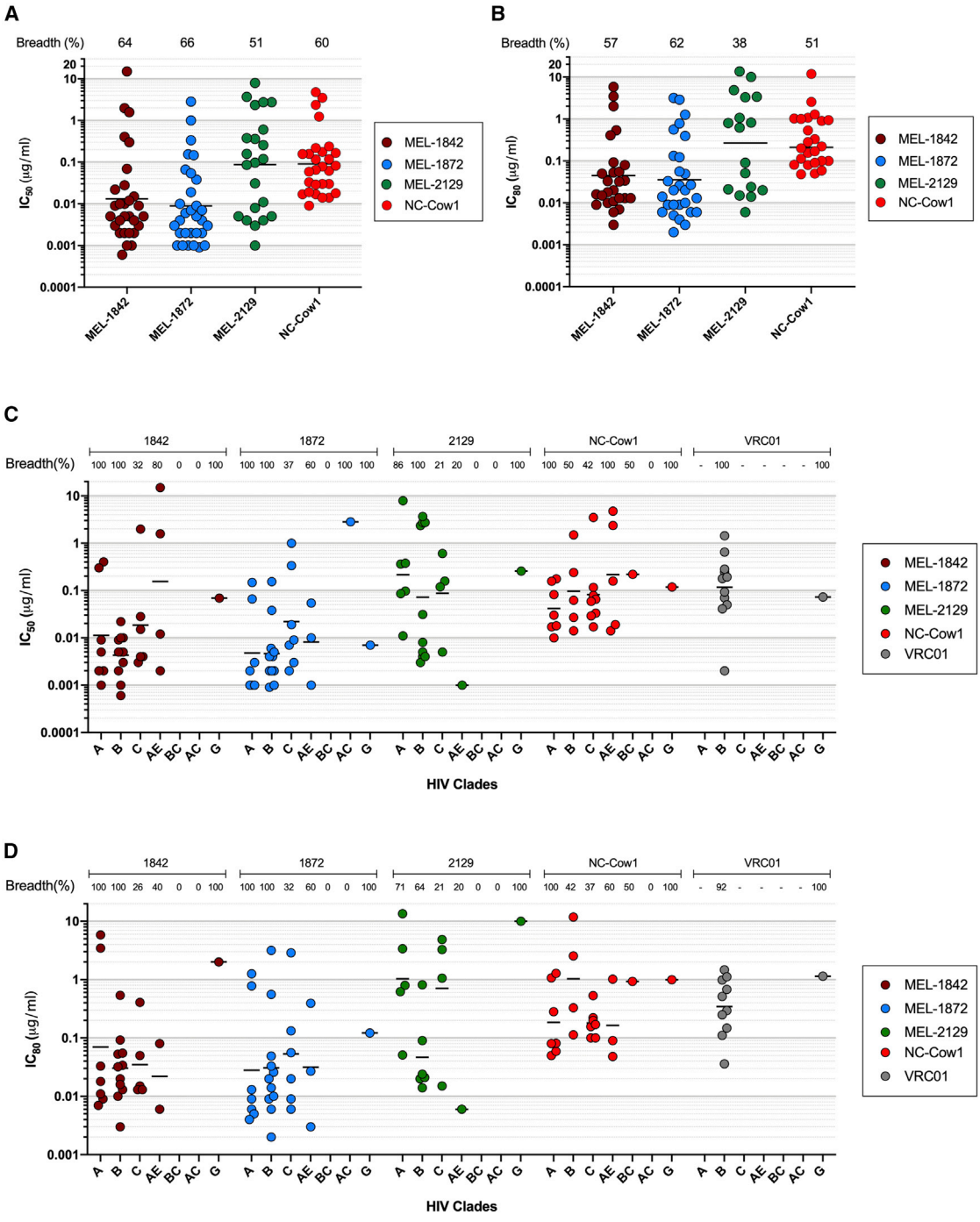


Figure 3. Neutralization profile of bNAb MEL-1842, MEL-1872, and MEL-2129

Comparison of IC_{50} (A) and IC_{80} (B) values of isolated mAbs with bNAb NC-COW1. Categorization and comparison of neutralization activity against different HIV clades using IC_{50} (C) and IC_{80} (D) values is shown. The black lines represent the geometric mean IC_{50} and IC_{80}. Neutralization assays were performed in duplicates with two independent biological replicates.

Bovine bNAbs bind to CD4bs on HIV-1 Env

A competition ELISA with reference human bNAbs targeting four known epitopes was performed to evaluate interference with bovine mAbs binding to AD8 SOSIP. Bovine mAbs 2129, 1842, and 1872 inhibited Env binding of human CD4bs bNAbs b12, VRC01, HJ16, and 3BNC117, except for 2129, which showed incomplete inhibition of HJ16 binding (Figure 5B). Other bovine mAbs demonstrated partial competition (25%–50%) with V2-apex human bNAbs (PGT145) and gp120-gp41 interface human bNAbs (PGT151). There was also strong

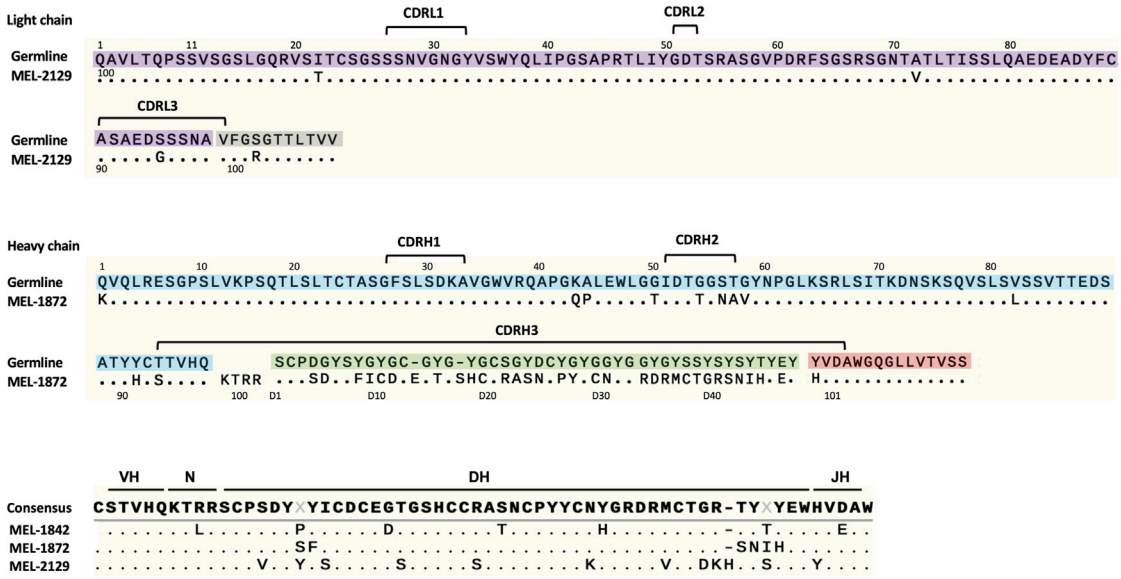


Figure 4. Amino acid alignment of heavy- and light-chain sequences of MEL-1872 mAb with the germline genes

Purple: IGLV1-47*01 germline gene; gray: IGLJ4*01 germline gene; blue: IGHV1-7*02 germline gene; green: IGHD8-2*01 germline gene; red: IGHJ2-4*01 germline gene.

competition between MEL-1842 and MEL-1872 mAbs, demonstrating that these mAbs may share a common or proximate binding site(s). On the other hand, MEL-2129 mAb showed lower inhibitory effect on Env binding of MEL-1842 and MEL-1872 mAbs.

Epitope mapping of bovine bNAbs with HIV-1 Env mutants

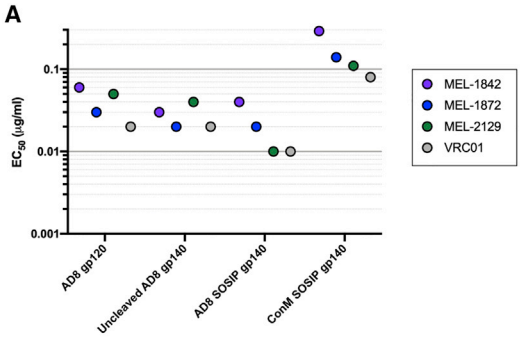
Affinity binding of bovine bNAbs to a panel of 33 AD8 Env mutants showed that mutations located primarily in the CD4bs, C4, and C5 regions of Env impeded the binding of these mAbs (Figures 5C and S6; Table S7). Using Swiss Modeller,[24] the binding site of bovine bNAbs is shown in Figure 6. For MEL-1842, MEL-1872, and MEL-2129 mAbs (which are from the same clonal lineage antibody family), the most significant loss of binding was observed for mutations G366A, I371A, D457A, and G471A, which resulted in $\leq$30% Env binding compared with wild-type (WT) Env. In previous studies, alanine mutation in G366, I371, and D457 was shown to prevent binding of human CD4bs bNAbs, such as N6, VRC01, VRC-PG04, and VRC27 to JRCSF gp120.[32] The mutations N262A, T455A, and G472A also inhibited the binding of MEL-1872 and MEL-2129 mAbs. Mutation in N262 was shown to inhibit binding of CD4bs bNAbs 3BNC117, VRC01, VRC-PG04, and VRC027, while substitution of G472 with alanine only hindered binding of VRC27.[32] In addition, mutations D279A, G473A, and D474A resulted in a substantial loss of binding for MEL-2129 (all $\leq$30% Env binding compared with WT AD8 Env). VRC01 and b12 were used as controls and exhibited the lowest binding percentage to mutations introduced in the CD4bs, as expected.[32] VRC01 showed a significant decrease in Env binding in response to mutations to the N-linked glycan at position 279 and the residues G367A and G368A ($\leq$30% binding). b12 also demonstrated a similarly low decrease in binding to G368A ($\leq$30% binding). For PGT121, the mutation of the glycan at position 332 resulted in the most substantial decrease in binding.

Neutralization activity of bovine bNAbs was also assessed in a TZM-bl neutralization assay using a panel of 27 mutated AD8 pseudoviruses. As shown in Figure 5C, the G471A mutation resulted in the most significant effect on neutralization of MEL-1842, MEL-1872, and MEL-2129 mAbs and increased IC_{50} and IC_{80} values to $\geq$4 μg/mL. I371A mutation also obstructed the neutralization activity of MEL-2129 and MEL-1872 mAbs effectively, while MEL-1842 mAb was insensitive to this mutation. G471 and I371 are also epitope residues for most of human CD4bs bNAbs, including HJ16, 8ANC131, CH103, and b12,[33] demonstrating the overlap between epitopes of bovine and human CD4bs bNAbs. In agreement with ELISA data and in addition to the mentioned mutations, mutations in C4 (T455A) and C5 (D474A) showed neutralization impediment for bovine MEL-2129 mAb. As expected, D279A mutation rendered VRC01 unable to neutralize AD8 mutated pseudovirus. In addition to G471A mutation, G366A and V372A mutations also inhibited neutralization activity of b12. PGT121 was affected by E370A and N332T mutations.

A neutralization assay with multi-clade panel of viruses and their corresponding CD4bs mapping mutations (N279A, N280D, and G458Y) showed evidence of CD4bs specificity for MEL-1842, MEL-1872, and MEL-2129 with at least three of the viruses (Table S8).

Bovine bNAbs are not polyreactive

Autoreactivity and polyreactivity of MEL-2129, MEL-1842, and MEL-1872 mAbs were evaluated in HEp-2 staining and ELISA assay against several human autoantigens. These bovine bNAbs



B

		MEL-1967	MEL-2000	MEL-2114	MEL-1905	MEL-2010	MEL-2028	MEL-1842	MEL-1872	MEL-2129	MEL-33	MEL-130	MEL-F2	MEL-D1	MEL-198	MEL-563	MEL-782	NC-COW1
CD4bs	Self	68	89	46	63	87	63	85	80	63	72	63	74	57	63	81	95	56
	b12	-47	-46	-1	-33	-8	-47	84	84	56	6	11	9	-14	5	14	0	9
	VRC01	0	12	1	2	-1	-8	87	76	45	3	3	-2	7	13	4	0	11
	HJ16	23	11	13	10	4	-14	75	73	26	6	9	4	-1	-4	2	12	16
	3BNC117	12	19	1	4	4	-3	89	74	54	0	-2	-1	1	16	-4	2	15
V3-glycan	PGT121	3	11	-6	-5	-3	-5	4	10	0	-12	-3	-5	-5	-8	-7	-3	-1
	10-1074	6	7	-2	-2	-2	-15	11	14	0	-8	-3	2	-12	-13	-4	-8	-6
V2-apex	PGT145	11	26	4	3	-5	-6	-11	-2	15	-6	3	-3	-8	-13	-2	-3	-15
Interface	PGT151	49	33	9	31	0	23	27	15	8	-4	1	2	-15	-18	-1	-6	-2
	1842	-	-	-	-	-	-	85	78	47	-	-	-	-	-	-	-	-
	1872	-	-	-	-	-	-	91	80	39	-	-	-	-	-	-	-	-
	2129	-	-	-	-	-	-	89	67	63	-	-	-	-	-	-	-	-

Inhibition (%) 0 100

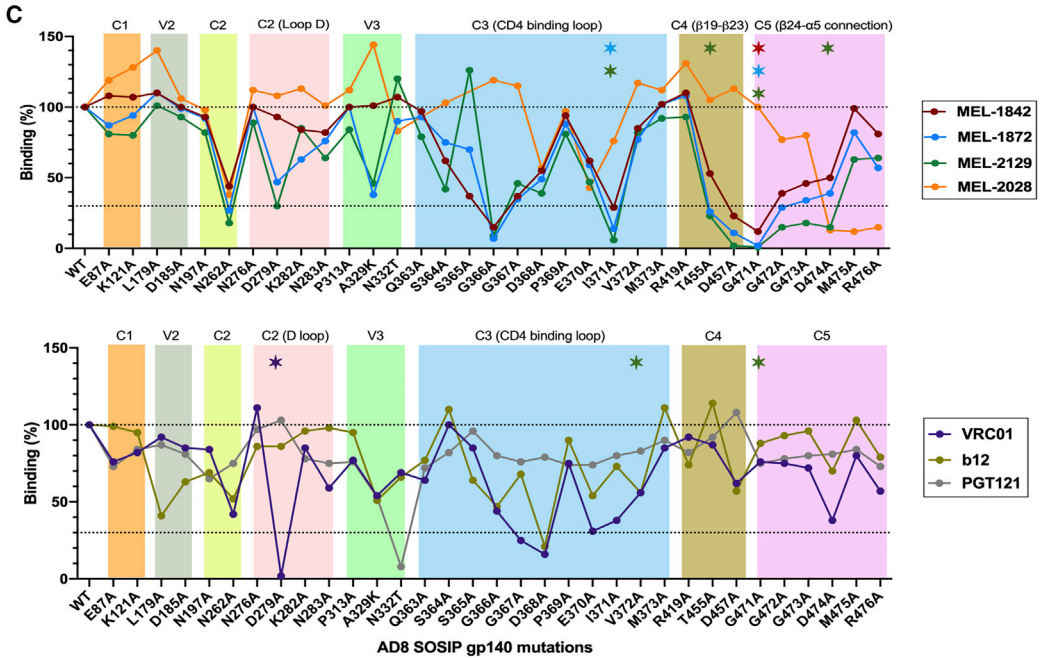


Figure 5. Epitope mapping of bovine monoclonal antibodies

(A) Binding of bovine bNAbs to HIV Envs. Bovine bNAbs were tested in direct ELISA assays to evaluate their binding to different forms of Env (monomeric gp120, uncleaved gp140, and SOSIP gp140) as well as ConM SOSIP, which is an Env trimer based on a consensus sequence of all HIV-1 group M isolates.

(B) Competition of bovine bNAbs with human bNAb for Env binding. The table shows the competition ELISA assay with values demonstrating Env binding inhibition (percentage) of human bNAbs by bovine antibodies. Competition ELISA between antibodies MEL-1842, MEL-1872, and MEL-2129 showed these antibodies bind to the same or proximate epitopes. Higher competition is shown in red, and lower inhibition values are in increasingly pale shades of orange. ELISA assays were performed in duplicates with two independent biological replicates.

(legend continued on next page)

showed no autoreactivity or polyreactivity against tested antigens (Figures 7A and 7B).

DISCUSSION

Despite decades of research, vaccination using HIV-1 Env formulations in humans and animal models have mostly yielded no or merely autologous, narrow-focused, neutralizing antibodies. In contrast, bovine antibodies naturally have some unique features, such as ultralong CDRH3 regions[11] or the presence of key amino acids[12] that are similar to those often observed in human bNAbs and promote development of bNAbs to HIV-1 Env.[8–10] Previous studies have shown that the bovine immune system does not require a long period of affinity maturation to induce bNAbs.[9,10] In this study, we vaccinated pregnant cows with different HIV-1 Env antigens, which induced bNAbs following vaccination. The pre-immune samples of bovines with no prior experience of HIV vaccines showed a low binding activity, despite no exposure to HIV antigen before the vaccination. We previously showed[8,9] that the genetic property of ultralong CDRH3 immunoglobulin domains close to the unmuted germ line in cows was mediating a low-level background of anti-HIV activity in unvaccinated cows.

In agreement with previous studies,[10] BG505 SOSIP vaccine was able to induce bNAbs. Although the serum samples showed low potency due to the transfer of bovine antibodies from serum to colostrum milk during pregnancy, we were able to isolate potent cross-clade bNAbs from this cow. While cell-mediated alloantigen rejection is suppressed at the maternal-fetal interface, further investigation is required to reveal whether these immunomodulatory effects impact on the attainment of anti-HIV bNAbs here after immunization of cows during pregnancy. In addition, different cell lines were used to produce HIV Env antigens in this study, and despite reports that glycosylation patterns of recombinant gp120 monomer from four HIV-1 clades produced in different cell lines revealed similar glycosylation profiles, we cannot exclude these small differences' impact on binding of antibodies targeting key immunological epitopes on gp120 monomer and on protein immunogenicity.[34]

Neutralizing mAbs to BG505 SOSIP have been reported in guinea pigs,[35] rabbits,[33] cows,[8–10] and rhesus macaques.[36] In BG505-SOSIP.664-immunized rabbits eliciting autologous neutralizing antibodies, the dominant epitope was the 241/289-glycan hole in approximately 50% of the animals, while the C3/465 epitope was frequent in 25% of sera.[7] On the other hand, the 241/289-glycan hole was targeted only in a minority of BG505-SOSIP.664-vaccinated rhesus macaques[7,19] and the dominant potent autologous neutralizing epitopes were V1/V3 and C3/V5 epitopes.[37] In this study, we evaluated binding and neutralizing activity of several mAbs to a panel of alanine mutants for HIV-1 AD8 isolate. The MEL-1872 mAb showed binding to CD4bs, and like CD4 and other CD4bs antibodies,[38] mutagenesis of conserved gp120 residues (366–368 from the CD4 binding loop and 473 from the β24-α5 region) reduced Env binding of this antibody. The mutations I371A, T455A, D457A, G471A, and G472A were also found to be of importance for resistance towards MEL-1872 mAb in AD8 strain. All mutated positions are part of, or in close vicinity to, the CD4bs, and most are highly conserved.

Among reported anti-HIV-1 neutralizing antibodies, CAP256-VRC26.25 (targeting V1V2 apex) has been the most potent antibody with geometric mean IC_{50} of 0.012 μg/mL (median IC_{50} = 0.006 μg/mL; breadth 59%) thus far.[39] The CDRH3 of CAP256-VRC26.25 comprises 36 amino acids and is one of the longest human CDRH3s identified.[39] As shown in previous studies,[39,40] there is a correlation between neutralization potency and CDRH3 length. CAP256-VRC26.25 has a protruding CDRH3 comprising a two-stranded antiparallel β sheet that is stabilized with a disulfide bond. Although CAP256-VRC26.25 has approximately 70% breadth on clade C viruses,[41–43] it shows limited breadth (<30%) on clade B viruses[39] due to the relative rarity of acidic and basic residues recognized by the antibody at positions 164 and 169, respectively.[39,41] NC-Cow1 is also a potent anti-HIV-1 bovine CD4bs bNAb with a protruding CDRH3 of 60 amino acids and broad neutralization against clade A viruses but moderate neutralization against clade B and C viruses.[10] The bovine antibody isolated in this study (MEL-1872) showed higher potency with geometric mean IC_{50} of 0.009 μg/mL (median IC_{50} = 0.006 μg/mL) and breadth (66%) than CAP256-VRC26.25. In addition, it showed more breadth and potency compared with NC-Cow1 against tested viruses (66% versus 60% breadth; geometric mean IC_{50} of 0.009 μg/mL versus 0.090 μg/0 mL). Although MEL-1872 and NC-Cow1 mAbs showed similar breadth against clade A and C viruses, the former antibody showed more potency (8-fold and 4-fold, respectively). MEL-1872 mAb also neutralized clade B viruses with more potency (above 23-fold) and breadth (100% versus 50%) than NC-Cow1. Using BG505 SOSIP as a sorting bait in a BG505-SOSIP-vaccinated cow resulted in isolation of one bnAb (NC-Cow1) out of seven strong Env-binding bnAbs (14%),[10] while use of AD8 SOSIP as the sorting bait in this study resulted in isolation of three bnAbs out of nine strong Env-binding bnAbs (33%) from cow no. 1, which was vaccinated with BG505 SOSIP. Thus, although like NC-Cow1, MEL-1872 mAb was isolated from a BG505 SOSIP (clade A)-vaccinated cow, the usage of a tier-2 HIV-1 Env with different clade (AD8 SOSIP; clade B) for B cell sorting might be the explanation of why MEL-1872 neutralization was broader and more potent against tested viruses than NC-Cow1 and other CD4bs antibodies that were isolated using Env-binding-based, HIV-1-specific B cell selection.[56,44] The approach of using mis-matched antigens has been used with other viral glycoproteins to isolate broad and potent antibodies. Sauer et al.[45] immunized mice and screened antigen-specific B cells using multiple variants of coronavirus Spike glycoproteins, which resulted in isolation of a broad coronavirus bNAb with high affinity.

(C) The effect of alanine mutagenesis on binding of bovine antibodies to AD8 gp120 captured from lysed virions. ELISA assay was performed using a constant half-maximal effective concentration (EC_{50}) of each antibody to AD8 WT Env. Stars also show significant neutralization IC_{50} increase of each antibody against mutated virus compared with WT virus (5-fold for all mAbs, except 198). PGT121 (V3-glycan epitope) and b12 and VRC01 (CD4bs epitope) were included for comparison. ELISA and neutralization assays were performed in duplicate with two independent biological replicates.

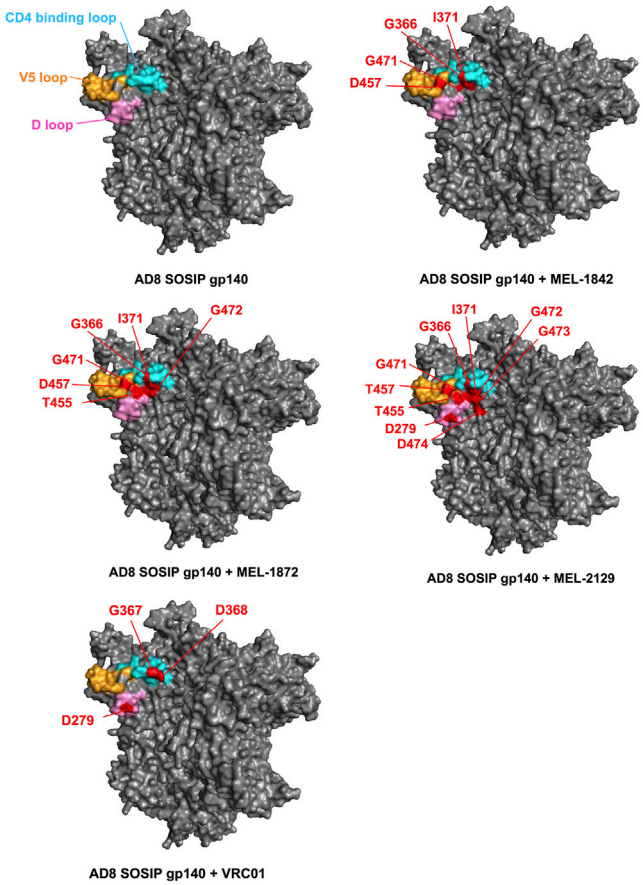


Figure 6. Binding of bovine bNAbs to CD4bs

Highlighted on the BG505 SOSIP.664 trimer (top left) are residues from the D loop (275–283: SNFTDNAKN, pale pink), CD4 binding loop (362–375: NQSSGGDPEIVMHS, cyan), and V5 loop (458–469: GGNNHNNDTETFR, pale orange). Binding site of antibodies MEL-1842, MEL-1872, MEL-2129, and VRC01 to AD8 SOSIP V4.1 are shown with red color, indicating the residues that binding of bovine antibodies to them was reduced through mutagenesis. Homology model of AD8 SOSIP was obtained using Swiss Modeller.

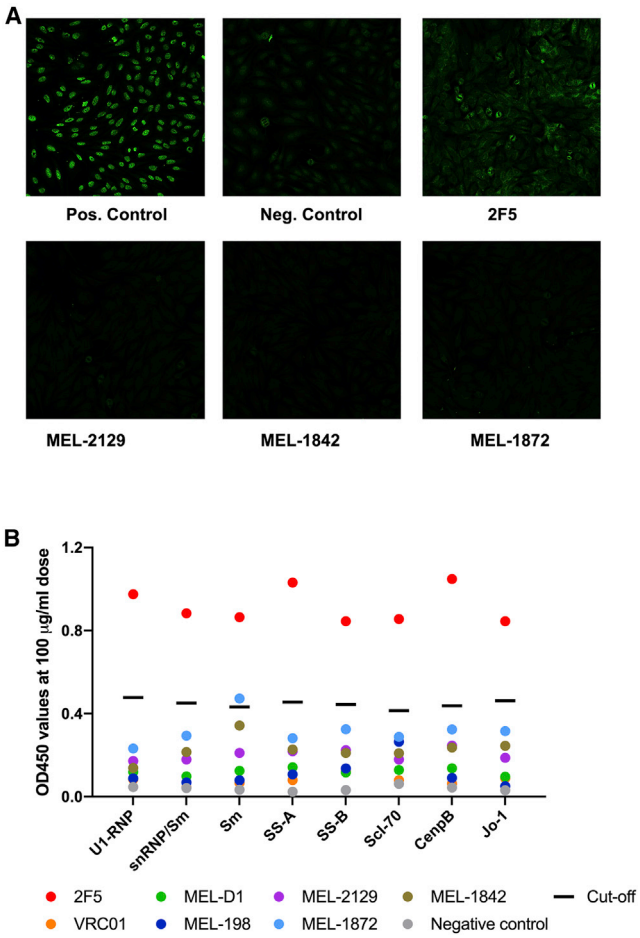


Figure 7. Assessment of bovine bNAb polyreactivity

(A) Polyreactivity test in Hep-2 cells. Bovine bNAbs MEL-1842, MEL-1872, MEL-2129, and MEL-198 did not show any polyreactivity against human Hep-2 cells. 2F5 is human anti-HIV bNAb with polyreactivity, while anti-HIV bNAb PGT121 is not polyreactive.

(B) Assessment of antibody polyreactivity against human antigens. ELISA assay was performed against human antigens using constant amount of 100 μg/mL from tested mAbs. Black line indicates cutoff values as indicated by the manufacturer. Each assay was repeated twice. snRNP, small nuclear ribonucleoprotein particle.

mAbs isolated from patient or vaccinated animals may guide vaccine design in an approach known as reverse vaccinology.[34,46] This has become a productive avenue for HIV-1 vaccine research.[46] A high-resolution structure of mAb-antigen complexes can facilitate immunogen design by disclosing the important details of epitopes.[47] A compelling HIV vaccine is required to induce bNAbs against conserved epitopes,[2] and many bNAbs target the CD4bs as a conserved region on HV Env.[56,48] Immunization of guinea pigs with epitope-focused antigenic domain of the CD4bs VRC01 antibody, called EAD-VRC01, induced CD4bs neutralizing antibodies against subtype B viruses.[49] Structure of MEL-1872 in complex with HIV Env would help reveal its mechanism of potent neutralization, and this could be useful in design of epitope vaccines.

Although there are numerous approved drugs against HIV-1 infection, they are limited to wealthy nations, and lifelong treatment requires diligent adherence and can be associated with some toxicity and economic cost.[50] Long-term use of anti-retroviral drugs could also introduce drug-resistant escape mutants.[51] Passive antibody prophylaxis and immunotherapy could hold a valuable place in both the prevention and treatment of HIV-1 infection. Disulfide bonds in bovine CDRH3 results in a rigid structure that might survive the acidic environment of mucosal environment better than human bNAbs.[10] This rigid structure in bovine CDRH3 provides an excellent opportunity to design small-molecule drug inhibitors accessing deep recessed epitopes on HIV-1 Env more efficiently than human bNAbs. Bovine bNAbs with broad diagnostic, therapeutic, and prophylactic potential can be used not only against HIV-1 but also to control or prevent other diseases. There is evidence showing the effectiveness of bovine IgG isolated from colostrum to improve the management of patients with HIV-1-associated diarrhea,[52] to boost the immune response in HIV-1-positive

children,[53] to neutralize respiratory syncytial virus (RSV) *in vitro*,[54] or to prevent influenza infection in healthy volunteers.[55] In a clinical trial, polyclonal IgG antibody produced from Middle East respiratory syndrome (MERS)-immunized cows was safe and well tolerated in healthy participants (up to 50 mg/kg). With the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there has been an urgent need for therapeutic and prophylactic treatments. In this regard, vaccination of cows against SARS-CoV-2 spike protein can induce bNAbs against recessed epitopes, and since bovine IgG-derived colostrum has been reported to be resistant to proteolysis, consumption of SARS-CoV-2 hyperimmune colostrum or immune milk could offer short-term protection to individuals until effective and safe vaccines are more widely available worldwide.

Studying bovine antibody responses in HIV-1-vaccinated cows will be very valuable, as the induced bNAbs may introduce new neutralizing epitopes and/or sites on Env protein that are not easily accessible to human antibodies. Anti-HIV-1 bovine antibodies, particularly those that share similarities to human bNAbs in terms of target epitopes and antibody molecular features, will provide extremely important research output in advancing rational design of HIV-1 vaccines and antibody-based therapeutics and prophylactics.

Limitation of the study

Different cell lines were used to produce HIV Env antigens in this study, and despite reports that glycosylation patterns of recombinant gp120 monomer from four HIV-1 clades produced in different cell lines revealed similar glycosylation profiles, we cannot exclude these small differences' impact on binding of bovine polyclonal antibodies targeting key immunological epitopes on gp120 monomer and on protein immunogenicity. While using cross-clade, B-cell-sorting probe resulted in isolation of potent bNAb, one limitation of the study is the small number of animals used. Vaccination of animals with HIV-1 Env and B cell sorting with different cross-clade antigen probes in future can lead to robust statistical analyses of the results. In addition, although MEL-1872 showed robust HIV-1 neutralization, the efficacy of protection *in vivo* needs to be further studied.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100635.

ACKNOWLEDGMENTS

This work was conducted under animal ethics approval 2015-17 from the Victorian State Government DEDJTR Research and Extension Animal Ethics Committee. Funding support was provided to D.F.J.P. from the Australian NHRMC - EU Horizons 2020 Grant (APP1115828), as a partner in the European AIDS Vaccine Initiative 2020 (EAVI 2020), from the Australian Centre for HIV-1 and Hepatitis Virology Research; to B.H. from the Melbourne HIV-1 Cure Consortium and the Australian Centre for HIV-1 and Hepatitis Virology Research; to A.S. from the University of Melbourne Early Career Research Grant; and to J.P.M. from grant P01 AI110657 and to D.M. from grant HSN272201800004C from the National Institutes of Health. This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement no. 681137. We acknowledge contributions of Leanne Horstman, Greg Morris, and Lianne Dorling (Department Jobs, Precincts and Resources, Victoria) for veterinarian support and animal husbandry and George Lovrecs and Brian Muller for technical support.

AUTHOR CONTRIBUTIONS

Conceptualization and oversight of the experiment, B.H. and D.F.J.P.; antigen B cell sorting, B.H.; PCR and antibody cloning, B.H. and J.M.E.; antibody gene analysis, B.H. and N.A.S.-Q.; antibody expression and purification, B.H., J.M.E., and S.G.; antibody characterization, neutralization, and mutagenesis experiments, B.H., D.S.F., N.A.S.-Q., H.G., and D.M.; provided proteins for immunization, R.W.S., M.J.v.G., J.P.M., A.C., and C.A.G.; structural work and analysis, A.S.; polyreactivity assays, B.H. and T.E.A.; cow immunization, D.F.J.P., W.J.W., and C.M.; analysis, figures, and original draft of manuscript, B.H., D.S.F., A.S., and N.A.S.-Q.; review and editing of the manuscript, B.H., D.S.F., H.G., N.A.S.-Q., J.M.E., C.A.G., S.G., T.E.A., C.M., W.J.W., M.J.v.G., A.C., I.R., P.R.G., J.P.M., R.W.S., D.M., A.S., and D.F.J.P.; funding acquisition, D.F.J.P., B.H., and A.S.

DECLARATION OF INTERESTS

B.H. and D.F.J.P. are inventors on a corresponding patent from University of Melbourne: HIV-1 antibodies PCT/AU2021/050593. The other authors declare no competing interests.

Received: October 21, 2021
Revised: January 27, 2022
Accepted: April 22, 2022
Published: May 17, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PGT121	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP-12343; RRID:AB_2491041
VRC01	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP-12033; RRID:AB_249101
b12	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP-2640
2G12	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP-1476
CHO1-31	Rosenberg et al., 2015	N/A
PGMD1400	Sok et al., 2014	N/A
3BNC117	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP12474, RRID:AB_2491033
HJ16	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP12138, RRID:AB_2491032
10-1074	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#12477 , RRID:AB_2491062
PGT145	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat# ARP12703, RRID:AB_2491054
PGT151	Falkowska ET AL., 2014	N/A
NC-Cow1	Sok et al., 2017	GenBank (MF167446.1 and MF167436.1)
HIVIG	AIDS Reagent Program, Division of AIDS, NIAID, NIH.	Cat#ARP-3957; RRID:AB_2890264
Mouse Anti-Bovine IgG Monoclonal Antibody, Unconjugated, Clone BG-18	Sigma-Aldrich	Cat# B6901, RRID:AB_258594
D7324 Sheep anti-gp120	Aalto Bio Reagents	Cat# D7324
Goat anti-human IgG HRP	KPL	Cat# 474-1002
6X His tag® antibody	Abcam	Ab9108
Sheep anti-bovine IgG-HRP	BioRad	Bio-Rad Cat# AAI23P, RRID:AB_323063
Biological samples		
Bovine PBMC samples	This paper	N/A
Bovine serum	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Monomeric AD8 gp120	This paper	N/A
Uncleaved AD8 gp140	This paper	N/A
AD8 SOSIP gp140. V4.1	This paper	N/A
ConM SOSIP gp140	This paper	N/A
AD8 6R SOSIP.664	This paper	N/A
D7324 tagged- AD8 SOSIP gp140. V4.1	This paper	N/A
Biotinylated AD8 SOSIP gp140. V4.1	This paper	N/A
6 His tagged- AD8 SOSIP gp140. V4.1	This paper	N/A
Biotinylated MONOMERIC AD8 gp120	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
KNH1144 SOSIP.v1	Kang et al., 2009	N/A
BG505 SOSIP.664	Sanders et al., 2013	N/A
BG505 Unc. SEKS	Ring et al., 2013	N/A
Critical commercial assays		
BirA Biotin-Protein Ligase Kit	Avidity	BirA-500
Britelite plus Reporter Gene Assay System	PerkinElmer	Cat# 6066761
RQ1 RNase-Free DNase	Promega	Cat# M6101
LIVE/DEAD™ Fixable Aqua Dead Cell Stain	Thermofisher	Cat# L34965
Streptavidin-APC	Life technologies	Cat# S868
Streptavidin-PE	Life technologies	Cat# S866
RNasin Plus Ribonuclease Inhibitor	Promega	Cat #N2615
SuperScript™ III First-Strand Synthesis System	Thermofisher	Cat# 18080051
MyTaq HS Red Mix	Bioline	Cat# BIO-25048
EcoRI-HF Restriction enzyme	New England Biolabs	Cat# R3101L
NheI-HF Restriction enzyme	New England Biolabs	Cat# R3131LL
AvrII Restriction enzyme	New England Biolabs	Cat# R0174L
ExpiFectamine™ 293 Transfection Kit	Thermofisher	Cat# A14524
Protein G Agarose Fast Flow	Millipore	Cat# A16266
Phusion® High-Fidelity DNA Polymerase	New England Biolabs	Cat# M0530S
EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit	Thermofisher	Cat# 21435
SureBlue™ TMB 1-Component Microwell Peroxidase Substrate	KPL	Cat# 5120-0075
ANA HEp-2 Standard Kit	Aesku Diagnostics	Cat # 51.101.US
AESKULISA ANA-8Pro	Aesku Diagnostics	Cat# 3101-AES
Montanide ISA206	Seppic	N/A
Ficol paque™ plus	GE Healthcare	Cat# Cytiva 17-1440-02
Casein Blocking Buffer 10x	Sigma-Aldrich	Cat# B6429
TMB	Sigma-Aldrich	Cat# T5525
Deposited data		
Heavy variable genes	This paper	GenBank: OM331742-OM331759
Light variable genes	This paper	GenBank: OM331760-OM331774
Experimental models: Cell lines		
HeLA-derived TZM-bl cells	NIH AIDS Reagent Program	Cat#8129; RRID:CVCL_B478
HEK293T	ATCC	Cat#CRL-3216; RRID:CVCL_0063
Expi293F cells	ThermoFisher Scientific Inc	Cat# A14525
Hela	ATCC	Cat#CCL-2
Oligonucleotides		
Primer for mAb generation	This study	Table S1
Primers for site directed mutagenesis	This study	Table S2
Recombinant DNA		
pFUSE2ss-CLIg-hL2	Invivogen	Cat#pfuse2ss-hcll2
pFUSEssCHIg-hG1	Invivogen	Cat# pfusess-hchg1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MN Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP900
6535 Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11017
HXB-2 Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11069
QH0692 Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11018
pREJO4541 Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11035
pRHPA4259 Env expression vector	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11036
AD8 Env expression vector	Purcell lab	N/A
JRCSF Env expression vector	Purcell lab	N/A
YU-2	Purcell lab	N/A
ZM53M.PB12	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP11313
BG505	Purcell lab	N/A
92RW020.2	Montefiori lab	N/A
Q259.d2.17	Montefiori lab	N/A
Q769.d22	Montefiori lab	N/A
Q842.d12	Montefiori lab	N/A
MS208.A1	Montefiori lab	N/A
Du156	Montefiori lab	N/A
ZM135M.PL10a	Montefiori lab	N/A
CAP210.2.00.E8	Montefiori lab	N/A
CAP45.2.00.G3	Montefiori lab	N/A
So431_C1_1	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13367
2969249	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13361
3728.v2.c6	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13362
CE703010010_C4	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13364
CE704810053_2B7	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13365
ZM215F.PB8	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13360
CE2103_E8	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13363
ZM233M.PB6	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13359
2759058_F10_B6	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13366
Ko243_H6.3	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13370
CAP382.2.00.D7.19	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13368
B005582-7_G7.8	AIDS Reagent Program, Division of AIDS, NIAID, NIH	Cat# ARP-13369

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
AE01	Montefiori lab	N/A
AE03	Montefiori lab	N/A
CNE5	Montefiori lab	N/A
X2278	deCamp et al., 2014	N/A
TRO11	deCamp et al., 2014	N/A
25710	deCamp et al., 2014	N/A
398F1	deCamp et al., 2014	N/A
BJOX2000	deCamp et al., 2014	N/A
CE1176	deCamp et al., 2014	N/A
CE0217	deCamp et al., 2014	N/A
CH119	deCamp et al., 2014	N/A
CNE8	deCamp et al., 2014	N/A
CNE55	deCamp et al., 2014	N/A
246F3	deCamp et al., 2014	N/A
X1632	deCamp et al., 2014	N/A
Mulv	Purcell lab	N/A
Software and algorithms		
IMGT	International ImMunoGeneTics Information System	http://www.imgt.org
GraphPad Prism Software	GraphPad Prism Software, Inc.	SCR_002798
FlowJo software v.10	BD biosciences	SCR_008520
PyMOL	Schrödinger, LLC	RRID: SCR_000305
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Damian Purcell (dfjp@unimelb.edu.au).

Materials availability

Material transfer agreements with standard academic terms will be established to document reagent sharing by the lead contact's institution.

Data and code availability

- All materials described in this manuscript are available via a material transfer agreement with the University of Melbourne. All data required to state the conclusions in the paper are present in the paper and/or the Supplementary data. Sequence data are provided in this paper or already published.[15,25–31,56] The GenBank accession codes for the heavy variable genes: OM331742-OM331759; light variable genes: OM331760-OM331774 are annotated at the bottom right of each box in Table S3.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and primary cells

The common laboratory cell lines HEK293T (ATCC CRL-11268), and Hela (ATCC CCL2) were originally purchased from ATCC. The source of other cell lines were: Expi293F cells (Thermo Fisher Scientific); and TZM-bl cells (AIDS Research and Reference Reagents Program, Division of AIDS ARP-8129). Master seed stocks of all cell lines were confirmed for identity through Short Tandem Repeat profiling and were negative for mycoplasma and were routinely tested bi-monthly. All cell lines were cultivated in complete Dulbecco's modified Eagle's medium (DMEM) media and 10% fetal bovine serum (FBS), except for Expi293F cells that were cultured in Expi293 Expression Medium (Gibco).

Primary bovine B-lymphocytes were prepared from bovine PBMCs obtained by centrifugation of cow blood on a Ficoll-Plaque PLUS step gradient (GE Healthcare).

Experimental animals

The Holstein Friesian cattle *(Bos Taurus)* used for the study were all females in the prime years for conception, 4–7 years of age. Cows were kept on a certified experimental open-gazing farm holding a Scientific Premises License from the Department of Economic Development, Jobs, Transport and Resources (DEDJTR) of the Victorian State Government that blocked any animal products from entering the food chain. Animal welfare was continuously evaluated and supervised by the Ellinbank Agricultural Research Farm Animal Husbandry Committee.

Ethics

This work was conducted under animal ethics approval 2015-17 from the Victorian State Government DEDJTR Research and Extension Animal Ethics Committee.

METHOD DETAILS

HIV-1 Env production, expression and purification

Vaccine proteins including HIV-1 NL AD8 (AD8) Unc gp140 Env (clade B) were expressed using stably-transfected Hela cell line and AD8 6R SOSIP.664 was produced by transient transfection of Expi293 cells. KNH1144 SOSIP.v1 (clade A),[57] and BG505 SOSIP.664 (clade A) were produced as previously described.[18]

AD8 SOSIP.v4.1 was produced using an Env-expression plasmid encoding the Env from AD8[58] modified according to the "v4.1" mutations described previously[59] (Figure S1A) with a C-terminal D7324 epitope-tag, 6His-tag or Avi-tag. Proteins were expressed in Expi293 cells by co-transfecting the Env-expression plasmid with a human furin protease expression plasmid. AD8 SOSIP Envs were purified from culture supernatant using a 2G12-sepharose affinity resin and eluted using 3M $MgCl_2$ (Figure S1B). The Env was immediately buffer exchanged into PBS and trimeric Env was further purified by size exclusion chromatography using a HiLoad 16/600 Superdex prep grade column (GE Healthcare Life Sciences). Avi-Tagged trimers were biotinylated using BirA enzyme according to manufacturer's instructions (Avidity, LLC). Monomeric AD8 gp120 was also produced as described previously.[60–62] BG505 Unc. SEKS (uncleaved gp140) was produced as described above for AD8 SOSIP gp140 protein, except that the Env expression plasmid[63] was not co-transfected with a furin expression plasmid.

Cow immunization

Three female Holstein cattle (*Bos taurus*) were vaccinated subcutaneously into the flank, using Seppic Montanide (ISA206) adjuvant. Cows were immunized prior to and during pregnancy and revaccinated after calving (Figure 1A). Cow #1 received 100μg KNH1144 SOSIP and BG505 SOSIP Env trimer in phase 1 and was revaccinated with 50μg BG505 SOSIP in phase 2. Cow #2 and #3 received 500μg AD8 Unc gp140 trimer during pregnancy and were revaccinated after calving with 50μg BG505 Unc gp140 and 100μg AD8 SOSIP. R6. 664, respectively. Sera samples were collected after calving at week 54 (phase 1) and week 59 (phase 2). Peripheral blood mononuclear cells (PBMC) from each cow were also isolated from bloods collected after phase 1 and phase 2 as described previously.[12]

Serum ELISA and neutralization of sera samples

IgG titres in sera against autologous Env vaccine antigens were measured by direct ELISA, with incubations performed at room temperature (RT) except when stated. Casein buffer 1x (Sigma) was used as sample diluent for each step. Briefly, 96-well plates were coated with 1 μg/mL recombinant Env gp140 proteins (BG505 SOSIP, AD8 Unc gp140) in coating buffer (200 mM Tris-HCl, 100 mM NaCl, pH 8.8) overnight at 4°C. The plate was washed four times with PBS+ 0.1% Tween and four times with PBS then blocked with casein buffer 1x (Sigma). Sera samples were added in half-log_{10} dilutions and incubated for 3 h at RT. Afterward, 1/1000 dilution of HRP-conjugated sheep-anti-bovine IgG (BioRad, #AAI23P) was loaded and incubated for 1 h at RT. Finally, color development was performed using TMB (Sigma, cat no: T5525) according to manufacturer instructions and the reaction was stopped using 1M H_2SO_4. Absorbance was measured at 450 nm against a reference of 690 nm.

For neutralization assay of sera samples, HIV-1 Env-pseudotyped viruses were produced as described previously.[9,64] Pseudoviruses were produced in HEK 293T cells by co-transfecting a backbone plasmid with one of Env expressing plasmids. TZM-bl neutralization assay was performed for sera samples collected pre-immunisation, at phase 1 (week 54) and phase 2 (week 59). The assay was performed as described previously.[64] Briefly, 50 μL of pseudovirus in complete growth medium (DMEM +10% FBS) was mixed with serial dilutions of serum samples in a final volume of 150 μL in 96-well plates (Corning, flat bottom, non-pyrogenic), and incubated for 1 h at 37 °C. Thereafter, 10^4 TZM-bl cells (containing DEAE-Dextran at pre-determined optimal concentration (Sigma)) was added to each well and plates were incubated for 48 h at 37 °C. Inhibition of infection was calculated by measuring relative luminescence units (RLUs) using Britelite plus (PerkinElmer) in a VICTOR Multilabel Plate Reader (PerkinElmer). ID50 neutralizing antibody titers are expressed as the reciprocal of the sample dilution required to reduce RLU by 50%.

Single particle negative stain electron microscopy

Purified AD8 SOSIP (100 ng/μL) was placed on glow-discharged carbon coated copper mesh grids and stained with 1% uranyl formate. Grids were screened for appropriate stain thickness and particle distribution and images were collected using an FEI Talos L120C electron microscope. Images were collected at 730,00X magnification with a −1.8 μm defocus for a final magnified pixel size of 1.9 Å/pix. Negatively stained AD8 particles were then automatically picked based on an empirical evaluation of maximum particle radius of 110 Å , characteristic particle radius of 80 Å, and with threshold peak high of 5 standard deviation above the noise using cisTEM software version 1.0.0-beta.[65] Further, a 2D classification was performed on 38,000 particles in cisTEM that resulted in 50 classes. The initial model was generated *ab initio* using the dataset and processed under the filter for particles from 20 Å to 8 Å in the first step of classification within cisTEM. Best 18 classes representing different orientations of AD8 were selected for further iterative 3D classification under C3 symmetry. This was followed by a local refinement and a final 3D refinement in cisTEM. UCSF Chimera was used to generate figures.[66]

Single cell sorting by fluorescence-activated cell sorting (FACS)

Sorting of bovine PBMCs was performed as described previously[12,64] with minor modifications (Figure 2B). In brief, 2.5 million cryopreserved PBMCs were thawed and resuspended in 10 mL pre-warmed 37°C RPMI 1640 medium (Life technologies) (containing 10% FBS, 20 μg/mL or 10 U/mL DNaseI) for 5 min at RT followed by centrifugation at 500 x g for 10 min at 4°C. The cells were resuspended in chilled PBS and LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific) was added and incubated for 10 min on ice. PBMCs were then stained with Alexa-fluor 488 conjugated anti-bovine IgG (Sigma, B6901) and 50 nM biotinylated AD8 SOSIP-avi gp140 coupled to streptavidin-APC and PE (Life Technologies) in equimolar ratios. The cells were incubated for 1 h at 4°C in PBS containing 1 mM EDTA and 1% horse serum (Sigma). Then, live IgG + AD8 SOSIP PE+/AD8 SOSIP APC + cells were single-sorted into 96-well plates containing lysis buffer (3.7μL/well PBS, 10 mM DTT and 8 U RNasin Ribonuclease Inhibitor (Promega)) on an ARIA III sorter using BD FACSDiva Software and were immediately frozen at −80°C until further use. Data were analyzed using FlowJo software v.10.

Single cell cDNA synthesis, RT-PCR, and cloning

cDNA was synthesized from mRNA of single cells as follows: Sorted cells were incubated with 200ng Random Hexamer Primer, 4 μL 5x RT Buffer (Thermo Fisher Scientific), 1 μL dNTPs mix (10 mM, Promega), 1 μL DTT (Thermo Fisher Scientific), 8U RNasin Ribonuclease Inhibitor (Promega), 0.25 μL Superscript III (200 U/mL, Thermo Fisher Scientific), and 6.5 μL of RNase-free H2O at 42°C for 10 min, 25°C for 10 min, 50°C for 60 min, and 94°C for 5 min[67] and antibody variable genes were amplified as described previously[12] with minor modifications. Briefly, antibody heavy gamma (γ) and light lambda (λ) variable genes were amplified independently in nested PCR using MyTaq HS Red Mix (Bioline) according to the manufacturer's instruction. The PCR reaction primers and conditions are listed in Table S1. PCR1 reactions were set up in 25 μL with 2.5μL cDNA, while PCR2 reaction volumes were 50μL using 5 μL PCR1 product. The PCR reaction cycle was performed as 94 °C 5 min, 50 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 45 s and final extension at 72 °C for 10 min. The annealing temperature of PCR1 for lambda gene was 58°C. The amplifid bovine VH/VL genes were cloned into the human constant heavy (CH) and constant light (CL) region expression vectors in pFUSEss-sCHIg-hG1 and pFUSE2ss-CLIg-hL2 (Invivogen), using *EcoRI*/*NheI* and *EcoRI*/*AvrII* restriction enzymes, respectively.

Antibody production and purification

Antibody plasmids containing heavy chain and light chain genes were co-transfected (2:3 ratio) into Expi293F cells using Expifectamine (Thermo Fisher Scientific) according to manufacturer instructions. Supernatants containing antibodies were harvested 4 days after transfection and filtered using 0.22 μm filters. NC-Cow1 antibody was produced as an anti-HIV-1 bovine antibody control by codon optimisation of genes available from the GenBank (MF167446.1 and MF167436.1). Antibody supernatants were purified using Protein G Agarose Fast Flow (Merck Millipore). Antibodies were eluted from chromatography columns using 50 mM glycine (pH 2.7) and immediately neutralised by addition of 1/10 volume of 1 M Tris (pH 8.0) before being buffer exchanged into PBS, concentration using Amicon 50 kDa spin membranes (Millipore) and sterilization using 0.22 μm filters.

Generation of HIV-1 AD8 pseudovirus mutant

Specific amino acid changes to HIV-1 AD8 gp160 Env were introduced using the following PCR reaction set up: 100ng Full-length AD8 gp160 Env plasmid, 5% Dimethyl sulfoxide (DMSO), 10 μL 5x Phusion Reaction buffer (New England BioLabs), 1 μL dNTP mix(10 mM, Promega), 3U Phusion HF DNA Polymerase (New England BioLabs, (MEL-2000 units/mL)), 0.5 μL from each forward and Reverse primer (20 μM) (Table S2) and nuclease-free H20 up to the total volume of 50 μL. The PCR reaction was performed as following: 95°C for 5 min, 30 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 8 min and final extension of 72°C 15 min. Mutations were confirmed by sequence analysis.

Neutralization assays of anti-HIV-1 monoclonal antibodies

The neutralization assay of TZM-bl was performed as described previously.[12] In brief, pseudoviruses and serial dilutions of mAbs were mixed and incubated for 1 h at 37°C. Then, 10^4 TZM-bl cells supplemented with DEAE-dextran at a final concentration of 10 μg/mL were added and the plate was incubated for 72 h at 37°C. The infectivity of pseudoviruses was calculated according to

luciferase relative light unit readout using either a Fluostar (BMG Labtech) or Victor XLight (Perkin-Elmer) luminometer. Human bNAbs were used as the controls in the assay. CH01-31 antibody was an equal concentration mixture of two bNAbs (CH01 and VRC-CH31).

ELISA assays of anti-HIV-1 monoclonal antibodies

To screen HIV-1 Env binding mAbs, ELISA plates were coated with 2 μg/mL D7324 Sheep anti-gp120 (Aalto Bio Reagents) at 4°C in 1X PBS overnight. Plates were washed four times with PBS +0.1% Tween and two times with PBS, then blocked with 5% Skim milk in 1× PBS at RT for 1 h. The plates were washed and 600 ng/mL D7324 tagged AD8 SOSIP trimer was added and incubated for 1 h at RT. Plates were washed and a 1/1000 dilution of goat anti-human IgG HRP (KPL Cat No. 474-1002) (pre-incubated with 2% normal sheep serum) was added to the wells. The plate was incubated at RT for 1 h, washed then developed by adding SureBlue TMB (KPL) according to the manufacturer's instructions. The absorbance was measured at 450 nm against a reference of 690 nm.

To assess the binding of mAbs to mutated HIV-1 Env gp160, harvested pseudoviruses were lysed with 1% Triton X-100 detergent (Astral Scientific). ELISA plates were coated with D7324 Sheep anti-gp120, blocked with skim milk and the lysed pseudoviruses were then captured on ELISA for 2 h at 37°C. Next, serial dilutions of mAbs were added before the addition of goat anti-human IgG HRP.

For experiments involving antibody binding to untagged HIV-1 Env (monomeric AD8 gp120, AD8 Unc gp140, AD8 SOSIP and ConM SOSIP), plates were coated directly with 1 μg/mL Env proteins at 4°C in PBS overnight then washed and blocked before addition of mAbs and goat anti-human gamma HRP.

Competition ELISA

To investigate the epitopes of AD8 Env-binding mAbs, a competition ELISA was performed using competing antibodies that were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific). The plates were coated with 1/1000 dilution of anti-6X His antibody (Abcam, #9108) and incubated overnight at 4°C. Then, the plates were washed and blocked with 5% skim milk in PBS +0.1% Tween (0.1%) for 1 h at RT. Following washing, 500 ng/mL His-tagged AD8 SOSIP trimer was added, and the plates were incubated for 2 h at RT. Then, bovine mAbs in the following amounts were added: 1 μg/mL (MEL-1842, MEL-1872, MEL-2129, MEL-2000, MEL-2028 and MEL-782), 2 μg/mL (MEL-2010, MEL-33, MEL-130, MEL-563 and MEL-663) and 5 μg/mL (MEL-1905, MEL-1967, MEL-2114, MEL-F2, MEL-198 and MEL-D1). After washing, the biotinylated human mAbs were added in the following amounts to give sufficient signal: 1 μg/mL (PGT121, PGT145, 10-1074, PGT151), 2 μg/mL (VRC01 and 3BNC117) and 5 μg/mL (b12, HJ16). Then, a 1/1000 dilution of Streptavidin HRP was added and incubated for 1 h at RT followed by addition of SureBlue according to the manufacturer's instructions.

For self-competition of bovine bNAbs, the assay was performed as above except using biotinylated bovine mAbs as the following amounts: 1 μg/mL for MEL-2028, MEL-2000, MEL-782, MEL-2129, MEL-1842, MEL-1872, 2 μg/mL for MEL-2010, MEL-33, MEL-130, MEL-563, 5 μg/mL for MEL-1905, MEL-1967, MEL-2114, MEL-F2, MEL-198 and MEL-D1.

Polyreactivity assay: HEp-2 cell staining assay

The HEp-2 cell-staining kit (Aesku Diagnostics) was used according to manufacturer's instructions. In brief, 2.5 μg of mAbs and controls were added to HEp-2 cell containing wells and incubated in a moist chamber at RT for 30 min. Slides were washed with PBS then 25 μL FITC-conjugated goat anti-human IgG was applied with incubation of 30 min at RT. The slide was washed and mounted on coverslips using the provided mounting medium. Slides were viewed at 20x magnification and imaged on the Zeiss LSM780 confocal microscope. All images were captured with the following conditions: digital gain 800, laser power 2.0%. Samples showing fluorescence greater than the negative control (provided by the vendor) were considered positive for HEp-2 staining.

Polyreactivity assay: single autoantigen reactivity

Single antigen ELISA assays were performed using AESKULISA ANA-8Pro (Aesku) for U1- ribonucleoprotein (RNP), SnRNP/Sm, Sm, SS-A, SS-B, Jo-1, Scl-70, and CenpB. The 96 wells were coated with these cellular and nuclear antigens for the qualitative detection of mAbs reactivity. The cut-off calibrator, negative control and positive control were provided by the manufacturer.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

Statistical analyses were performed with Graphpad Prism 9. Correlations between the neutralizing breadth and neutralization activity (IC_{50}) of AD8 SOSIP binding antibodies were assessed using the non-parametric Spearman test.

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Supplemental information

Broad and ultra-potent cross-clade neutralization of HIV-1 by a vaccine-induced CD4 binding site bovine antibody

Behnaz Heydarchi, Danielle S. Fong, Hongmei Gao, Natalia A. Salazar-Quiroz, Jack M. Edwards, Christopher A. Gonelli, Samantha Grimley, Turgut E. Aktepe, Charlene Mackenzie, William J. Wales, Marit J. van Gils, Albert Cupo, Isabelle Rouiller, Paul R. Gooley, John P. Moore, Rogier W. Sanders, David Montefiori, Ashish Sethi, and Damian F.J. Purcell

Table S1: Primers used for producing chimeric bovine- human full antibodies. Related to STAR Methods (Single cell cDNA synthesis, RT-PCR and cloning)

Primer code	Sequence 5'-3'		PCR Reaction
odp 2569	ATGAACCCACTGTGGACCCTC	Forward	H1
odp 2570	AGAACTCAGAGGGTAGACTTTCGG	Reverse	H1
odp 3667	CTTTCGGGGCTGTGGTGGAGGC	Reverse	H1
odp 3668	**GAATTC**GMAGGTGCAGCTGCRGGAGTC	Forward (EcoRI)	H2
odp 2568	**GCTAGC**TGAGGAGACGGTGACCAGGAG	Reverse (NheI)	H2
odp 3670	CACCATGGCCTGGTCCCCTCTG	Forward	L1
odp 3671	GACCCCAGACTCACCATCTC	Forward	L1
odp 3672	AGGGCTGCGGGCTCAGAAGGCAGC	Forward	L1
odp 3673	CTGCCCCTCCTCACTCTCTGC	Forward	L1
odp 3674	GGAACCTTTCCTGCAGCTC	Forward	L1
odp 3675	GCTTGCTTATGGCTCAGGTC	Forward	L1
odp 2573	ATGTCCACCATGGCCTGGTCC	Forward	L1
odp 2574	CTTGTTGCCGTTGAGCTCCTC	Reverse	L1
odp 2571	**GAATTC**GCAGGCTGTGCTGACTCAG	Forward (EcoRI)	L2
odp 3677	**CCTAGG**ACGACKGTCAGTGTGGTSCC	Reverse (AvrII)	L2
odp 2781	CTCAACTCTACGTCTTTGTTTC	Forward	Sequencing

Restriction enzyme sites are in bold and the nucleotides inserted to keep the frame reading in the expression vector are in Italic and underlined. H: heavy chain, L: light chain. Reaction number shows whether the primer was used in nester RT-PCR reaction 1 or reaction 2.

Table S2: Primers used for site directed mutagenesis of full length AD8 gp160 Env. Related to STAR Methods (Generation of HIV-1 AD8 pseudovirus mutant)

Primer code	Sequence 5'-3'		Mutation
odp 3770	cacaagaagtagtattgg**C**aaatgtgacaga	Forward	E87A
odp 3771	attttctgtcacattt**G**ccaatactacttc	Reverse	E87A
odp 3772	taaagccatgtgta**GC**attaaccccactctgtg	Forward	K121A
odp 3773	acacagagtggggttaa**TGC**tacacatggc	Reverse	K121A
odp 3774	aagactatgcacttttttataga**GC**tgatgtagtaccaatag	Forward	L179A
odp 3775	tcattatctattggtactacatct**GC**tctataaaaaagtgcatag	Reverse	L179A
odp 3776	ttgatgtagtaccaatag**C**taatgataatactagctatagg	Forward	D185A
odp 3777	acctatagctagtattatcatta**G**ctattggtactaca	Reverse	D185A
odp 3778	tataggttgataaattgt**GC**tacctcaaccattacacagg	Forward	N197A
odp 3779	tgtgtaatggttgaggta**GC**acaatttatcaacctatagc	Reverse	N197A
odp 3782	tcaactcaactgctgtta**GC**tggcagtctagc	Forward	N262A
odp 3783	ttcttctgctagactgcca**GC**taacagcagttgag	Reverse	N262A
odp 3784	agaggtagtaattagatctagt**GC**tttcacagacaatgc	Forward	N276A
odp 3785	ttgcattgtctgtgaaa**GC**actagatctaattactacctc	Reverse	N276A
odp 3786	tctagtaatttcacag**C**caatgcaaaaaacataatagtac	Forward	D279A
odp 3787	atgtttttgcattg**G**ctgtgaaattactagatctaattactac	Reverse	D279A
odp 3788	atttcacagacaatgca**GC**aaacataatagtacagttg	Forward	K282A
odp 3789	aactgtactattatgtt**TGC**tgcattgtctgtgaaattac	Reverse	K282A
odp 3790	ttcacagacaatgcaaaa**GC**cataatagtacagttg	Forward	N283A
odp 3791	ttcaactgtactattat**GGC**ttttgcattgtctgtg	Reverse	N283A
odp 3792	agtatacatatagga**G**caggaagagcattttatac	Forward	P313A
odp 3793	tgttgtataaaatgctcttcctg**C**tcctatatgtatac	Reverse	P313A
odp 3794	aggagatataagacaa**AA**acattgcaacattagtagaac	Forward	A329K
odp 3795	ttgttctactaatgtt**TT**aatgtgcttgtcttatatctcc	Reverse	A329K
odp 3796	aagacaagcacattgca**C**cattagtagaacaaaatgg	Forward	N332T
odp 3797	tgttattccattttgttctactaatg**G**tgcaatgtgcttgtcttatatc	Reverse	N332T
odp 3800	aataaaacaatagtctttaat**GC**atcctcaggaggggaccc	Forward	Q363A
odp 3801	acaatttctgggtcccctcctgaggat**GC**attaaagactattgttttattatt ccc	Reverse	Q363A
odp 3802	aacaatagtctttaatcaa**G**cctcaggaggggacccagaaattg	Forward	S364A
odp 3803	ttctgggtcccctcctgagg**C**ttgattaaagactattgttttattattcc	Reverse	S364A
odp 3804	aatagtctttaatcaatcc**G**caggaggggacccagaaattgtaatgc	Forward	S365A
odp 3805	aatttctgggtcccctcctg**C**ggattgattaaagactattgttttattattcc	Reverse	S365A
odp 3806	tagtctttaatcaatcctcag**C**aggggacccagaaattgtaatgc	Forward	G366A
odp 3807	attacaatttctgggtcccct**G**ctgaggattgattaaagac	Reverse	G366A
odp 3808	tagtctttaatcaatcctcaggag**C**ggacccagaaattgtaatgc	Forward	G367A
odp 3809	tgcattacaatttctgggtcc**G**ctcctgaggattgattaaagac	Reverse	G367A
odp 3810	ttaatcaatcctcaggaggggg**C**cccagaaattgtaatgcac	Forward	D368A
odp 3811	aactgtgcattacaatttctggg**G**cccctcctgaggattg	Reverse	D368A

Table S2: Continued.

Primer code	Sequence 5'-3'		Mutation
odp 3812	aatcaatcctcaggaggggac**G**cagaaattgtaatgcacag	Forward	P369A
odp 3813	aactgtgcattacaatttctg**C**gtcccctcctgagg	Reverse	P369A
odp 3814	aatcaatcctcaggaggggacccag**C**aattgtaatgcacag	Forward	E370A
odp 3815	taaaactgtgcattacaatt**G**ctgggtcccctcctgagg	Reverse	E370A
odp 3816	aatcctcaggaggggacccagaa**GC**tgtaatgcacagttttaattgtgg	Forward	I371A
odp 3817	aattaaaactgtgcattaca**GC**ttctgggtcccctcc	Reverse	I371A
odp 3818	atcaatcctcaggaggggacccagaaattg**C**aatgcacagttttaattgtgg	Forward	V372A
odp 3819	ttcccctccacaattaaaactgtgcatt**G**caatttctgggtcccctcc	Reverse	V372A
odp 3820	acccagaaattgta**GC**gcacagttttaattgtggaggg	Forward	M373A
odp 3821	ttcccctccacaattaaaactgtgc**GC**tacaatttctggg	Reverse	M373A
odp 3822	atgacactatcacactcccatgt**GC**aataaaacaaattataaacatgtg	Forward	R419A
odp 3823	atgtttataatttgttttatt**GC**acatgggagtgtgatagtgtc	Reverse	R419A
odp 3824	atattacagggctgatatta**G**caagagatggtggaaataacc	Forward	T455A
odp 3825	ttgtggttatttccaccatctcttg**C**taatatcagccctg	Reverse	T455A
odp 3826	tacagggctgatattaacaagag**C**tggtggaaataaccacaataatgatac	Forward	D457A
odp 3827	ttgtggttatttccacca**G**ctcttgttaatatcagccctg	Reverse	D457A
odp 3828	taccgagacctttagacctg**C**aggaggagatatgagggac	Forward	G471A
odp 3829	ttgtccctcatatctcctcct**G**caggtctaaaggtctcgg	Reverse	G471A
odp 3830	agacctttagacctggag**C**aggagatatgagggacaattgg	Forward	G472A
odp 3831	attgtccctcatatctcct**G**ctccaggtctaaaggtctcgg	Reverse	G472A
odp 3832	acctttagacctggaggag**C**agatatgagggacaattggag	Forward	G473A
odp 3833	ttctccaattgtccctcatatct**G**ctcctccaggtctaaaggtctcgg	Reverse	G473A
odp 3834	tagacctggaggaggag**C**tatgagggacaattggagaagtg	Forward	D474A
odp 3835	ttctccaattgtccctcata**G**ctcctcctccaggtctaaagg	Reverse	D474A
odp 3836	ttagacctggaggaggagat**GC**gagggacaattggagaagtg	Forward	M475A
odp 3837	ttctccaattgtccctc**GC**atctcctcctccaggtctaaagg	Reverse	M475A
odp 3838	tggaggaggagatatg**GC**ggacaattggagaagtg	Forward	R476A
odp 3839	acttctccaattgtcc**GC**catatctcctcctccaggtctaaagg	Reverse	R476A
odp 1377	ggtacataatgtttgggccac	Forward	Sequencing
odp 1379	gctgttaaatggcagtctagc	Forward	Sequencing
odp 1441	ctactgtaattcaacacaactg	Forward	Sequencing

Nucleotides to be mutated are shown in capital letters and bold font.

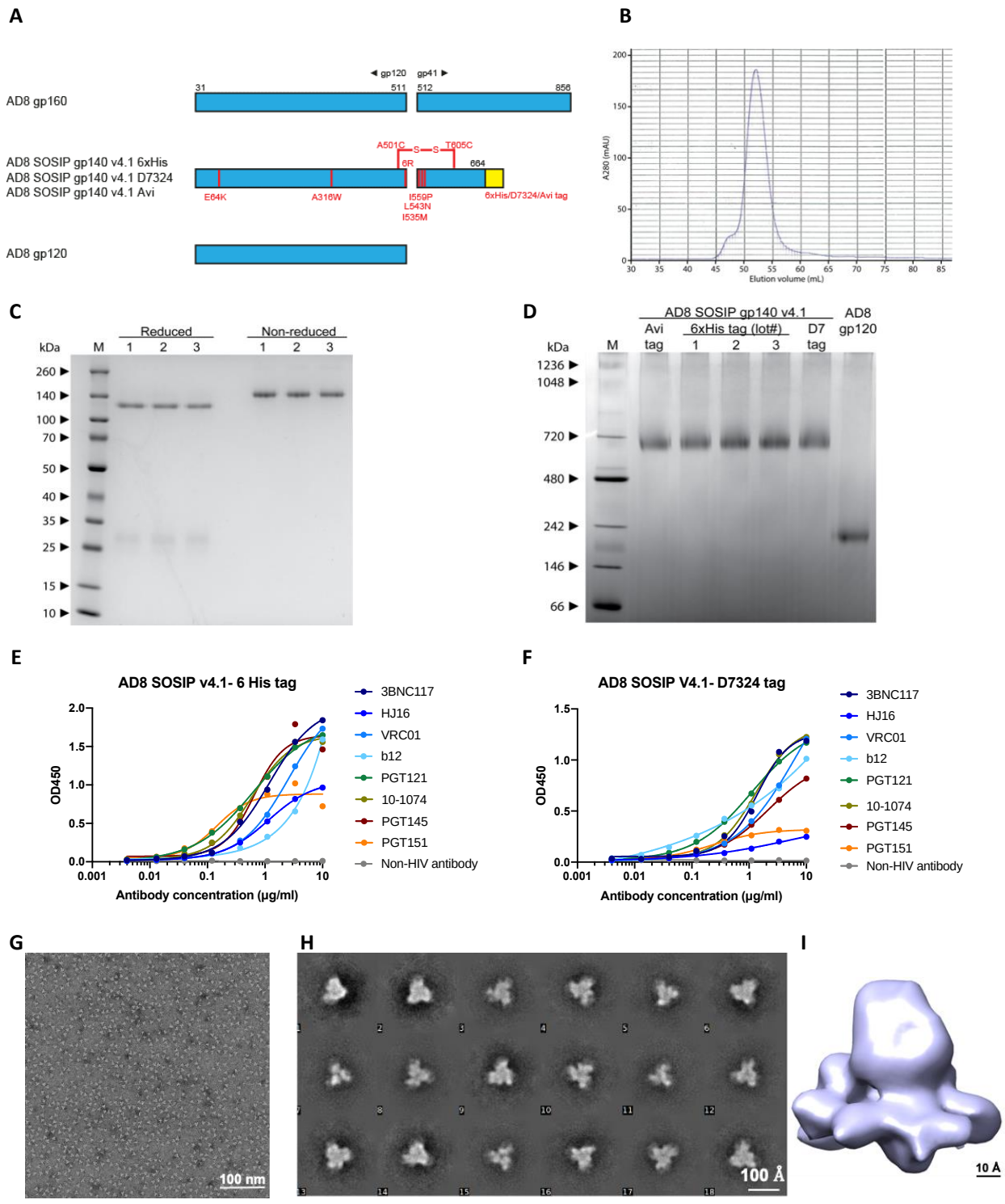

Figure S1: Design and characterization of AD8 SOSIP trimers. (A) Linear representation of mature AD8 gp160, SOSIP gp140 v4.1 (with either a 6xHis, D7324, or Avi tag at the C-terminus at position 664), and gp120. All Envs were expressed with their wild-type signal peptide. SOSIP v4.1 mutations were introduced as previously described [ref v4.1 paper SW de Taeye 2015 Cell]. (B) SEC profile of 2G12-purified AD8 SOSIP gp140 v4.1 6xHis run on a Superdex S200 16/600 column.

(C) SDS-PAGE analysis using an 8-16% Tris-glycine gel of 3 separate lots (numbered 1-3) of 2G12/SEC purified 6xHis-tagged AD8 SOSIP gp140 v4.1. Proteins were running with or without reducing agent. Lane M was loaded with Spectra Multicolor Broad Range Protein Ladder. (D) BN-PAGE analysis using a 4-16% Bis-Tris NativePAGE gel. Trimeric AD8 SOSIP gp140 v4.1 with Avi, 6xHis (3 separate lots), or a D7324 tag were analysed as well as monomeric AD8 gp120. Lane M was loaded with NativeMark Unstained Protein Standard. For both (C) and (D), gels were stained with Coomassie Blue. Capture ELISA on AD8 SOSIP v4.1 His tag (E) and D7324 tag (F) using human bNAbs. G) Negative staining using 1% Uranyl Acetate on freshly glow discharged carbon coated copper grids. Images were taken on FEI Talos L120C microscope. Pixel size: 1.9Å 73000X magnification. (H) 2D classes showing different views and (I) 3D volume map for AD8-SOSIP. Related to Figure 2.

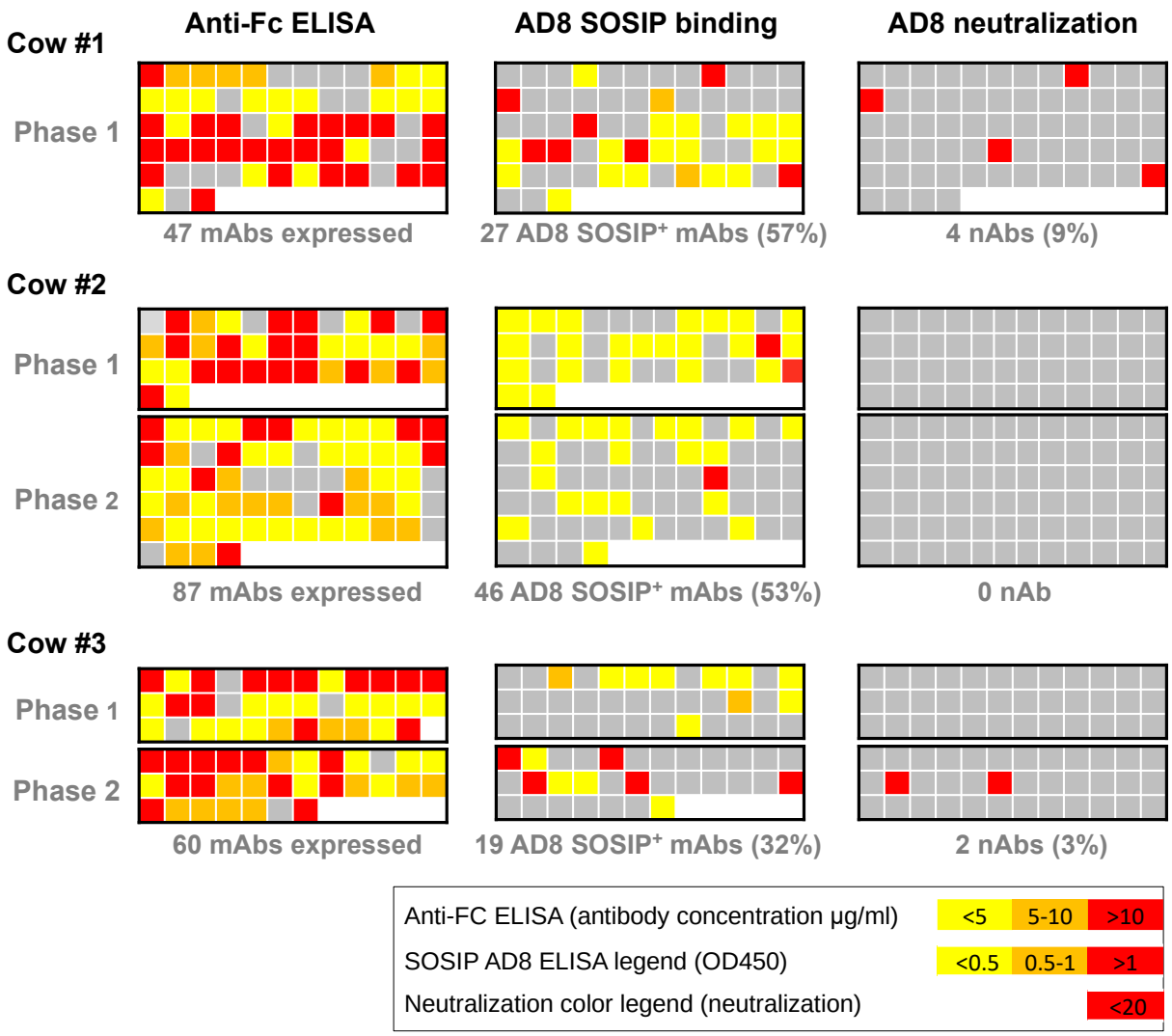

Figure S2. Workflow of isolating anti-HIV bovine antibodies. Amplified heavy chains were their light chain or with MEL-2129 antibody light chain and tested for expression (anti-Fc ELISA), antigen binding was assessed in capture ELISA using AD8 SOSIP gp140 v4.1 and autologous neutralization (AD8 pseudovirus) was investigated using TZM-bl cells. Related to Figure 2.

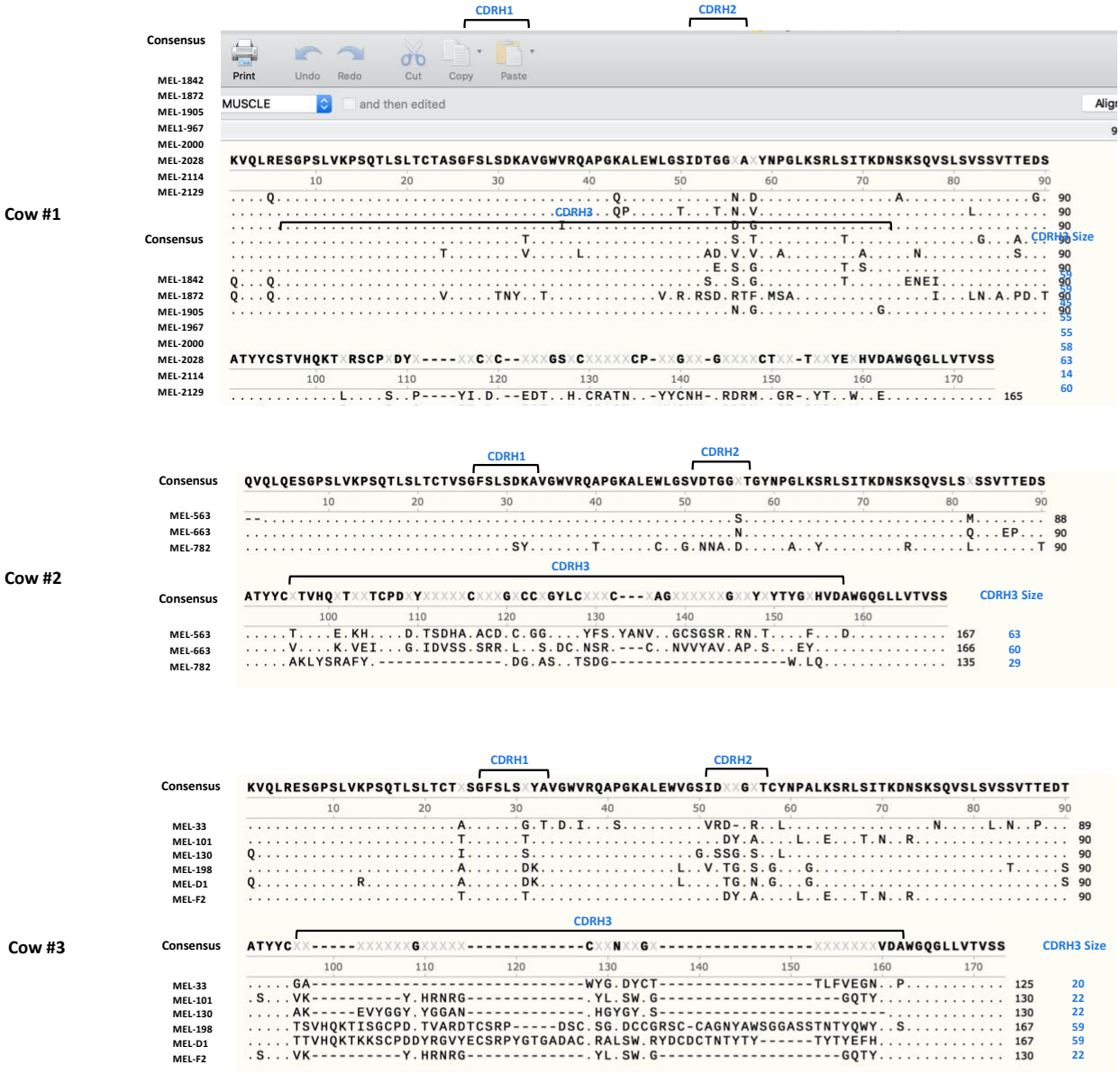

Figure S3. Amino acid alignment of heavy chain sequences of isolated monoclonal antibodies. Related to Figure 2.

Geometric mean IC50

Clade	n	VRC01		NC-Cow1		MEL-1842		MEL-1872		MEL-2129	
		Breadth	IC50	Breadth	IC50	Breadth	IC50	Breadth	IC50	Breadth	IC50
A	7	NA	NA	100%	0.040	100%	0.011	100%	0.005	86%	0.215
B	12	100%	0.0117	50%	0.094	100%	0.004	100%	0.004	100% *	0.072
C	19	NA	NA	42%	0.081	32%	0.018	37%	0.021	21%	0.087
AE	5	NA	NA	100%	0.216	80%	0.154	60%	0.008	20%	0.001
BC	2	NA	NA	50%	0.219	0%	-	0%	-	0%	-
AC	1	NA	NA	0%	-	0%	-	100%	2.830	0%	-
G	1	100%	0.073	100%	0.118	100%	0.069	100%	0.007	100%	0.257
	47										

IC50 (µg/ml) <0.01 0.01-1 1-20

* 11 clade B tested.

Geometric mean IC80

Clade	n	VRC01		NC-Cow1		MEL-1842		MEL-1872		MEL-2129	
		Breadth	IC80	Breadth	IC80	Breadth	IC80	Breadth	IC80	Breadth	IC80
A	7	NA	NA	100%	0.185	100%	0.070	100%	0.028	71%	1.030
B	12	92%	0.346	42%	1.029	100%	0.030	100%	0.031	64% *	0.047
C	19	NA	NA	37%	0.181	26%	0.035	32%	0.053	21%	0.710
AE	5	NA	NA	60%	0.164	40%	0.022	60%	0.032	20%	0.006
BC	2	NA	NA	50%	0.934	0%	-	0%	-	0%	-
AC	1	NA	NA	0%	-	0%	-	0%	-	0%	-
G	1	100%	1.146	100%	0.997	100%	2.005	100%	0.122	100%	10.015
	47										

IC80 (µg/ml) <0.01 0.01-1 1-20

* 11 clade B tested.

Figure S4. Categorization of neutralization activity of bovine bNAbs against clade A, B, C, AE, BC, AC and G HIV viruses. Values in red color show low IC_{50} and IC_{80} and better neutralization while, those in yellow color show high IC_{50} and IC_{80} values and less neutralization activity. Neutralizatoin assays were performed in duplicates with two independent biological replicates. Related to Figure 3.

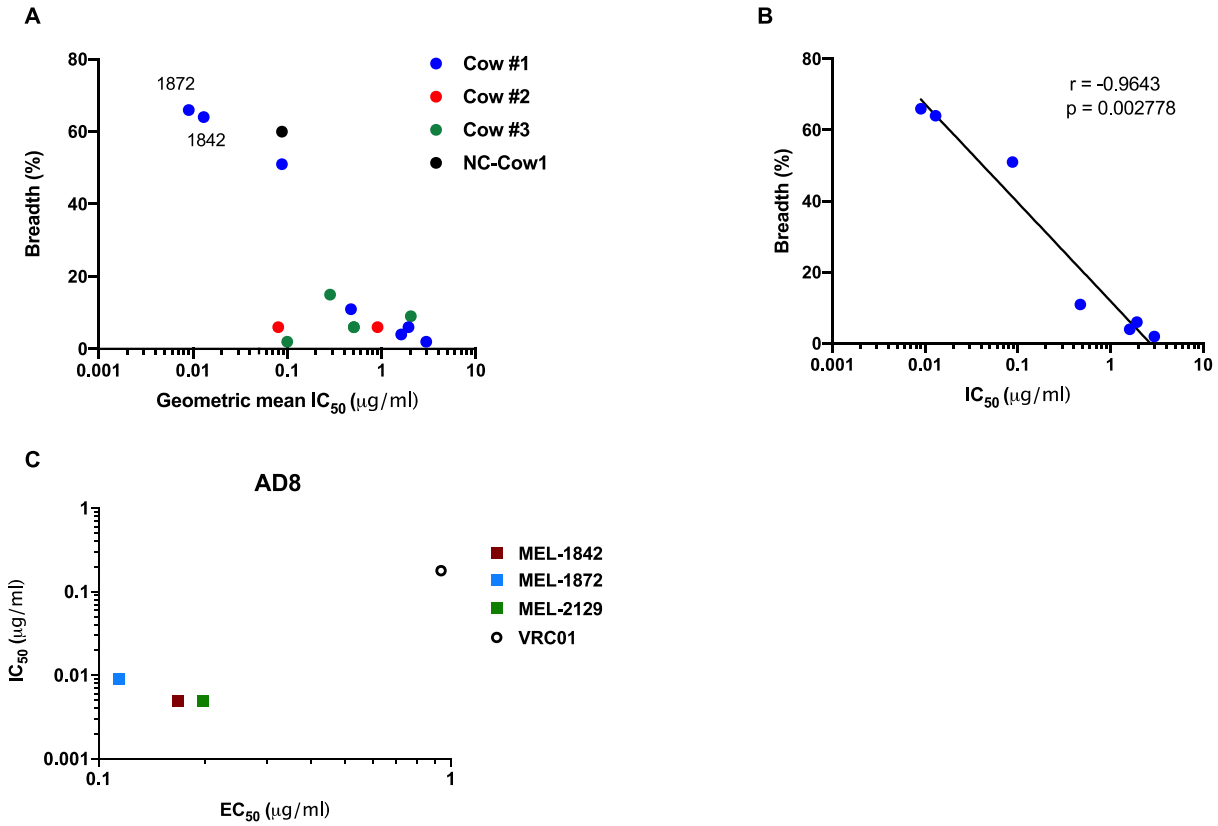

Figure S*5*. Correlation of neutralization and Env binding in isolated monoclonal antibodies. A) Correlations between the neutralizing breadth and neutralization activity (IC_{50}) of AD8 SOSIP binding antibodies, B) Correlations between the neutralizing breadth and neutralization activity (IC_{50}) of bNAbs from cow #1, C) Correlation between IC_{50} and EC_{50} of bNAbs of cow#1 against AD8 strain. Related to Figure 2 and 3.

IC50

	MEL-1842	MEL-1872	MEL-2129	VRC01	b12	PGT121
WT	0.004	0.004	0.006	0.183	0.263	0.016
E87A	0.006	0.005	0.009	0.429	0.469	0.020
K121A	0.003	0.004	0.005	0.272	0.212	0.014
L179A	0.003	0.003	0.005	0.099	0.192	0.007
D185A	0.003	0.002	0.004	0.132	0.294	0.006
N197A	0.003	0.002	0.003	0.082	0.030	0.014
N262A	0.005	0.003	0.004	0.118	0.189	0.010
N276A	0.005	0.004	0.006	0.035	0.238	0.017
D279A	0.002	0.003	0.011	> 4	0.528	0.012
K282A	0.002	0.003	0.002	0.114	0.176	0.011
N283A	0.005	0.005	0.011	0.196	0.171	0.018
P313A	0.004	0.004	0.005	0.173	0.243	0.016
N332T	0.005	0.004	0.005	0.132	0.465	0.110
Q363A	0.004	0.003	0.007	0.161	0.386	0.013
S364A	0.004	0.003	0.016	0.063	0.070	0.012
G366A	0.014	0.029	0.015	0.129	1.285	0.010
E370A	0.005	0.003	0.005	0.183	0.446	0.066
I371A	0.008	0.293	3.620	0.167	0.225	0.011
V372A	0.004	0.003	0.006	0.215	2.460	0.014
M373A	0.004	0.003	0.005	0.231	0.183	0.016
R419A	0.003	0.003	0.005	0.152	0.268	0.009
T455A	0.005	0.004	0.105	0.125	0.143	0.009
G471A	> 4	> 4	> 4	0.657	3.004	0.039
G472A	0.002	0.002	0.004	0.173	0.369	0.013
G473A	0.003	0.002	0.007	0.104	0.158	0.009
D474A	0.009	0.003	0.056	0.302	0.366	0.014
M475A	0.004	0.004	0.006	0.196	0.242	0.024
R476A	0.003	0.001	0.002	0.101	0.186	0.017

IC80

	MEL-1842	MEL-1872	MEL-2129	VRC01	b12	PGT121
WT	0.020	0.014	0.021	0.514	0.919	0.060
E87A	0.017	0.015	0.024	1.642	3.234	0.063
K121A	0.013	0.013	0.020	0.702	0.744	0.058
L179A	0.009	0.007	0.014	0.429	2.020	0.022
D185A	0.007	0.008	0.013	0.570	1.303	0.030
N197A	0.007	0.006	0.008	0.250	0.165	0.035
N262A	0.012	0.007	0.013	0.585	0.498	0.024
N276A	0.012	0.012	0.023	0.107	0.732	0.044
D279A	0.006	0.007	0.058	> 4	1.477	0.054
K282A	0.009	0.004	0.010	0.297	0.594	0.027
N283A	0.013	0.016	0.048	0.527	0.672	0.064
P313A	0.012	0.011	0.024	0.699	0.733	0.042
N332T	0.016	0.011	0.016	0.462	0.722	1.840
Q363A	0.018	0.009	0.028	0.414	1.681	0.075
S364A	0.015	0.012	0.069	0.185	0.215	0.034
G366A	0.054	0.117	0.071	0.331	> 4	0.028
E370A	0.037	0.015	0.027	0.932	0.717	0.033
I371A	0.057	> 4	> 4	0.751	0.954	0.039
V372A	0.014	0.010	0.021	0.584	> 4	0.054
M373A	0.013	0.011	0.020	0.712	0.582	0.044
R419A	0.010	0.009	0.023	0.451	1.817	0.032
T455A	0.031	0.100	1.201	0.344	0.688	0.040
G471A	> 4	> 4	> 4	> 4	> 4	0.115
G472A	0.007	0.004	0.012	0.423	> 4	0.038
G473A	0.011	0.009	0.037	0.603	0.741	0.028
D474A	0.060	0.027	0.829	2.830	2.910	0.045
M475A	0.011	0.009	0.018	0.619	0.643	0.094
R476A	0.028	0.014	0.013	0.427	1.289	0.059

Figure S6. Heatmap of relative neutralization activity of monoclonal antibodies against AD8 Env mutants. The colors refer to the changes in IC_{50} and IC_{80} ranging from 0.001 ug/ml (yellow) to 4ug/ml (red). Values in yellow color show low IC_{50} and better neutralization while, those in red color show high IC_{50} values and less neutralization activity. White indicates values of >4, meaning that the IC_{50} could not be achieved for the viruses with that particular mutation to Env. PGT121 (V3-glycan epitope) and b12 and VRC01 (CD4bs epitope) were included for comparison. Neutralization assays were performed in duplicates with two independent biological replicates. Average IC_{50} values were used for drawing heatmaps. Related to Figure 5.