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Functional Interrogation and Mining of Natively-Paired Human V_H:V_L Antibody Repertoires

Bo Wang[1], **Brandon J. DeKosky**[2,3,4], **Morgan R. Timm**[2], **Jiwon Lee**[1], **Erica Normandin**[2], **John Misasi**[2], **Rui Kong**[2], **Jonathan R. McDaniel**[1], **George Delidakis**[1], **Kendra E. Leigh**[2], **Thomas Niezold**[2], **Chang W. Choi**[2], **Elise G. Viox**[2], **Ahmed Fahad**[4], **Alberto Cagigi**[2], **Aurélie Ploquin**[2], **Kwanyee Leung**[2], **Eun S. Yang**[2], **Wing-Pui Kong**[2], **William N. Voss**[1], **Aaron G. Schmidt**[5], **M. Anthony Moody**[6,7], **David R. Ambrozak**[2], **Amy R. Henry**[2], **Farida Laboune**[2], **Julie E. Ledgerwood**[2], **Barney S. Graham**[2], **Mark Connors**[8], **Daniel C. Douek**[2], **Nancy J. Sullivan**[2], **Andrew D. Ellington**[9,10], **John R. Mascola**[2], and **George Georgiou**[1,9,10,11]

[1]Dept. of Chemical Engineering, The University of Texas at Austin, Austin, TX, USA

[2]Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

[3]Dept. of Chemical & Petroleum Engineering, The University of Kansas, Lawrence, KS, USA

[4]Dept. of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS, USA

[5]Laboratory of Molecular Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

[6]Duke Human Vaccine Institute, Duke University Medical School, Durham, NC, USA

[7]Department of Pediatrics, Duke University Medical School, Durham, NC, USA

[8]National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

[9]Center for Systems and Synthetic Biology, The University of Texas at Austin, Austin, TX, USA

[10]Dept. of Molecular Biosciences, The University of Texas at Austin, Austin, TX, USA

[11]Dept. of Bioengineering, The University of Texas at Austin, Austin, TX, USA

Abstract

Correspondence should be addressed to J.R. Mascola (jmascola@mail.nih.gov) or G.G. (gg@che.utexas.edu).

Data availability

Antibody sequences for VRC34.YD.01-VRC34.YD.07 have been deposited in GenBank (MF990262-MF990275). NGS datasets for EBOV and HIV-1 library screening have been deposited in the NCBI Short Read Archive.

Contributions

B.W. and B.J.D. contributed equally to this study. B.W., B.J.D., J.R. Mascola, and G.G. conceived the study and designed the experiments. B.W. and B.J.D. conducted the experiments with help from M.R.T., J.L., E.N., J.M., R.K., J.R. McDaniel, G.D., K.E.L., T.N., C.W.C., E.G.V, A.F., A.C., A.P., K.L., E.S.Y., W-P.K., W.N.V., A.G.S., M.A.M., D.R.A., A.R.H., F.L., J.E.L., B.S.G., M.C., and D.C.D. N.J.S, A.D.E, J.R. Mascola, and G.G. supervised the study. B.W., B.J.D., J.R. Mascola, and G.G. wrote the manuscript with input from all authors.

Competing financial interests

G.G., B.J.D. and A.D.E. declare competing financial interests in the form of a patent filed by the University of Texas at Austin.

We present a technology to screen natively-paired human antibody repertoires from millions of B cells. Libraries of natively-paired variable region heavy and light (V_H:V_L) amplicons are expressed in a yeast display platform that is optimized for human Fab surface expression. Using our method we identify HIV-1 broadly neutralizing antibodies (bNAbs) from an HIV-1 slow progressor and high-affinity neutralizing antibodies against Ebola virus glycoprotein and influenza hemagglutinin.

The human B cell receptor (BCR) repertoire constitutes an invaluable resource for discovery of therapeutic antibodies[1,2]. Cloning from individual B cells obtained via immortalization and expansion *in vitro*, or from single B cells obtained by limiting dilution or FACS, has been used extensively to discover anti-infective antibodies, including broadly neutralizing antibodies (bNAbs) to HIV-1, influenza, etc.[3,4]. In parallel, over the last 25 years the screening of combinatorial libraries generated by random pairing of amplified V_H and V_L genes from human B cells has yielded numerous antibodies, leading to dozens of experimental or approved drug products[5,6]. However, single cell cloning is time- and resource-intensive, and is therefore limited to analysis of a small fraction of the human antibody repertoire[7,8], whereas combinatorial library screening has the capacity to interrogate antibody function from millions of B cells, but the non-cognate pairing of V_H and V_L sequences in these libraries frequently gives rise to antibodies with lower selectivity and inferior biophysical properties compared to authentic human immunoglobulins[9,10].

We report a technology for large-scale functional interrogation of the natively paired V_H:V_L antibody repertoire (Fig. 1). Because V_H and V_L genes are encoded by separate mRNA transcripts, they are first physically linked into a single amplicon for subsequent cloning into an expression vector. V_H and V_L linkage is accomplished by a two-step single cell emulsion lysis and oligo-dT capture of V_H and V_L mRNAs from the same B cell, followed by a second reverse transcription (RT) and overlap-extension (OE)-PCR step to create contiguous V_H:V_L amplicons[11,12]. In these amplicons the V_H and V_L genes are joined through a linker designed to enable one step sub-cloning into a yeast Fab surface expression vector, whereby the V_H and V_L are transcribed from a galactose-inducible bidirectional promoter with C_{H1} (human IgG1 isotype) and C_L (human κ and λ2 isotypes) at the C-terminus of the V_H and V_L respectively (Fig. 1a, b, Supplementary Fig. 1, Supplementary Table 1).

Human antibodies often express poorly in microbial hosts[13], and while expression efficiency in yeast is substantially higher relative to *E. coli* or phage[13–15], examination of a panel of 13 previously reported human influenza hemagglutinin (HA)-specific antibodies revealed that only 7/13 antibodies (53%) bound antigen when displayed on yeast (Supplementary Fig. 2)[16]. Consistent with earlier reports[17], co-expression of protein disulfide isomerase (PDI) increased display efficiency, as monitored by 2-color flow cytometry, to 10/13 antibodies (Fig. 1c). In addition to PDI expression, the enhanced dimerization of heavy and light chains via fusion to C-terminal leucine-zipper (LZ) domains resulted in the display of the full set of 13/13 human anti-HA antibodies (Fig. 1c, Supplementary Fig. 2)[18]. We then tested the display efficiency of three other human antibodies (2 anti-Ebola virus (EBOV) antibodies: c13c6 and KZ52, and the anti-HIV-1 bNAb N123-VRC34.01 that targets the HIV-1 fusion peptide[19]). All three antibodies displayed efficiently and were shown to bind selectively to

their respective antigens in the optimized system (Supplementary Fig. 2). Extensive earlier studies demonstrated that yeast display enables interrogation of the human antibody repertoire based on affinities or off rates, for epitope specificity, and for other properties including stability[15] (Fig. 1d). Clones of interest can then be expressed either as Fab or as IgG for detailed functional and biochemical assays (Fig. 1d).

We used this approach to analyze the antibody repertoire of an individual 6 days after immunization with an experimental EBOV vaccine[20]. This peak plasmablast V_H:V_L repertoire was displayed in yeast, and cells were analyzed and sorted for binding to EBOV mucin-like domain deleted glycoprotein ($GP_{\Delta muc}$). We used high-throughput sequencing (HTS) to track antibody lineages throughout the screening progress. Of 1,189 unique CDRH3:CDRL3 nucleotide clusters obtained from 5,002 plasmablasts after highly stringent sequence quality filtering, 828 were verified as cloned and displayed in the system using HTS (70% overall efficiency for library construction and display). As expected for the peak post vaccination plasmablast response, an appreciable (6%) fraction of repertoire-expressing yeast cells in the pre-sort library bound to antigen, and antigen-specific clones were highly enriched after the third round of sorting (Fig. 2a, Supplementary Fig. 3). Single colony analysis of yeast yielded 7 antibody lineages that bound to $GP_{\Delta muc}$ (EBOV.YD.01-EBOV.YD.04, EBOV.YD.09-EBOV.YD.11, Supplementary Table 2, Supplementary Fig. 4). Comparison of HTS datasets for the pre-sort library and the sorted library after three rounds of screening revealed that all 7 clones isolated above had been enriched by ≥120-fold. Four of these antibodies were randomly selected (EBOV.YD.01-EBOV.YD.04) and expressed as IgG1s in HEK293 cells, then digested to generate Fabs, which were shown to bind $GP_{\Delta muc}$ with nM affinities by biolayer interferometry (BLI) (Supplementary Fig. 5, Supplementary Table 2). All four antibodies blocked infection by EBOV GP-pseudotyped lentiviral particles, with neutralization ranging from 55% to 99% at 10 μg/ml (Fig. 2b, Supplementary Fig. 6). Competition assays revealed that these antibodies targeted distinct non-overlapping epitopes (Fig. 2c). EBOV.YD.03 competed with the well-characterized neutralizing antibody KZ52, indicating that it binds an epitope similar to antibodies generated during natural infection[21] (Fig. 2c).

HTS surveillance of antibody clonal prevalence during screening enabled us to retrieve other antibodies that were enriched across rounds, but not identified by single colony picking. We synthesized 4 additional antibody lineages that had been enriched >8-fold in HTS datasets after multiple rounds of FACS. 3/4 antibodies identified by HTS bound to $GP_{\Delta muc}$ with single-digit nM K_D (EBOV.YD.06-EBOV.YD.08, Supplementary Fig. 5, Supplementary Table 2) while another clone, EBOV.YD.05, bound weakly (~3 μM K_D as a Fab).

We then applied this yeast display technology to assess an antibody lineage in an HIV-1 infected donor. Kong and coworkers recently identified N123-VRC34, an HIV-1 bNAb lineage that binds to the fusion peptide (FP)[19]. We interrogated the antibody repertoire of 1.42 million peripheral B cells from this donor (N123) and amplified the V_H:V_L repertoire with a unique human FR1 primer set that was also supplemented with lineage-specific primers. Only the inclusion of lineage-specific primers enabled us to successfully recover the N123-VRC34 lineage, which contains several reported FR1 mutations (Supplementary Tables 3 and 4) and is extremely rare at this time point within the donor (roughly 0.003% of

all B cells[19]). Yeast libraries were sorted using an epitope protein scaffold containing the 8 terminal AA of the fusion peptide (VRC34-epitope scaffold-FP-APC) and a version of the scaffold alone without the fusion peptide (VRC34-epitope scaffold-KO-PE)[19]. As with EBOV antibody libraries, the HIV-1 antibody libraries were highly enriched for FP-specific clones by the third round of sorting (Fig. 2d, Supplementary Fig. 7).

HTS revealed that after three rounds of screening, VRC34-lineage antibodies far outcompeted other antibody lineages, constituting 98.7% of high quality sequences and suggesting that the VRC34 lineage dominated the FP-specific repertoire in this donor. Three prevalent V_H:V_κ clones were expressed and the respective Fabs were shown to bind to the HIV-1 fusion peptide probe with high affinity (Fig. 2f, Supplementary Fig. 8). To further "bin" FP binding clones based on affinity, during the third round of sorting the yeast population was gated by increasing fluorescence intensity to FP; Fabs of four clones restricted to a high, medium, or low-affinity gated population had K_D values consistent with their respective FACS profile (Fig. 2e,f, Supplementary Fig. 8). In total, seven unique VRC34 lineage antibodies were identified and all were broadly neutralizing (Supplementary Fig. 9). Notably, three double-nucleotide changes within a codon were observed that resulted in nonsynonymous amino acid substitutions, which are highly unlikely to have resulted from PCR or other artifacts and thus likely arose from somatic hypermutation (SHM) in the donor, suggesting site-specific selection *in vivo* (Supplementary Fig. 10). These results suggest that genetic lineage-targeting coupled with yeast display can be useful for antibody discovery against HIV-1 or other difficult pathogens for which bNAbs are reported to have specific genetic requirements[22,23].

Finally, we constructed a paired V_H:V_L library from 12 million peripheral B cells harvested 270 days after immunization with seasonal, trivalent inactivated influenza vaccine (IIV3)[24], when HA-specific B cells occur at a frequency of ~0.01%[25]. We isolated single yeast colonies after 1 round of sorting for antibody-expressing cells and 4 rounds of screening with Group 1 HA (18 clones) and separately, Group 2 HA (16 clones) included in IIV3 (Group 1: H1 from A/Solomon Islands/3/2006; Group 2: H3 from A/Wisconsin/67/2005); decreasing concentrations of antigen were used across rounds to increase selection stringency (Fig. 2g, h, Supplementary Fig. 11). Of these, 15/34 (44%) colonies encoded four unique antibody lineages to HA (one targeting H1 and three targeting H3) that bound to recombinant HAs with affinities ranging from 0.35 to 39.9 nM when expressed as IgGs (Fig. 2i, Supplementary Fig. 12, Supplementary Table 5); an additional 7/34 colonies (21%) recognized HAs but with lower affinities. Two of the 4 antibodies reported in Fig. 2i neutralized influenza with picomolar IC_{50} (Supplementary Fig. 13).

The display of a properly folded, functional antibody repertoire in yeast constitutes a renewable resource for the isolation of human antibodies and also for repeated analyses of the antibody response based on properties such as affinity, epitope coverage (e.g., by sorting in the presence of competitor antibodies), and stability[15]. Yeast surface display has been reported to have a lower expression bias relative to other microbial display technologies[13–15] and the yeast display optimization reported here further ensured *bona fide* expression of human antibody repertoires. We expect native V_H:V_L antibodies to show superior selectivity and biophysical properties compared to randomly paired V_H and V_L antibodies isolated

using other display platforms[9,10]. Native antibody libraries displayed on yeast can also be screened for antigens that bind to B cell surface ligands (e.g., sialic acid[26] or CR2[27]) and are therefore not suitable for single B cell sorting, and separately to discover antibodies targeting insoluble antigens, including membrane proteins[28–30].

Methods

A **Life Sciences Reporting Summary** is available.

Strain and media

The yeast strain AWY101 (MATα *AGA1::GAL1-AGA1::URA3 PDI1::GAPDH-PDI1::LEU2 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL)* (kind gift from Eric Shusta, University of Wisconsin-Madison) was used for library construction and screening. EBY100 (MATa *AGA1::GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) was used for initial native human antibody display. Yeast cells were maintained in YPD medium (20 g/l dextrose, 20 g/l peptone, and 10 g/l yeast extract); after library transformation, yeast cells were maintained in SDCAA medium (20 g/l dextrose, 6.7 g/l yeast nitrogen base, 5 g/l casamino acids, 8.56 g/l $NaH_2PO_4.H_2O$, and 10.2 g/l $Na_2HPO_4.7H_2O$). SGDCAA medium (SDCAA with 20 g/l galactose, 2 g/l dextrose) was used for library induction.

Antigens and antibodies

Recombinant Ebola virus glycoprotein with the mucin-like domain deleted ($GP_{\Delta muc}$), and HIV-1 fusion peptide probe (VRC34-epitope scaffold-FP) and knock-out scaffold probe (VRC34-epitope scaffold-KO) were produced as described previously[19,31,32]. Proteins were biotinylated and conjugated with streptavidin-APC ($GP_{\Delta muc}$ and VRC34-epitope scaffold-FP) or streptavidin-PE (VRC34-epitope scaffold-KO) (Thermo Fisher Scientific), respectively. Recombinant hemagglutinins (A/California/07/2009, A/Solomon Islands/3/2006, and A/Wisconsin/67/2005) were produced as before[33] or acquired from BEI Resources (A/Victoria/210/2009, and B/Brisbane/60/2008) and were biotinylated using an EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific). Anti-flag FITC antibody was purchased from Sigma-Aldrich (clone M2).

Optimization of native human antibody display

Vectors encoding previously reported anti-influenza virus HA monoclonal antibodies with or without leucine zipper (LZ) domains were transformed into EBY100 or AWY101 using a Frozen-EZ Yeast Transformation II kit (Zymo Research)[16]. After culturing in SDCAA to an OD_{600} of 2 at 30°C, Fab surface expression was induced by transferring cells to SGDCAA medium at OD_{600} of 0.5. After two days of induction at 20°C, 10^6 cells were collected and washed twice with PBS+0.5% BSA+2mM EDTA, and incubated with 100nM biotinylated hemagglutinin at room temperature for 30min, followed by staining with 2μg/ml anti-flag FITC and 2μg/ml streptavidin-APC at 4°C for 15min. Cells were washed twice with ice-cold PBS+0.5% BSA+2mM EDTA and analyzed on a FACS Aria II (BD Biosciences).

Analysis of anti-EBOV $GP_{\Delta muc}$ (c13c6, KZ52) and anti-HIV-1 FP (VRC34.01) antibodies was performed as above[19,34,35], except 23 nM $GP_{\Delta muc}$-APC or 50 nM VRC34-epitope scaffold-FP-APC and 50 nM VRC34-epitope scaffold-KO-PE, respectively, were used for antigen staining.

Generation of natively paired V_H:V_L from peripheral B cells, library construction, yeast display and FACS screening

Human subject protocols were approved by the NIAID Institutional Review Board. For anti-EBOV $GP_{\Delta muc}$ antibody isolation, peripheral blood mononuclear cells (PBMCs) were isolated from a healthy human volunteer after immunization with a Phase I Ebola GP vaccine (NCT02408913)[20]. The volunteer was first immunized with chimpanzee-derived replication-defective adenovirus encoding EBOV GP, then boosted 30 weeks and 5 days later with modified vaccinia Ankara encoding EBOV GP. Previous studies showed that plasmablasts in peripheral blood peak around six days post-boost immunization[36]. 10 ml of blood were collected and PBMCs isolated using Ficoll-Paque™ PLUS (GE Healthcare) six days post-boost. PBMCs were stained with a multi-color flow cytometry panel consisting of fluorophore-labeled antibodies against CD3 (Brilliant Violet 421, clone SP34-2, BD Biosciences), CD19 (PE-Cy7, clone HIB19, BD Biosciences), CD4 (Brilliant Violet 421, clone OKT4, BioLegend), CD8a (Brilliant Violet 421, clone RPA-T8, BioLegend), CD14 (Brilliant Violet 421, clone M5E2, BioLegend), CD20 (Brilliant Violet 605, clone 2H7, BioLegend), CD27 (Brilliant Violet 711, clone O323, BioLegend), and CD38 (Alexa Fluor® 680, clone OKT10, custom-conjugated at the Vaccine Research Center, NIAID), and 7-aminoactinomycin D (7-AAD, Thermo Fisher Scientific) to exclude dead cells. 5,002 $CD3^-CD4^-CD8^-CD14^-CD19^+CD20^-CD27^+CD38^+$ plasmablasts were isolated using a FACS Aria sorter (BD Biosciences) and subsequently utilized for emulsion V_H:V_L overlap extension RT-PCR.

For HIV-1 FP antibody isolation, PBMCs were collected from donor N123 on June 22nd, 2009. This donor is a chronically HIV-1 infected individual enrolled in NIAID under a clinical protocol approved by the NIAID Institutional Review Board[37,38]. This donor was diagnosed with HIV-1 in 2000. After more than nine years of infection, this donor showed a CD4 T cell count of 463 cells/ml and a plasma HIV-1 viral load of 4,920 RNA copies/ml. This donor was not on antiretroviral treatment. 1.42×10^6 peripheral B cells were isolated from 25 million PBMCs using a human B cell selection kit (Stemcell Technologies).

For the isolation of influenza HA-specific antibodies, a healthy donor was vaccinated with a trivalent inactivated influenza vaccine (IIV3: A/Solomon Islands/3/2006, A/Wisconsin/67/2005, B/Malaysia/2508/2004)[24]. Subsequently, 270 days after vaccination, 1.2×10^7 B cells were isolated from blood leukapheresis using a human pan B cell isolation kit (Miltenyi Biotec).

We utilized a flow-focusing nozzle to rapidly compartmentalize B cells in single cell emulsion droplets, followed by single B cell lysis inside droplets and single-cell mRNA capture with oligo(dT)-coated magnetic beads[11,12]. Overlap extension RT-PCR was then performed to link heavy and light chains using a Superscript III RT-PCR kit (Thermo Fisher Scientific)[11,12]. We included NcoI and NheI restriction sites in the linker region of the

overlap-extension RT-PCR primers that link V_H and V_L into an ~850 bp amplicon (Supplementary Table 3). For HIV-1 experiments, we also included additional primers specific to the VRC34 lineage (Supplementary Table 4). For library construction, 100 ng of V_H:V_L cDNA was amplified under the following conditions with AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) to introduce NotI and AscI sites, respectively (Supplementary Table 1): 2 min initial denaturation at 95 °C, denaturation at 95 °C for 20 s for 20 cycles, annealing at 60 °C for 20 s and extension at 68 °C for 60 s, final extension at 68 °C for 5 min. The DNA product was digested and ligated into pCT-VHVL-K1 (for V_H:V_κ libraries) and pCT-VHVL-L1 (for V_H:V_λ libraries), and transformed into electrocompetent *E. coli* for library cloning *en masse*. Plasmid DNA encoding V_H:V_L libraries was miniprepped, digested with NcoI and NheI, ligated with the bidirectional promoter, and transformed into *E. coli* again. The final library DNA was miniprepped, then amplified using the transformation primers (Supplementary Table 1) to generate library inserts with homologous ends to NotI and AscI double digested vectors, and then inserts were co-transformed into electrocompetent AWY101 together with the digested vectors to generate libraries via yeast homologous recombination[39]. Library sizes for EBOV, HIV-1 and flu repertoires were EBOV_κ: $2x10^7$, EBOV_λ: 10^7, HIV-1_κ: 10^7, HIV-1_λ: $9x10^6$, flu_κ: $7x10^7$, and flu_λ: $3x10^7$, respectively, as determined by colony counting.

For library screening, natively paired human V_H:V_L libraries were displayed on yeast by growing cells resuspended in SGDCAA medium at 20°C for 2d to induce Fab expression. The fraction of Fab-expressing cells 2d post induction were consistent with previous reports for yeast displaying naïve human scFv[15]. For EBOV vaccinee and HIV-1 donor libraries, three rounds of sorting were performed against $GP_{\Delta muc}$, or VRC34-epitope scaffold-FP-APC and VRC34-epitope scaffold-KO-PE, respectively. The VRC34-epitope scaffold-FP was designed to present the FP in an optimal conformation and provide a glycan in a similar context as that presented by the native HIV-1 trimer[19]. In the 1st round of screening, at least 10-fold coverage in yeast clones relative to library size were labeled with 2 μg/ml anti-FLAG-FITC and either (i) 23 nM $GP_{\Delta muc}$-APC for isolating of EBOV $GP_{\Delta muc}$-specific antibodies, or (ii) 50 nM VRC34-epitope scaffold-FP-APC and 50 nM VRC34-epitope scaffold-KO-PE for the isolation of HIV-1 FP-specific antibodies. For EBOV antibody libraries, the PE channel was also included to correct for yeast autofluorescence. Cells were stained at room temperature for 30 min and washed twice with ice-cold PBS+0.5% BSA +2mM EDTA, then analyzed by FACS. $FITC^+APC^+PE^-$ cells were selected and recovered in SDCAA medium at 30°C. Subsequent screening rounds were performed similarly, except that for the EBOV GP antibody library, at least 5×10^5 cells were screened in Round 2 and 3, and for HIV-1 FP antibody library, at least 10^7 cells were screened in Round 2 and 3. Affinity binning of anti-EBOV $GP_{\Delta muc}$ and anti-HIV-1 FP antibody repertoires was performed similarly as described[40].

Influenza HA-specific antibodies were isolated following 5 total rounds of sorting (two rounds of MACS and 3 rounds of FACS enrichment for binding to fluorescent HAs) as follows: For the 1st round, at least 10-fold coverage in yeast clones relative to library size were labeled with 2 μg/ml anti-FLAG-FITC at room temperature for 30min. After washing, cells were labeled with anti-FITC microbeads (Miltenyi Biotec) at 4°C for 15min, and Fab expressing cells were selected by MACS. For the 2nd round of sorting, cells were labeled

with 1 μM biotinylated recombinant HA (H1 A/Solomon Islands/3/2006, H3 A/Wisconsin/67/2005), and then selected with streptavidin microbeads (Miltenyi Biotec) using MACS as previously described[41]. Subsequently the library was screened using 3 rounds of FACS by labeling $5x10^6$ cells with 2 μg/ml anti-FLAG-FITC together with 1 μM HA (1st FACS round), 200 nM HA (2nd FACS round), or 40 nM HA (3rd FACS round), at room temperature for 30 min, followed by incubation with 2 μg/ml streptavidin-APC at 4°C for 15 min prior to sorting.

Sequencing of the natively paired antibody repertoire from B cells was performed as previously described[42]. Bioinformatic analysis was performed as previously described[42].

Recovery and expression of antibody clones from enriched libraries

Yeast cells from the final round of sorting were plated on SDCAA plates. A minimum of 10 colonies were selected following the last round of each screening campaign. Colony PCR was performed on heavy and light chain variable regions of each clone. Clones were sequenced, and the unique antibodies were named as *project_name.YD.unique_clone_number*. For antibody expression, restriction sites were incorporated for insertion into the VRC8400 IgG1, and Igκ or Igλ expression vectors (for anti-EBOV $GP_{\Delta muc}$ and HIV-1 FP antibodies), or Gibson assembly was used to clone the variable regions into modified pcDNA3.4 IgG1, and Igκ or Igλ vectors (for anti-HA antibodies). Heavy and light chain plasmids for each antibody were co-transfected into Expi293 cells, and secreted antibodies were purified on a Protein A column[43]. Fabs were produced by digestion of IgG1 with Lys-C Protease (Thermo Fisher Scientific) and separated from Fc using Protein A or Protein G columns.

For EBOV vaccinee libraries, the population of sorted $FITC^+$ Fab-expressing yeast cells in the first round of FACS were recovered in SDCAA medium at 30°C. Plasmid DNA was extracted using high-efficiency yeast plasmid recovery protocols as reported previously[44] and V_H genes in sorted libraries were PCR-amplified using primers that targeted the yeast expression plasmid vector backbone (2YDrec_heavy_Vfor_MSrev1: TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNCTGTTATTGCTAGCGTTTTAGCA, and 2YDrec_huIgH_Crev_MSfor1: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNAAGGCGCGCCTGTACTTGC). Libraries were prepped by an additional round of PCR-based primer extension and Illumina adapter addition to incorporate unique DNA barcodes for each sample, and sequenced using the Illumina 2x300bp MiSeq platform. Similarly, FACS-sorted yeast from EBOV vaccinee libraries analyzed for binding to $GP_{\Delta muc}$ and FACS-sorted yeast from HIV-1 donor libraries analyzed for binding to VRC34-epitope scaffold-FP-APC were recovered, mini-prepped, and sequenced in the same way after each round of sorting to quantify V_H gene clonal enrichment across library sorting rounds.

For determining the EBOV vaccinee antibody library display efficiency, the original plasmablast V_H:V_L repertoire underwent highly stringent quality filtering (≥15 CDRH3:CDRL3 reads, 96% CDRH3 nt clustering) and CDR-H3 nucleotide junctions were mapped to CDR-H3 junctions recovered in the $FITC^+$ Fab-expressing yeast library. Mapping was performed using usearch v5.2.32 with an exact nucleotide length match requirement and

a 96% cutoff threshold for CDR-H3 junction nucleotide sequence match. For EBOV $GP_{\Delta muc}$ library antibody discovery via NGS clonal lineage tracking, CDR-H3 amino acid sequences from NGS datasets that were enriched >8-fold across multiple rounds of screening were synthesized, expressed in HEK293 cells, and tested for soluble binding to $GP_{\Delta muc}$ as Fabs (EBOV.YD.05-EBOV.YD.08). Consensus sequences for NGS-discovered antibodies were generated based on exact CDR-H3 and CDR-L3 nucleotide junction matches between the originally paired V_H:V_L, separate V_H, and separate V_L sequencing libraries (prior to yeast display screening), as previously described for antibody discovery from paired heavy:light sequence datasets[42,45,46]. Briefly, consensus sequences were generated using usearch v5.2.32 from exact match reads to the CDR-H3 nucleotide or CDR-L3 nucleotide junctions for heavy or light chains, respectively, and plasmids containing antibody heavy and light chain sequences were expressed via transient transfection in HEK293 cells for soluble antibody generation as previously described[32].

Affinity characterization

Binding kinetics of anti-EBOV $GP_{\Delta muc}$ and anti-HIV-1 FP Fabs were determined using biolayer interferometry on a FortéBio Octet HTX instrument[32]. For EBOV $GP_{\Delta muc}$-targeting antibodies, AR2G biosensors were coupled with $GP_{\Delta muc}$ (10 μg/ml in 10 mM Acetate, pH 4.5) for 600 s. Typical capture levels after quenching with 1 M ethanolamine (pH 8.0) for 300 s were between 2 and 2.5 nm, and variability within the same protein did not exceed 0.25 nm. Biosensors were then equilibrated for 420 s in PBST-BSA (PBS+1% BSA+0.01% Tween+0.02% sodium azide) prior to binding assessment of the Fab. Association of Fab was measured for 300–600 s and dissociation was measured for 300–600 s, both in PBST-BSA. Correction to subtract non-specific baseline drift was carried out by subtracting the measurements recorded for a sensor loaded with unrelated antigen (HIV-1 gp120).

For HIV-1 FP-targeting antibodies, streptavidin biosensors were used to capture VRC34-epitope scaffold-FP at 0.5 μg/mL in PBST-BSA. Typical capture levels for FP probe were between 0.4 and 0.7 nm. Biosensors were then equilibrated for 60 s in PBST-BSA prior to binding assessment of the Fab. Association of Fab was measured for 150 s and dissociation was measured for 150 s, both in PBST-BSA. Correction to subtract non-specific baseline drift was carried out by subtracting the measurements recorded for a sensor loaded without Fab. All assays were performed with agitation set to 1,000 rpm at 30°C. Data analysis and curve fitting were carried out using the Octet analysis software, version 9.0. Experimental data were fitted using a 1:1 binding model for all experiments. Global analyses of the complete data sets assuming binding was reversible (full dissociation) were carried out using nonlinear least-squares fitting allowing a single set of binding parameters to be obtained simultaneously for all concentrations used in each experiment.

For anti-HA IgGs, recombinant HAs (H1 A/Solomon Islands/3/2006, H3 A/Wisconsin/67/2005)[33] were immobilized in separate channels by amine-coupling at pH 6.0. BSA was immobilized in the reference channel, to correct for buffer effects and non-specific binding signal. All SPR measurements were performed in HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% vol/vol surfactant P20; GE Healthcare). Serially diluted antibodies were injected in triplicates at 30 μl/min for 2 min and allowed a

dissociation time of 10 min. The chip was regenerated after each binding event with 50 mM Tris, pH 11.5, with a contact time of 15 sec. The resulting sensorgrams were fitted with a two-state model (with conformation change) using Biaevaluation 3.0 software. The K_D values reported are the average of the three technical replicates ± SD.

For EBOV $GP_{\Delta muc}$ antibody cross-competition assays, 10 μg/mL of $GP_{\Delta muc}$ was loaded onto Octet HTX biosensors using amine coupling (AR2G, FortéBio) for 600 s. The newly identified antibodies, the KZ52 antibody as a positive control[21], and the isotype negative control (VRC01[47]) were diluted to 50 μg/mL in PBST-BSA. Binding of competitor and analyte mAbs were each assessed for 1,800 s. The assay was performed in duplicate with agitation at 1,000 rpm at 30°C. The percent inhibition (PI) was calculated by the equation: PI = 100 − [(probing mAb binding in the presence of competitor mAb)/(probing mAb binding in the absence of competitor mAb)] × 100.

Neutralization assays

For EBOV neutralization, GP-pseudotyped lentiviral particles expressing a luciferase reporter gene were produced as described previously[48], and were incubated 1h at 37˚C with serially diluted purified mAbs. HEK293T cells were infected with the lentivirus:mAb mixture for 72h in presence of polybrene (5μg/ml, Sigma-Aldrich). Luciferase expression was assessed with Bright Glo (Promega) using a Victor X3 Plate Reader (PerkinElmer). Cell infection was calculated relative to the negative control antibody VRC01.

HIV-1 neutralization was assessed in TZM-bl cells as described previously[19]. Briefly, 293T cells were co-transfected by a pSG3ΔEnv backbone and an HIV-1 Env expression plasmid to produce Env-pseudotyped virus stocks. Viruses were mixed with 5-fold serially diluted mAbs starting at 50 μg/ml, and incubated at 37°C for 1h before adding to the cells. After incubation at 37°C for 48h, the supernatants were removed and the cells were lysed. Luciferase activity was measured. 50% inhibitory concentrations (IC_{50}) were determined as described[19].

For flu neutralization, influenza pseudotyped lentiviral vectors expressing a luciferase reporter gene were produced as described[49]. Briefly, the following plasmids: 17.5μg pCMVΔR8.2, 17.5μg pHR'CMV-Luc, 0.3μg pCMV Sport/h TMPRSS2, and 1μg CMV/R-HA and 0.125μg corresponding CMV/R-NA of a given strain of influenza virus, were transiently co-transfected into 15cm TC plate of 293T cells using Fugene6 (Promega). Cells were transfected overnight and replenished with fresh medium. Forty-eight hours later, supernatants were harvested, filtered through a 0.45-μm PES membrane filter, aliquoted, and frozen at −80°C. For neutralization assays, monoclonal antibodies at various dilutions were mixed with pseudoviruses and incubated at 37°C for 1h before adding to 293A cells in 96-well plates (10,000 cells per well). Seventy-two hours later, cells were lysed in cell culture lysis buffer (Promega, Madison, WI) before mixing with luciferase assay reagent (Promega). Light intensity was quantitated with a Perkin Elmer microplate reader and antibody neutralization results in lower light intensity.

Statistical analysis

Refer to the **Life Sciences Reporting Summary**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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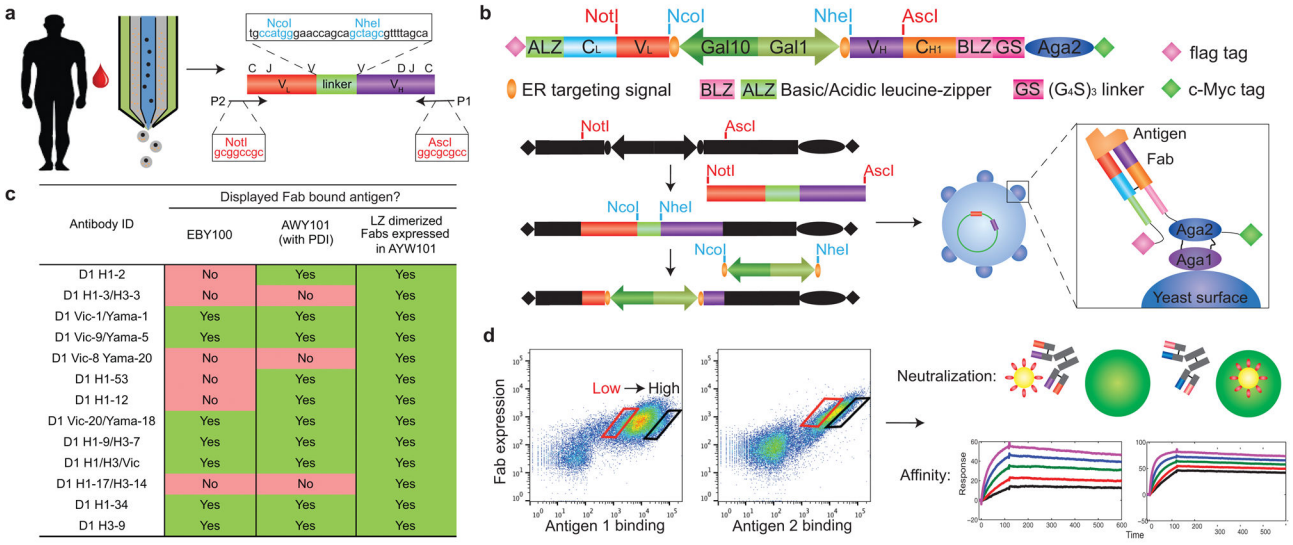
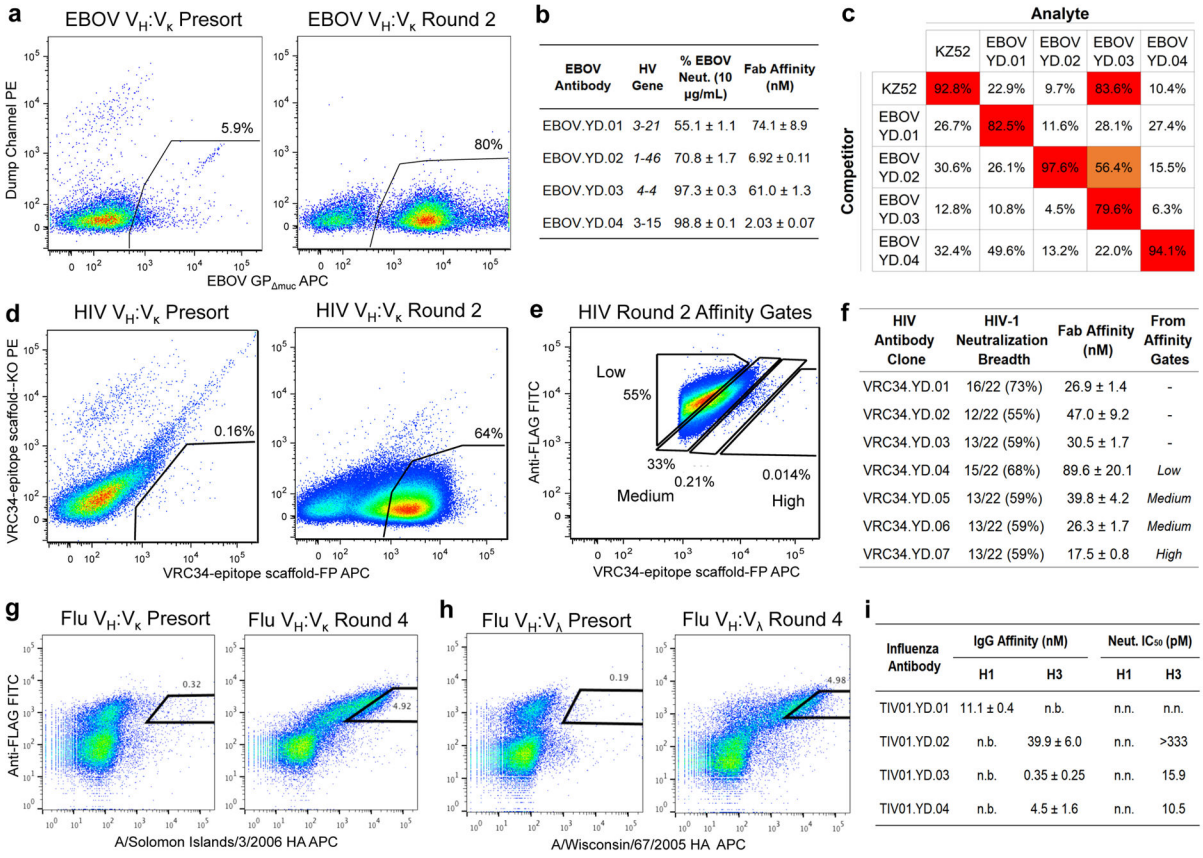


Figure 1. High-throughput cloning, yeast display, and functional analysis of the human natively paired V_H:V_L antibody repertoire

(**a**) Using an axisymmetric flow focusing device, peripheral blood B cells are compartmentalized inside microdroplets, lysed, and single-cell mRNA is captured as overlap extension PCR template to produce V_H and V_L cDNAs joined by a 32-nucleotide linker sequence containing NcoI and NheI restriction sites. (**b**) Natively paired V_H:V_L amplicon libraries are subcloned *en masse* into a Fab expression vector with a galactose-inducible bidirectional promoter Gal1/Gal10 for transcription of C_{H1}-V_H and $C_{\kappa/\lambda}$-$V_{\kappa/\lambda}$, along with expression tags (c-Myc, flag, respectively) and leucine-zipper (LZ) dimerization domains. (**c**) Display characteristics for a panel of 13 anti-HA antibodies in yeast strains EBY100, AWY101 that overexpresses PDI, and AWY101 with LZ-forced dimerization. (**d**) Sequential rounds of FACS under increasingly stringent conditions (e.g., lower antigen concentrations, co-incubation with competitor antibodies) are used to bin libraries within various windows of affinity. Antibodies are recovered from sorted yeast, expressed, and characterized.



EBOV Antibody	HV Gene	% EBOV Neut. (10 µg/mL)	Fab Affinity (nM)
EBOV.YD.01	*3-21*	55.1 ± 1.1	74.1 ± 8.9
EBOV.YD.02	*1-46*	70.8 ± 1.7	6.92 ± 0.11
EBOV.YD.03	*4-4*	97.3 ± 0.3	61.0 ± 1.3
EBOV.YD.04	3-15	98.8 ± 0.1	2.03 ± 0.07

Competitor \ Analyte	KZ52	EBOV YD.01	EBOV YD.02	EBOV YD.03	EBOV YD.04
KZ52	92.8%	22.9%	9.7%	83.6%	10.4%
EBOV YD.01	26.7%	82.5%	11.6%	28.1%	27.4%
EBOV YD.02	30.6%	26.1%	97.6%	56.4%	15.5%
EBOV YD.03	12.8%	10.8%	4.5%	79.6%	6.3%
EBOV YD.04	32.4%	49.6%	13.2%	22.0%	94.1%

HIV Antibody Clone	HIV-1 Neutralization Breadth	Fab Affinity (nM)	From Affinity Gates
VRC34.YD.01	16/22 (73%)	26.9 ± 1.4	-
VRC34.YD.02	12/22 (55%)	47.0 ± 9.2	-
VRC34.YD.03	13/22 (59%)	30.5 ± 1.7	-
VRC34.YD.04	15/22 (68%)	89.6 ± 20.1	*Low*
VRC34.YD.05	13/22 (59%)	39.8 ± 4.2	*Medium*
VRC34.YD.06	13/22 (59%)	26.3 ± 1.7	*Medium*
VRC34.YD.07	13/22 (59%)	17.5 ± 0.8	*High*

Influenza Antibody	IgG Affinity (nM)		Neut. IC$_{50}$ (pM)	
	H1	H3	H1	H3
TIV01.YD.01	11.1 ± 0.4	n.b.	n.n.	n.n.
TIV01.YD.02	n.b.	39.9 ± 6.0	n.n.	>333
TIV01.YD.03	n.b.	0.35 ± 0.25	n.n.	15.9
TIV01.YD.04	n.b.	4.5 ± 1.6	n.n.	10.5

Figure 2. Examples of natively paired antibody repertoire analysis and functional characterization

(**a**) The natively paired repertoire of peripheral plasmablasts from an EBOV vaccinee was cloned into yeast and screened for binding to EBOV GP$_{\Delta muc}$. (**b**) Neutralization and affinity of GP$_{\Delta muc}$ antibodies randomly selected after the 3rd round of sorting. (**c**) Competition analysis of anti-GP$_{\Delta muc}$ antibodies from (**b**) and the EBOV-neutralizing antibody KZ52. (**d**) FACS analysis of the yeast display repertoire from 1.42×10^6 peripheral B cells for binding to HIV-1 FP probe. (**e**) Diagonal FACS gates were used to bin the sorted (Round 2) repertoire based on affinity. (**f**) K_D values and HIV-1 neutralization breadth of purified antibodies following screening. (**g**) The steady-state peripheral B cell repertoire was interrogated with A/Solomon Islands/3/2006 H1 HA. (**h**) The same B cell repertoire was mined with A/Wisconsin/67/2005 H3 HA. (**i**) Affinity and neutralization of anti-HA antibodies isolated after the 4th round of antigen screening. n.b., no binding, n.n., no neutralization. Affinity values and EBOV neutralization are reported as average ± standard deviation from three technical replicates. Influenza neutralization is reported as average from three technical replicates.