



## Research paper

# Proteins mimicking epitope of HIV-1 virus neutralizing antibody induce virus-neutralizing sera in mice



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## ABSTRACT

**Background:** The development of an effective vaccine preventing HIV-1 infection is hindered by the enormous antigenic variability and unique biochemical and immunological properties of HIV-1 Env glycoprotein, the most promising target for HIV-1 neutralizing antibody. Functional studies of rare elite neutralizers led to the discovery of broadly neutralizing antibodies.

**Methods:** We employed a highly complex combinatorial protein library derived from a 5 kDa albumin-binding domain scaffold, fused with support protein of total 38 kDa, to screen for binders of broadly neutralizing antibody VRC01 paratope. The most specific binders were used for immunization of experimental mice to elicit Env-specific antibodies and to test their neutralization activity using a panel of HIV-1 clade C and B pseudoviruses.

**Findings:** Three most specific binders designated as VRA017, VRA019, and VRA177 exhibited high specificity to VRC01 antibody. Immunized mice produced Env-binding antibodies which neutralize eight of twelve HIV-1 Tier 2 pseudoviruses. Molecular modelling revealed a shape complementarity between VRA proteins and a part of VRC01 gp120 interacting surface.

**Interpretation:** This strategy based on the identification of protein replicas of broadly neutralizing antibody paratope represents a novel approach in HIV-1 vaccine development. This approach is not affected by low immunogenicity of neutralization-sensitive epitopes, variability, and unique biochemical properties of HIV-1 Env used as a crucial antigen in the majority of contemporary tested vaccines.

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## 1. Introduction

HIV/AIDS is a global pandemic resulting in an estimated 35·4 million deaths worldwide. Irrespective of intensive research, no commercial vaccine is available. The most important obstacles are an enormous HIV-1 antigenic variability and unique biochemical, biological, and immunological properties of the most promising vaccine candidate, HIV-

1 envelope (Env) glycoprotein which is responsible for HIV-1 attachment to and the entry into the host cell [1,2].

New strategies in HIV-1 vaccine development were encouraged after identification of a few antibodies able to neutralize a broad range of HIV-1 Env variants (bn-mAbs) which limit viraemia, as shown for elite neutralizers [2–4]. The generation of HIV-1-specific bn-mAbs under natural conditions is a long-term process lasting years and difficult to be elicited by conventional vaccination. The majority of identified bn-mAbs exhibit unique properties including a long HCDR3, extraordinary frequencies of V(D)J mutations and poly- or autoreactivity with human lipids and proteins, which seem to be a crucial obstacle for the development of a successful vaccination strategy [5–7].

Despite growing knowledge about the molecular structure of HIV-1 Env, its interaction with bn-mAbs, and mechanisms of immune

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### Research in context

#### Evidence before this study

The development of effective vaccine preventing HIV-1 virus infection is hindered by enormous antigenic variability of the virus envelope glycoprotein (Env) and unique biological and immunological properties of Env as the most promising target for HIV-1 neutralizing antibody. Functional studies of a rare population of patients who control the infection even without antiretroviral therapy led to the discovery of monoclonal antibodies neutralizing a broad spectrum of Env variants (bn-mAb). The generation of such bn-Abs is a long-term process lasting years and difficult to elicit by conventional vaccination.

#### Added value of this study

High complexity combinatorial non-glycosylated protein library is promising for identification of protein variants mimicking Env domains recognized by HIV-1 bn-mAb such as VRC01. Such variants are potentially effective immunogens for elicitation of antibodies analogous in epitope recognition to initially used bn-mAb. We identified the three most VRC01-specific protein variants designated VRA017, VRA019, and VRA177. Molecular modelling revealed a shape complementarity between VRA proteins and a part of VRC01 gp120 interacting surface. Vaccination of experimental mice with VRA variants succeed to elicit Env binding- and panel of HIV-1 pseudoviruses-neutralizing antibodies.

#### Implications of all the available evidence

Strategy based on the identification of small proteins mimicking epitopes recognized by selected bn-mAb represents a promising approach for the development of vaccines able to induce antibodies analogous to known protective ones identified in infection-controlling subjects. This approach is not affected by HIV-1 Env low immunogenicity of neutralization epitopes, variability, and unique biochemical and immunological properties of protective epitopes, such as those on HIV-1 Env. Furthermore, such proteins are easy to express with high yield in a prokaryote expression system, are conformationally stable, and are not modified by post-translational modification such as glycosylation which in the HIV-1 Env exhibit substantial micro- and macro-heterogeneity.

response development, currently tested vaccine induce immune response with still limited efficacy and breadth [1,8–11].

We developed a new strategy for elicitation of bn-mAbs based on directed evolution of proteins, and identification and preparation of recombinant protein replicas of epitopes recognized by well-characterized bn-mAbs. These proteins can be used as antigens for construction of a vaccine, inducing production of serum antibodies with specificity and neutralization potential analogous to the used bn-mAb. The strategy utilizes engineered artificial binding proteins identified using a highly complex combinatorial library derived from albumin-binding domain (ABD) scaffold of streptococcal protein G [12], the approach employed to identify several collections of binders raised to a portfolio of important protein targets [13–16].

Herein, we describe an innovative approach employing a highly complex combinatorial library and ribosome display (RD) to identify VRC01 paratope-specific binders as HIV-1 gp120 mimicking candidates. Immunization of mice with recombinant VRA binders confirmed their ability to elicit Env-binding serum antibodies neutralizing panel of Clade B and Clade C pseudotyped HIV-1 viruses. Thus, this strategy

proved to be a promising approach to HIV-1 and other infection-preventing vaccine development (Fig. 1).

## 2. Materials and methods

### 2.1. Construction and production of recombinant gp120

Recombinant multimerizing Clade B gp120 V5- and HIS-Tagged protein were prepared and characterized as previously [17–20] at a stock concentration (1.2 mg/mL) and used for ABD competition experiments.

### 2.2. Antibodies used for preselection, selection and further characterization

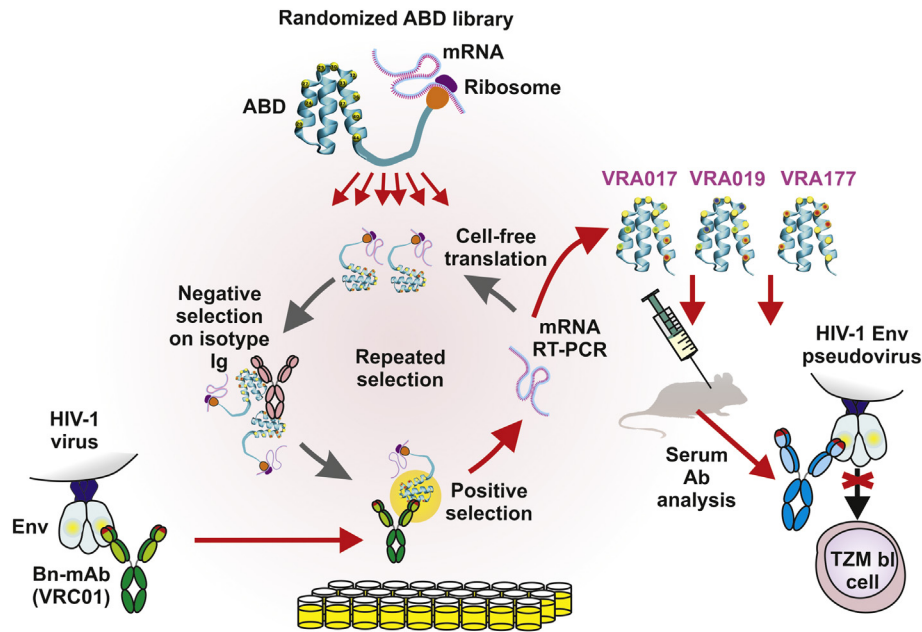
Broadly neutralizing human anti-HIV-1 gp120 monoclonal antibody VRC01 (RRID: AB\_2491019) was obtained from Dr. John Mascola (cat# 12033) [21] through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Fab fragment of VRC01 was prepared using Fab Micro Preparation kit (Pierce, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. 125  $\mu$ L of VRC01 antibody (100  $\mu$ g) was applied onto a column containing equilibrated immobilized papain and incubated for 5 h at 37 °C. The digested antibody was eluted by centrifugation and added onto the column with equilibrated immobilized Protein A. The column was centrifuged to collect Fab fragment. The concentration of purified Fab fragment was measured. VRC01 IgG protein, as well as its Fab, were tested for its activity to bind gp120 when immobilized on Polysorp plate (NUNC, Roskilde, Denmark) in ELISA. Human IgG kappa 1 mg/mL (purified myeloma protein, Sigma-Aldrich, St. Luis, MO) was also tested as a negative control. VRC01 mAb was used for ribosome display as a target protein, stored as 3 mg/mL source stock in PBS (pH 7.2) at –80 °C. Human IgG1 (Sigma-Aldrich) was used as an isotype control for preselection in ribosome display, stored as 1 mg/mL source stock at –20 °C.

### 2.3. ABD library assembly and ribosome display selection

ABD-derived combinatorial library was assembled by PCR [12] and used for *in vitro* translation and further ribosome display selection [14]. Three- and five-round RD selections were performed, 96-well Polysorp plates (NUNC) were coated by VRC01 IgG diluted in coating 100 mM bicarbonate/carbonate solution (pH 9.6) at a concentration according to the adjusted stringency in each round of ribosome display selection procedure: 1st round - 50  $\mu$ g/mL, 2nd round - 25  $\mu$ g/mL, 3rd round - 10  $\mu$ g/mL, 4th round - 5  $\mu$ g/mL and 5th round - 5  $\mu$ g/mL. Preselection procedure was performed in wells coated with human IgG1 kappa antibody (Sigma-Aldrich) at a constant concentration of 25  $\mu$ g/mL in each round. Final cDNA after the third and fifth round of the selection was amplified by PCR and introduced into a pET-28b vector carrying cloned toIA-AviTag sequence [15] and introduced into *E. coli* XL1 blue host cells.

### 2.4. Production of ABD-derived protein variants

Protein variants were produced in the form of fusion recombinant proteins His<sub>6</sub>-VRA-TolA-AVI allowing *in vivo* biotinylation of the binding proteins at C-terminus [15]. Truncated fusion VRA proteins were constructed by replacement of full-length toIA (UniProt accession number: P19934, NCBI Reference Sequence: NC\_000913.3) with its truncated toIS form as a product of PCR with primers toIA-C-end 5'-ATTA GGATCCCCGTCAGGGCCGATATCAATAACTATGC -3' and toIA-AVI\_rev1 5'-TTTCCGCTCAGCTATTCGTGCCATTCGATTTCTGAGCCTCGAAGATG TCGTTTAGCCCCGTTTGAAGTCCAATGCGCG-3'. The fusion VRA protein binders were produced as *in vivo* biotinylated proteins in *E. coli* BL21 (DE3) BirA strain with added 50  $\mu$ M d-biotin (prepared 5 mM solution in 10 mM bicine buffer, pH 8.3) in LB medium containing kanamycin (60  $\mu$ g/mL) and chloramphenicol (30  $\mu$ g/mL). Protein production was induced by 1.5 mM IPTG after the culture reached the density



**Fig. 1.** Principle of elicitation of Env-specific neutralizing serum antibodies using protein binders selected from ABD library. Bn-mAb VRC01 was used as a target for the selection of binders from a combinatorial ABD library with a theoretical complexity  $10^{14}$  variants. The negative selection was used to minimize binders not involved in epitope recognition. Positive selection was performed in 96-well plates with immobilized VRC01 bn-mAb, followed by mRNA isolation, reverse transcription to DNA, and ribosome display selection. After several selection rounds, a library of cDNA variants called VRA binders was introduced into a plasmid vector. Three VRA variants VRA017, VRA019, and VRA177 were identified as the most promising candidates and in the form of fusion proteins, including a truncated VRA0175 version, were used for the immunization of experimental mice followed by an analysis of their ability to elicit HIV-1 Env-specific and HIV-1 pseudovirus-neutralizing serum antibodies.

$OD_{600} = 0.6$ . Cells were harvested 4 h after induction, sonicated in TN buffer (50 mM Tris, 150 mM NaCl, pH 8.0), centrifuged (40,000  $\times$ g, 20 min, 4 °C) and subsequently, bacterial lysates were analyzed or protein was purified on Ni-NTA agarose column.

### 2.5. ELISA for ABD screening

Cell lysates of clones of *E. coli* BL21 BirA host cells producing biotinylated protein variants were prepared using lysozyme solution (PBS buffer, 0.05% Tween, 1% lysozyme, 25 U/mL benzonase, pH 7.4) or using sonicator (Misonix 3000). Polysorp plate (NUNC) was coated with VRC01 IgG1 (5  $\mu$ g/mL) or IgG1 kappa (5  $\mu$ g/mL) in coating buffer at 7 °C overnight. Next day, the plate was washed by PBST solution (PBS buffer containing 0.05% Tween, pH 7.4) and wells were blocked by PBSTB (PBS buffer pH 7.4, containing 0.05% Tween and 1% BSA). The lysate samples (diluted 33 $\times$ ), purified protein variants as well as wild type ABD (ABDwt), serving as a negative control, diluted in PBSTB were applied and their binding was detected using streptavidin Poly-HRP conjugate diluted in PBSTB 1:10,000 (Pierce). The V5-tagged gp120 recombinant protein was diluted in PBSTB and detected by anti-V5 tag – HRP conjugate in PBSTB (1:10,000). Results were visualized by the enzymatic reaction of HRP with OPD substrate (Sigma-Aldrich) in citrate buffer (3.31% sodium citrate tribasic dihydrate, phosphoric acid until pH 5.0), or TMB-Complete 2 substrate (TestLine Clinical Diagnostics s.r.o., Brno, Czech Republic) and the reaction was stopped by 2 M sulphuric acid and absorbance at 492 or 450 nm was measured respectively.

### 2.6. Competition ELISA for ABD

The wells of Polysorp plate (NUNC) were coated with VRC01 IgG antibody or Fab fragment, diluted in coating buffer (5  $\mu$ g/mL) and V5-tagged gp120 was applied as a serially diluted competitor and binding of *in vivo* biotinylated VRA017, VRA019, and VRA177 (as His6-VRA-TolA-AVI fusion protein, 5  $\mu$ g/mL) was detected by streptavidin–HRP conjugate. Alternatively, VRA017 protein was applied as a serially

diluted competitor in PBSTB at the constant concentration of V5-tagged gp120 (1.2  $\mu$ g/mL). Detection of bound gp120 was performed using anti-V5 tag – HRP antibody conjugate. Results were visualized by the enzymatic reaction of HRP with OPD substrate (Sigma-Aldrich) in citrate buffer, or TMB-Complete 2 substrate (TestLine) and the reaction was stopped by 2 M sulphuric acid and absorbance at 492 or 450 nm was measured, respectively.

### 2.7. Sequence analysis of selected variants

Plasmid DNA coding for full-length protein variants was sequenced. Multiple alignments of amino acid sequences were performed using NCBI BLAST (National Center for Biotechnology Information, <https://www.ncbi.nlm.nih.gov/>).

### 2.8. Fluorescence-based thermal-shift assay

Protein samples (0.2 mg/mL) in PBS and 5 $\times$  Sypro Orange dye (Sigma-Aldrich) were mixed in a total volume of 25  $\mu$ L and measured using the real-time PCR Detection System CFX96 Touch (Bio-Rad Laboratories, Hercules, CA) as described previously [14].

### 2.9. Modelling of VRA-VRC01 interactions

The structure of studied ABD-derived VRA017, VRA019, and VRA177 variants was modelled using the MODELLER 9v14 suite of programs [22] based on the ABDwt structure (PDB id 1gjt [23], residues 20–66). Amino acid sequences of the ABD variants were aligned with the Clustal Omega program [24]. The structure of VRC01 broadly neutralizing antibody was obtained from the available crystal structure of the HIV-1 gp120/VRC01 complex (PDB ID 3ngb) [25]. The flexible side chain protein-protein global docking was performed using a local copy of the ClusPro server [26,27], docking the VRA variants (as ligands) to the interacting domains of VRC01, residues 1–113 of chain H and residues 3–107 of chain L from the 3ngb crystal structure (as the receptor).

## 2.10. Immunization of experimental mice

All experiments were performed on 6- to 8-weeks old female BALB/c mice purchased from AnLab (Brno, Czech Republic) under standard housing conditions reported elsewhere [28] according to ARRIVE guidelines [29]. The vaccination experiments were approved by the Ethics Committee of the Faculty of Medicine and Dentistry (Palacky University Olomouc, Czech Republic), and the Ministry of Education, Youth and Sports, Czech Republic (MSMT-15434/2015-7). Preimmune serum samples (130  $\mu$ L per animal) were obtained using tail vein blood sample collection approach. All mice were immunized three times with individual ABD variant. Two independent immunization experiments were performed. The first experiment used VRA017, VRA019, and VRA177 variants and ABDwt for evaluation of immunogenicity and specificity of elicited murine serum antibodies. The second experiment tested VRA017, VRA177, and a truncated version of VRA017 designated as VRA017S with eliminated C-terminal TolS-AVI segment to allow narrower focusing the immune response against VRC01 epitope and ABDwt control. All immunizations were performed by intradermal route with equal doses 20  $\mu$ g of ABD (diluted in 50  $\mu$ L of sterile PBS) per mouse per one immunization, mixed 1:1 (v:v) with Freund adjuvant as detailed before [28].

### 2.11. Env-specific serum antibody determination by ELISA

To determine the reactivity of mice sera with HIV-1 Env, a recombinant trimeric Clade B gp120 MBL [17,19] devoid of all tags [30] was used in ELISA assay [19] with the following modifications: Maxisorp plates (NUNC) were coated with gp120 MBL (50 ng/well) overnight at 4 °C. Plates were washed and blocked with 1% BSA/PBS/Tween20 for 3 h at room temperature. Sera were serially diluted (starting from dilution 1:100) in blocking buffer (in duplicates) and incubated overnight at 4 °C, to identify single dilution corresponding to the linear proportion of titration curves, obtained from the majority of VRA-immunized animal, used for final comparison. Final serum dilution was set to 1:400. To detect the bound antibodies specific to gp120, plates were washed and incubated with rabbit anti-mouse IgG, IgG1, IgG2a, and IgM secondary antibody conjugated with horseradish peroxidase (Sigma-Aldrich) diluted in blocking buffer for 3 h at room temperature. Signal was developed with *O*-phenylenediamine-H<sub>2</sub>O<sub>2</sub> substrate. The reaction was stopped with 1 M sulphuric acid, the absorbance was measured at 492 nm.

### 2.12. Competition of VRC01 with hyperimmune mice sera for gp120 binding by ELISA

Plates were coated as described above for Env-specific serum antibody determination by ELISA. VRC01 serially diluted in blocking buffer (in doublets) was applied with mouse sera diluted 1:400. To detect bound mice antibodies, plates were washed and incubated with rabbit anti-mouse IgG secondary antibody conjugated with horseradish peroxidase diluted in blocking buffer for 3 h at room temperature. Plates were developed and measured as mentioned above.

### 2.13. Virus neutralization assay

Neutralization assay was performed using various pseudoviruses from clade B and clade C produced in HEK293/17 cell line. Cells at 60–90% confluency in 75 cm<sup>2</sup> culture flask were co-transfected using transfection reagent FuGene6 (Promega, Madison, WI). Before transfection, 8  $\mu$ g of plasmid pSG3deltaEnv, 4  $\mu$ g of plasmid encoding Env and 48  $\mu$ L of FuGene6 were mixed with DMEM culture medium in a total volume of 800  $\mu$ L and incubated 30 min at room temperature. Then, the mixture was added to 12 mL of RPMI-1640 in a flask with cells. After 2 days, culture medium with produced pseudoviruses was harvested, aliquoted and stored at –80 °C until used. Neutralization assay was

performed as described previously [31] using TZM-bl cell line stably expressing CD4 receptor, CCR5, and CXCR4 co-receptors and containing genes for luciferase and  $\beta$ -galactosidase under control of HIV-1 long-terminal-repeat promotor (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). Serially diluted serum samples in duplicates were incubated with pseudoviruses at approximately 150,000 RLU in 150  $\mu$ L of DMEM. After 90 min' incubation at 37 °C, 100  $\mu$ L of cells at a density of 10<sup>5</sup> cells/mL was added. The plate was incubated at 37 °C in 5% CO<sub>2</sub> atmosphere for 48 h. Then, 150  $\mu$ L of culture medium was removed and 100  $\mu$ L of lysis buffer containing luciferin (Promega) was added. After 2 min, 100  $\mu$ L of lysed cells were transferred into black 96-well plates and luminescence was measured using HP luminometer.

### 2.14. Statistics

Differences between groups and statistical significance were determined by analysis of variance (ANOVA), Kruskal-Wallis test and Dunn's post-test. All statistical analyses were performed using SPSS v. 21 statistical packages (IBM Corp., Armonk, NY) or GraphPad Prism 5 Software (GraphPad Software Inc., San Diego, CA).

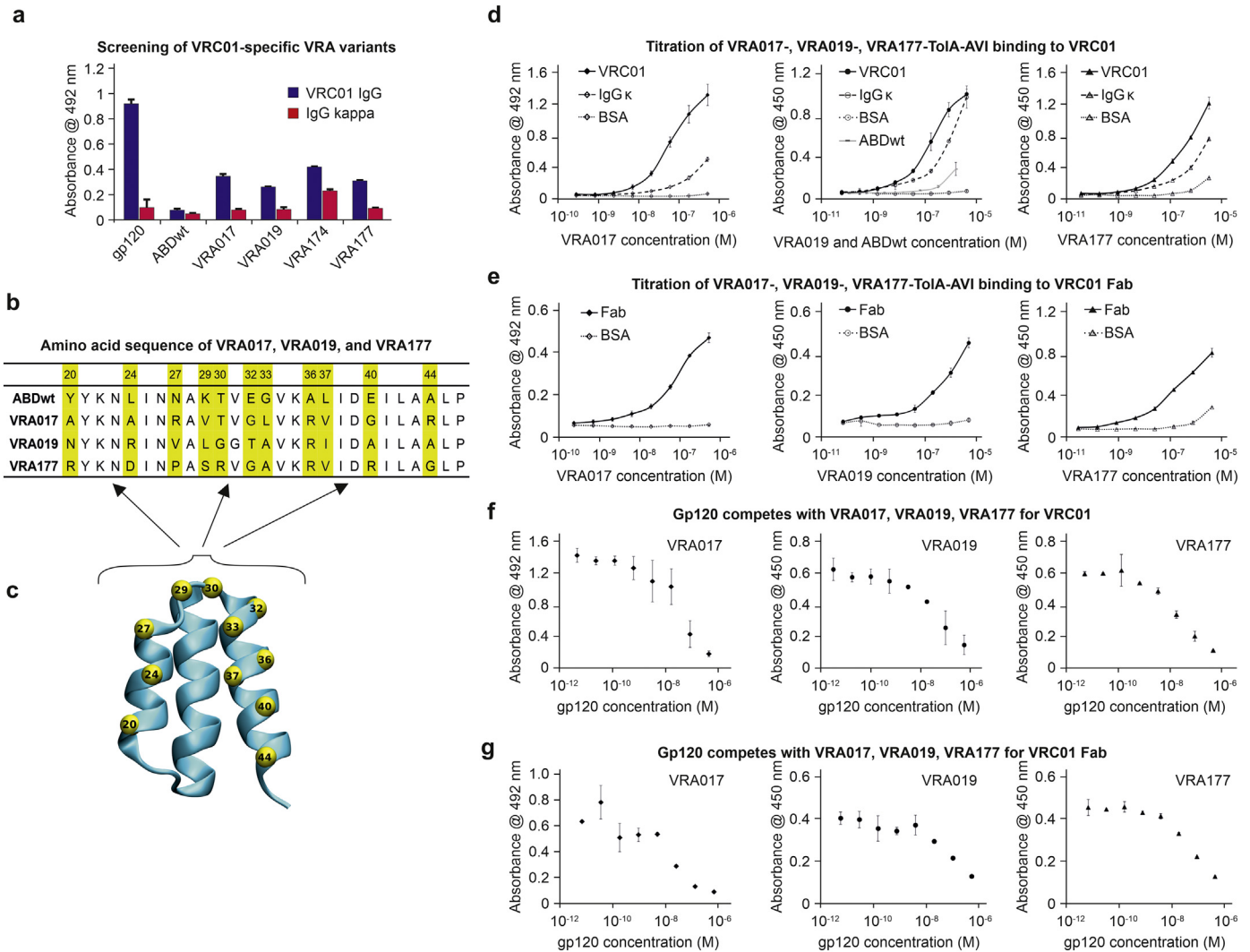
## 3. Results

### 3.1. Generation and identification of VRC01-binding VRA proteins

VRC01 bn-mAb belongs to the most attractive HIV-1 Env-specific antibody due to its high binding affinity to Env glycoprotein and high neutralization breadth. Therefore, we used VRC01 as a target for the selection of protein binders from a highly complex ABD-derived combinatorial library in 5-round ribosome display (RD). Since an increasing stringency during 5-round RD significantly limits the complexity of the acquired protein collection in favour of high-affinity binders, we also performed 3-round RD. Bacterial lysates of almost 800 clones were screened and four VRC01-targeted variants preferentially binding bn-mAb IgG in comparison to IgG-kappa isotype control were identified, and called as VRA proteins. Two of them, VRA017 and VRA019, originate from 5-round RD collection and two, VRA174 and VRA177, resulted from 3-round RD selection (Fig. 2a). As the following experiments revealed a sequence identity of VRA174 and VRA177, only VRA177 variant was used further. VRA proteins were expressed in *Escherichia coli* (*E. coli*) BL21(DE3) BirA as biotinylated 38 kDa fusion proteins (His<sub>6</sub>-ABD-TolA-AVI). Purified proteins of all variants confirmed their specificity for VRC01 IgG and low binding to bovine serum albumin (Fig. 2d,e). Further, we studied the binding of VRA017, VRA019, and VRA177 to Fab fragment of the VRC01 IgG. As presented in Fig. 2e, VRA017, VRA019, and to a lesser extent VRA177 retains the binding preference to VRC01 Fab IgG, supporting expectations that these protein variants might recognize variable regions on the VRC01 target.

### 3.2. VRA017, VRA019, and VRA177 protein binders compete with HIV-1 gp120 for binding to VRC01 IgG

To further test whether VRA017, VRA019, and VRA177 proteins bind to variable domains of the VRC01 IgG, we performed competition ELISA experiments in which recombinant gp120 glycoprotein competed with individual binders for binding to VRC01 IgG or its Fab fragment (Fig. 2f, g). We used two experimental setups. In the first one, increasing concentration of a trimeric form of the V5-tag-gp120 as a competitor was tested with the constant concentration of the biotinylated His<sub>6</sub>-VRA017-TolA-AVI protein. The substantial inhibitory effect was found for the immobilized VRC01 IgG as well as for Fab (Fig. 2f,g). This suggests that VRA017 and similarly VRA019 and VRA177 recognize the binding area on VRC01 as it does the gp120. In addition, the best binder VRA017 when serially diluted, it competed with trimeric gp120 for



**Fig. 2.** Identification of VRA ligands of VRC01 paratope derived from ABD library. **a**, Variants preferentially binding to VRC01 IgG identified by ELISA. Cell lysates of VRA clones were screened for binding to VRC01 IgG and isotype IgG control. The proteins were produced as biotinylated His6-VRA-ToIA-AVI fusions and binding to IgG was visualized by the streptavidin-HRP conjugate. Parental His6-ABDwt-ToIA-AVI biotinylated protein was used as a negative control. V5-tag-gp120 glycoprotein binding to VRC01 antibody was used as a positive control, detected by anti-V5 Ab-HRP conjugate. **b**, Sequence comparison of the VRA binders with ABDwt. Yellow boxes indicate the 11 positions at which the residues of ABD (aa 20–46) were randomized. **c**, ABD scaffold with 11 randomized aa. **d**, Binding of VRA017-, VRA019- and VRA177-ToIA-AVI proteins to VRC01, isotype control IgG kappa and BSA in ELISA. Binding of ABDwt-ToIA-AVI as an original default protein scaffold to VRC01 is shown in comparison to VRA019-ToIA-AVI. **e**, Binding of VRA017-, VRA019- and VRA177-ToIA-AVI proteins to VRC01/Fab fragment and BSA in ELISA. **f**, The VRA proteins compete with gp120 for binding to VRC01. Increasing concentration of gp120 inhibits binding of the VRA017-, VRA019-, and VRA177-ToIA-AVI proteins (at a constant concentration around  $2.0 \times 10^{-7}$  M) to VRC01 IgG and **g**, VRC01 Fab. All the VRA and ABDwt proteins were biotinylated and detected by the streptavidin-HRP conjugate. Each experiment is shown as the mean value of triplicates (duplicates in cases of the VRA019 and VRA177 competition experiments) with standard deviation (SD).

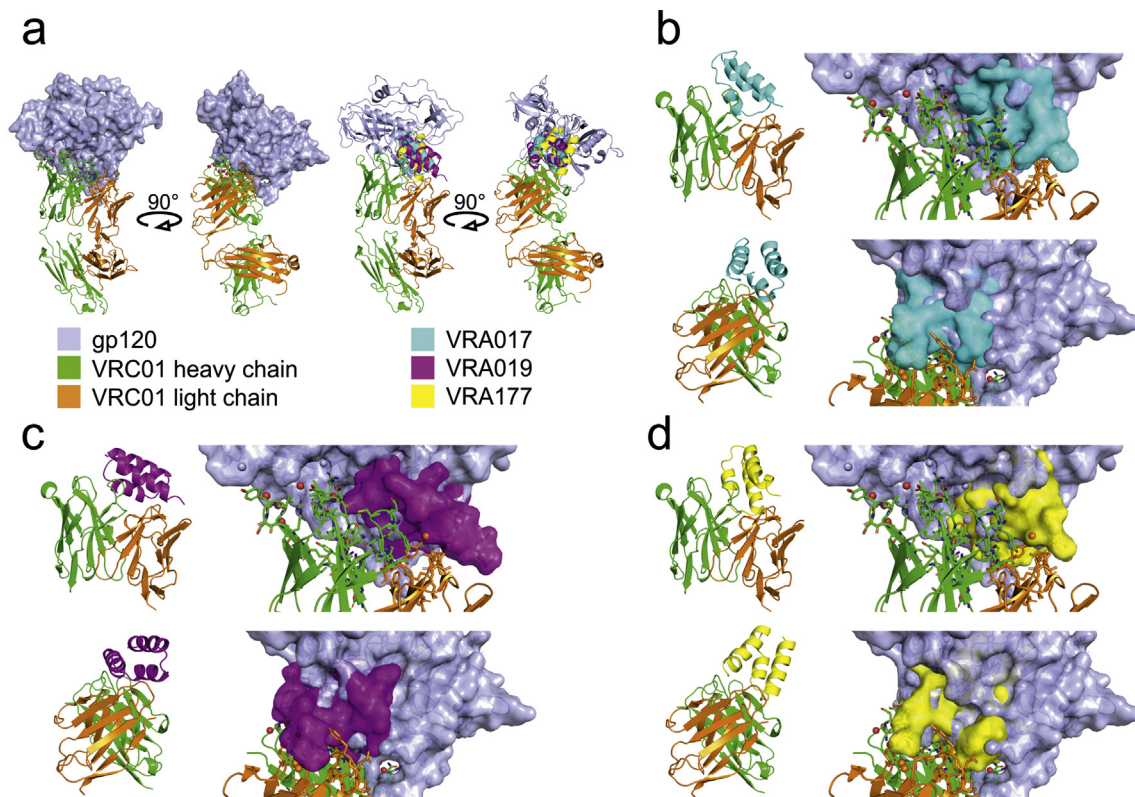
binding to immobilized VRC01 antibody target. We found that increasing concentration of VRA017 protein suppresses the binding of recombinant gp120 to VRC01 IgG as well as to Fab (Fig. S1). Thus, these results predict that VRA017 is our best-mimicking candidate for VRC01 bn-mAb.

### 3.3. Truncated VRA variants maintain the ABD thermal stability

To analyze the thermal stability of the VRA proteins, we used fluorescence-based thermal shift assay performed with truncated His<sub>6</sub>-VRA-ToIA-AVI proteins. Temperature melting point ( $T_m$ ) for these variants was found to be 50 °C (VRA017 and VRA019) and 58.5 °C (VRA177) as shown in Fig. S3. In our previous studies [12,14,15] we provided  $T_m$  58 °C for parental ABD-wild type protein measured in the form of ToIA fusion variant and here we present  $T_m$  55.5 °C for parental non-mutated ABD-wild type protein in fusion with ToIA helper protein (ABDwtS). We conclude that randomization of the master ABD scaffold does not disrupt a basic three-helix structure and retains the stability.

### 3.4. Modelling of VRA-VRC01 interactions

To evaluate whether selected VRA protein variants can mimic epitopes on the HIV-1 Env glycoprotein, crystal structure of VRC01 antibody in complex with HIV-1 gp120 (PDB ID 3ngb) was used. As shown in Fig. 3a, both the heavy and light IgG chains contribute to the gp120 recognition, the interacting residues are shown as sticks. Interestingly, the interaction of gp120 with VRC01 is mediated by a higher number of contacts with IgG heavy chain (Fig. 3a) where an interface is formed not only by the variable loops but also through the heavy chain  $\beta$ -sheet domain. The binding is achieved by a relatively small number of directly interacting residues involved in hydrogen bonds, salt bridges and hydrophobic contacts. Especially in the region of variable loops of the antibody, a significant number of gp120-antibody contacts include a water molecule bridging the protein residues (water shown as spheres). For the antibody, these interface water molecules contribute to the distribution of charges and shape of the antigen and can be considered as an integral part of the recognized gp120 binding



**Fig. 3.** Modelling of VRA-VRC01 interactions by docking. a, A crystal structure of the HIV-1 gp120/VRC01 complex (PDB ID 3ngb) and cartoon representation of VRA proteins (right) binding to VRC01 antibody demonstrated for VRA017 (cyan), VRA019 (magenta) and VRA177 (yellow), VRC01 antibody heavy chain is shown in green colour, light chain in orange and gp120 glycoprotein in light blue. b, Front (upper) and side (bottom) view to the binding mode of VRA017 protein on the variable domains of VRC01 IgG $\kappa$  are presented (left), a detailed view on interaction surfaces of VRA017 (cyan) and gp120 with VRC01 demonstrates the similarity of both surfaces (right). c, Result of docking for VRA019. d, Docking of VRA177 variant. Pymol session is available on [http://www.ibt.cas.cz/vyzkum/laboratore/laborator-inzenyrstvi-vazebynych-proteinu/VRA\\_gp120\\_mimicking.pse.zip](http://www.ibt.cas.cz/vyzkum/laboratore/laborator-inzenyrstvi-vazebynych-proteinu/VRA_gp120_mimicking.pse.zip)

surface (Fig. 3a). The N276-linked glycan (shown as lime yellow surface) was experimentally identified as crucial for gp120 recognition by VRC01. The sugar moiety interacts with variable loops of both heavy and light IgG chains by direct as well as water-mediated contacts, enhancing the binding affinity and specificity. A shape complementarity between VRA proteins and a part of VRC01 gp120 interacting surface is a key property of such proteins. A VRA variant expected to elicit antibodies recognizing the gp120 should mimic the gp120 protein including its hydration layer and N276-linked glycan moiety.

To gain a rational background for a mimicking potential of the particular VRA binding variants, we used the known crystal structure of the HIV-1 gp120/VRC01 complex (PDB ID 3ngb) for docking. As shown in Fig. 3a, the binding of VRA017, VRA019, and VRA177 to the VRC01 shares a similar region of the VRC01 interface and differs mostly in details at the level of residues involved in the interaction (see also supplementary PyMOL session for all presented cases). In the case of the VRA017 variant (Fig. 3b), the shape of gp120 (including the hydration layer and N276-linked glycan) and VRA017 interfaces is similar, however, there are two important differences. Two Arginine residues are protruding from the VRA017 surface. The Arg27 interacts directly with two Aspartate residues on top of heavy chain loops and contributes to a chain of salt bridges orienting the loops into a nearly identical position as in the gp120/VRC01 crystal structure. The second (Arg44) residue is involved in a set of interactions effectively substituting the N276-linked glycan of the gp120, including its coordinated water molecule. It orients and stabilizes loop residues at the heavy/light chain border by van der Waals interaction, and forms an additional stabilizing salt bridge with Asp100C originally involved in a water bridge mediated interaction. The gp120 N276-linked glycan interactions are further

substituted by the C-terminal part of the third helix of the VRA017 protein, while the second helix interacts with a set of hydrophobic residues. The cavity formed between VRA017 helices two and three stabilizes interaction and orientation of light chain by interaction with two light chain loop Tyrosine residues. This combined effect offers a rational background to the VRA017 potential for mimicking the gp120 binding interface as recognized by the VRC01 antibody.

In the VRA019 (Fig. 3c), there is a similar situation as in VRA017, but the N276-linked glycan with water is surprisingly substituted by Alanine (Ala40) residue involved in a vdW interaction with neighbouring heavy chain Tyrosine residue. The binding pocket for light chain loop Tyrosine between the second and third VRA helices is preserved. The difference is that the position occupied originally by Arg27 in VRA017 is substituted by Leu29, resulting in a slightly different shifted position of VRA019 with respect to the VRA017. In this way, the Leucine stabilizes the binding by a vdW interaction with the side chain of the heavy chain Lysine residue. The interaction is further stabilized by a hydrogen bond of the terminal ammonium group of the Lysine to the backbone of the VRA019. The specificity is dictated by Arg36 interaction with one of the heavy chain loop Aspartates, forming a salt bridge. In summary, VRA019 binding mode is very similar to that of VRA017. Although the binding details differ, the shape of the interacting surface supports the interactions.

In contrast to both above mentioned VRA protein variants, the VRA177 (Fig. 3d) differs by the orientation of helices, yet the shape of the interacting surface and distribution of charges might simulate the gp120 surface even better. The charged residues of the heavy chain interact with VRA177 Arg40 and Asp24. The Arg40 residue plays an equivalent role as the Lysine on gp120. The Asp24 forms a new salt bridge with Lysine residue of the antibody improving the original hydrogen

bond to gp120 backbone carbonyl. This results in the best mimicking potential of the VRA177 to the gp120. The N276-linked glycan with coordinated water is mimicked by Leu45, similarly to the Ala40 residue of VRA019. The N276-glycan substitution by C-terminus of the third helix of the VRA177 and the cavity for Tyrosine residues between the second and third helix stabilizing the interaction and orientation of the light chain is shared among all VRA variants.

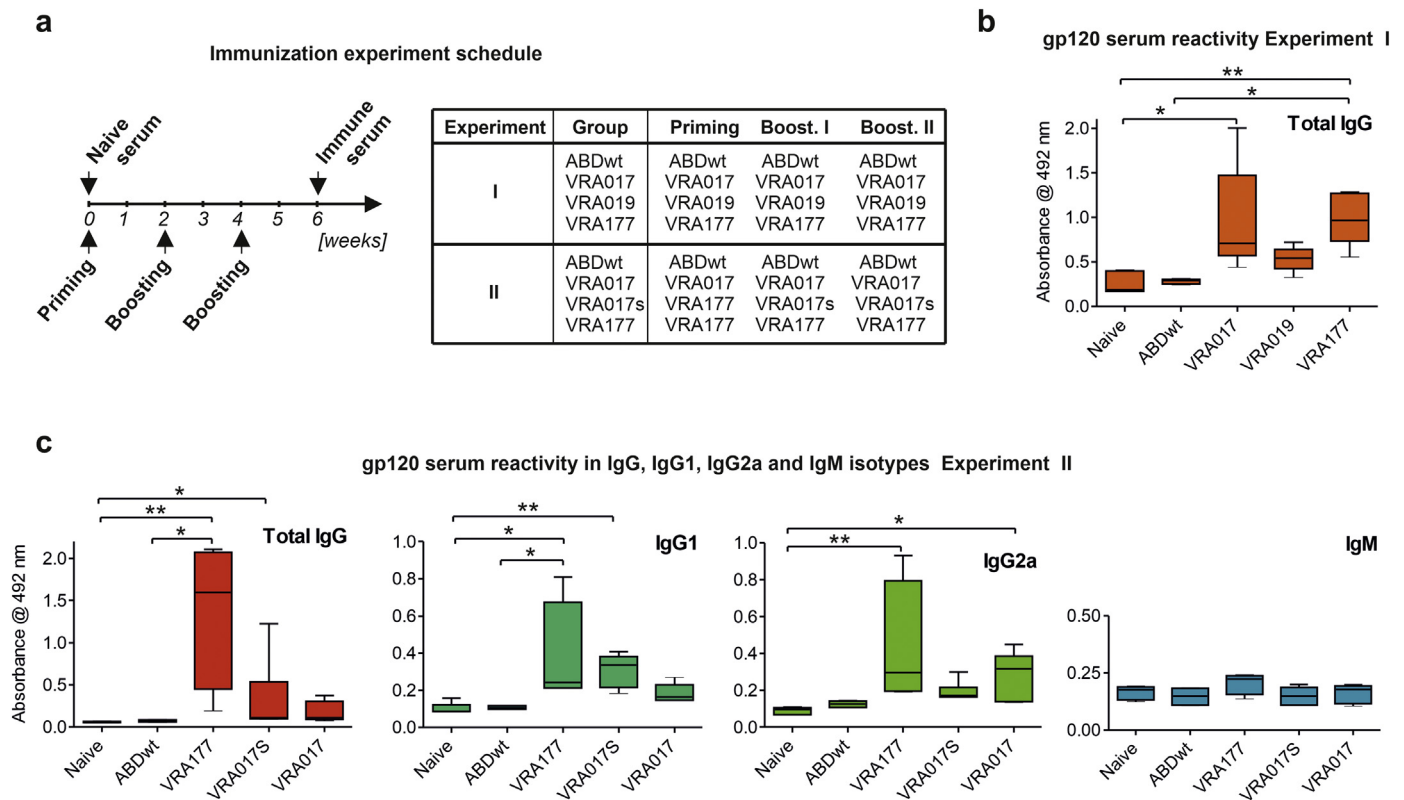
In summary, appropriately positioned charged residues of VRA proteins interact directly with two Aspartate residues on top of heavy chain loops and contribute to a chain of salt bridges orienting the loops into a nearly identical position as in the gp120/VRC01 crystal structure. In addition, Arg, Ala, and Leu residues (VRA017, 019, and 177, respectively) are involved in a set of interactions effectively substituting the N276-linked glycan of the gp120, including its coordinated water molecule, which orients and stabilizes loop residues at the heavy/light chain border by van der Waals interaction. The gp120 N276-linked glycan interactions are further substituted by the C-terminal part of the third helix of the VRA proteins, while the second helix interacts with a set of hydrophobic residues. This combined effect offers a rational background to the VRA protein potential for mimicking the gp120 binding interface as recognized by the VRC01 antibody.

3.5. VRA017, VRA019, and VRA177 induce serum antibodies recognizing HIV-1 Env glycoproteins

Following VRA isolation, *in vitro* and *in silico* characterization, three ABD candidates – VRA017, VRA019, and VRA177 – were expressed and purified as recombinant proteins and used for immunization of experimental mice by i.d. route with Freund's adjuvant. Two independent immunization experiments were performed both by three consecutive

antigen injections, according to the schedule presented in Fig. 4a. In the first experiments we tested VRA017, VRA019, and VRA177 (Fig. 4b) whereas the following experiment tested VRA017, VRA177, and a truncated version of the VRA017 designated VRA017s (Figs. S2, 4c) with a partial elimination of C-terminal ToLA-AVI sequences, thus allowing to narrowly focus of the immune response against VRC01 epitope. VRA017s was expressed and purified identically to the VRA017. The initial experiment confirmed the ability of all identified VRA variants (VRA017, VRA019, and VRA177) to elicit HIV-1 Env-specific serum antibodies as confirmed by ELISA (Fig. 4b) with recombinant tag-free multimerizing CladeB gp120 MBL antigen characterized extensively earlier [17–20]. The most dominant was Env-specific response elicited by VRA017 variant, followed by VRA177 and VRA019. In contrast, wild-type ABD precursor did not elicit detectable Env-specific serum response when compared to preimmune sera from naive mice.

The following immunization experiment was performed only with the most promising ABD variants VRA017 and VRA177, and VRA017s (Fig. 4c). Immunization confirmed sufficient immunogenicity of both VRA017 and VRA177 fusion proteins and short VRA017s variant when used for boosting after VRA177 priming (Fig. 4c). We compared serum antibody response in total IgG isotype, IgG1, IgG2a, and IgM isotypes. In contrast to a very minute response to VRA variant in IgM isotype, IgG response both in IgG1 and IgG2a was well demonstrated (Fig. 4c) including that induced by boosting with the short VRA017s protein. To further confirm the gp120 specificity of sera from immunized mice, we performed a competition assay where the binding of sera was competed with a serial dilution of VRC01 in ELISA (Fig. S5). VRC01 at concentration 4 µg/mL completely inhibited all hyperimmune sera reactivity.



**Fig. 4.** Sera from mice immunized with VRA017, VRA177, VRA019, and VRA017s specifically recognized HIV-1 Env. a, Mice were immunized in two independent experiments by the administration of three doses of individual ABD variant including wild-type (ABDwt) and VRC01-binding variants VRA017, VRA019, VRA177, and the truncated version VRA017s. Each individual group consisted of five animals. Following immunization, sera were collected and Clade B multimerized recombinant Env variants (gp120 MBL), without any purification and identification tags, was used for testing of Env-specific serum antibody titres of IgM, all IgG subclasses (IgG<sub>tot</sub>), IgG1, and IgG2a by ELISA. b, Env-specific serum IgG<sub>tot</sub> from experiment I. c, Env-specific serum IgG<sub>tot</sub>, IgG1, IgG2a, and IgM from experiment II. Statistical comparison was performed by ANOVA Kruskal-Wallis test with Dunn's post-test (\* P < 0.05, \*\* P < 0.01).

**Table 1**  
Neutralization activity of sera from mice immunized in Experiment II against selected HIV-1 pseudoviruses.

		Reciprocal serum dilution resulted in 50% virus neutralization					
	Pseudovirus	Tier	Naive	ABDwt	VRA177	VRA177 + VRA017S + VRA017S	VRA017
Clade B	pWITO	2	<30	<30	<30	<30	<30
	pREJO	2	<30	<30	31	61	<30
	AC10.0	2	<30	<30	<30	48	<30
	TRO	2	<30	<30	<30	37	<30
	SC 422664	2	<30	<30	57	71	34
	pRHPA	2	<30	<30	84	90	40
	PVO	3	<30	<30	<30	<30	<30
Clade C	Du422	2	<30	<30	<30	<30	<30
	Du172	2	<30	<30	<30	<30	<30
	Du156	2	<30	<30	34	65	<30
	ZM53M	2	<30	<30	32	87	<30
	ZM214	2	<30	<30	<30	32	<30
	CAP210.2	2	<30	<30	<30	<30	<30

Results are expressed as the reciprocal serum dilution which resulted in 50% virus neutralization. Virus-free cells luminescence value reached approximately 15,000–20,000 RLU. Cells with optimized virus concentration exhibit luminescence value of approximately 150,000 RLU.

### 3.6. VRA017S elicits serum antibodies able to neutralize part of the panel of Clade B and Clade C pseudoviruses

Subsequently, we tested the biological activity of serum antibodies using neutralization assay with the available set of Clade B and Clade C pseudoviruses. Due to the limited volume of post boost sera, we tested thirteen pseudoviruses in two independent experiments and expressed values as reciprocal serum dilution resulting in 50% virus neutralization (Table 1). Titration curves are provided for pseudoviruses exhibiting 50% neutralization titers higher than 30 (Fig. S6). Interestingly, in contrast to VRA177 and VRA017 variants which induced well-detected serum binding antibodies and poor neutralization, sera from mice primed with VRA177 and twice boosted with VRA017S exhibited well-detected neutralization activity to eight of twelve tested Tier 2 HIV-1 pseudoviruses. One HIV-1 pseudovirus of Tier 3 was not neutralized by any tested sera. Repeatedly VRA177- and VRA017-immunized mice sera neutralized only five and two of total thirteen tested pseudoviruses respectively. Thus, shortening of VRA variant VRA017 was effective in the elicitation of neutralization antibodies. This indicates, in agreement with molecular modelling, that shortening of VRA017 further improved the mimicking capacity of the identified VRA017 variant.

## 4. Discussion

HIV-1 attachment to, and entry into, a host cell requires interaction between viral envelope glycoprotein (Env) and the host cell CD4 receptor and CCR5 or CXCR4 co-receptor [32]. Antibodies neutralizing interaction of HIV-1 Env with cell receptors may limit viraemia, as shown for elite neutralizers whose antibodies exhibit strong and exceptionally broad neutralizing activity *in vitro*. [4]. The HIV-1 Env, a trimer of gp120/gp41, is, therefore, a relevant target for vaccine design [1,33].

Currently, there is no available robust and versatile system inducing a strong epitope-specific antibody-mediated immune response, mimicking that identified in elite neutralizers. Until now, the most effective HIV-1 vaccination trial RV144 lowered the rate of HIV infection only modestly, by 31.2% compared with placebo ( $p = 0.04$ ) [34] and further development of an effective HIV-1 vaccine have had limited success [1]. Several factors act against development of bn-Ab after the vaccine administration, including a) enormous Env variability, metastability, and extensive *N*-glycosylation hampering identification and preparation of optimal and universal Env, and b) uncommon properties of identified

HIV-1 bn-mAbs including a long HCDR3, extraordinary frequencies of V(D)J mutations, and/or poly- or autoreactivity [7].

Some limitations have been solved by designing structurally modified recombinant soluble Env variants such as gp120 with multimerizing partner MBL [18,19], gp140 trimers as cleaved stabilized SOSIP [35,36], uncleaved NFL [37], uncleaved stabilized UFO [38], or UFO-BG [11] and single-chain gp140 (sc-gp140) [39], all produced as secreted glycoprotein or prepared as glycoproteins exposed on virus-like particles or liposomes. Another strategy was focused on the preparation of Env mutants recognized by a germline B cell receptor for stimulation of specific B cells able to somatically hypermutate their Ig gene, thus generating potentially broadly neutralizing antibodies with a high binding affinity [40–44]. On the other hand, some questions have not yet been completely answered, including the selection of Env variant able to induce broadly neutralizing antibodies [1,7,33,45,46].

Successful identification of ABD-derived VRC01 binders is the outcome of an experimental approach and the modelling helps to uncover molecular mechanisms governing the experimentally observed mimicking function. The docking suggested that VRA interactions are mediated predominantly by the randomized residues of helices two and three, while residues of non-mutated helix one did not contribute to the interaction. The docking based on the published crystal structure of VRC01 in complex with an HIV-1 gp120 core [25] suggested that the VRA and gp120 share similar binding regions at the VRC01 surface and that the shape complementarity and distribution of charges between the interacting surfaces of VRA/VRC01 and gp120/VRC01 form a mimicking potential of the VRA binders.

Three consecutive immunizations of experimental mice with VRC01-specific VRA binders elicited both binding and HIV-1 Clade B and C pseudoviruses neutralizing antibodies. These results indicate that identified VRA variants such as HIV-1 Env evolutionary unrelated proteins are effective as potential antigens for the development of HIV-1 vaccine. This observation offers a potential alternative to studies aiming to elicit HIV-1 CD4bs-specific bn-Ab by immunization with structurally modified HIV-1 Env antigen either to target the germline BcR in wild-type mice or in transgenic mice with VRC01 IgH or IgL precursor genes integrated into the corresponding mouse Ig loci [41,47–49]. Furthermore, the majority of these studies exhibited very limited HIV-1 antibodies with neutralizing activities being induced [41,47,48]. In addition, some elicited VRC01-like antibodies remained unable to neutralize wild-type HIV-1 strains with glycan residues adjacent to the CD4bs [49]. Therefore, the VRA antigen strategy could provide a viable alternative to the vaccination approaches based on the modification of a native HIV-1 Env antigen.

Although the levels of HIV-1 Env-specific serum antibodies elicited by immunization with VRA177 followed by two VRA017S boosters were lower than those after three VRA177 immunizations, sera elicited by VRA177-VRA017S-VRA017S exhibited broader and stronger neutralizing activity against Clade B and C Tier 2 pseudoviruses, which indicates a higher proportion of neutralizing antibodies in this particular group of mice. Moderate non-specific neutralization was detected for some pseudoviruses when sera from naive and ABDwt-immunized mice were tested at dilution 1:30 (Fig. S6). Nevertheless, measured neutralizations were always weaker in comparison to sera from VRA-immunized mice. Non-specific weak neutralization could be further analyzed using Murine leukemia virus assay [50], but due to limited volume of murine sera we preferred to perform HIV-1 pseudoviruses neutralization controls as mentioned above. In the presented work we tested pools of Clade B and C pseudoviruses available from NIH AIDS Reagent Program. Phylogenetic tree analysis (Fig. S7) indicated that selected Env sequences cover two of four major Clade B clusters and three of four dominant Clade C clusters. To further confirm the ability of VRA immunogens to elicit neutralization breadth and potency, experiments in other animal models (such as Guinea pigs and rabbits) should be performed, because mice are known for their poor potency to develop robust neutralizing antibody responses to HIV-1 [51]. This is, at



least partially, due to shorter heavy-chain CDRH3 lengths in comparison to humans, macaques, or rabbit, which compromise them from neutralization of HIV-1 Env typical for its extensive glycan shield [52,53]. Indeed, we initiated experiments with best VRA antigens and their combination VRA177-VRA017S-VRA017S using experimental rabbits to address this issue and to characterize VRA immunogens in more details. This will allow to perform more detailed analyses of VRA specificities including comparison of VRA interaction with VRC01 class antibodies (such as 3BNC117, 12A12, N6) [54,55], with CD4bs antibodies (such as b12, 8ANC131, CH103) [55,56], or to test more diverse and larger panel of HIV-1 pseudoviruses.

There are several ways how to enhance immunogenicity and neutralization titres of sera after immunization with identified VRA ligands. This includes modifications in dosage, application of adjuvant, a fusion of VRA with immunomodulating protein and corpuscular formulation of antigens which could enhance vaccine efficacy as we and others have confirmed for HIV-1 and other antigens [19,48,57].

Collectively, the approach described herein can significantly contribute to the elimination of current obstacles in vaccine development, allowing to elicit antibody response in quality similar to that identified in elite HIV-1 neutralizers, but without the need of a long term exposition of vaccinee to several immunogens with the hope to induce broadly neutralizing serum antibodies against conserved neutralizing epitopes identified in naturally infected subjects. Selection of antigen mimetics recognized by well-characterized bn-mAb such as VRC01 could lead to the identification of protein variants able to induce antibodies of the same specificity as an original antibody with a well detectable neutralization activity. Thus, within the field of reverse vaccinology, this approach represents a viable route for the development of improved vaccination strategies preventing infections such as HIV-1.

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### Author contributions

M.R., P.M. and J.T. designed the study. M.K. and M.M. performed the selection and characterization of VRA variants. J.C. performed *in silico* modelling by docking. H.P. performed VRA stability analyses. V.L. constructed truncated versions of VRA fusion proteins. P.K. and L.C. performed the immunological analyses. J.M., P.K. and M.R. performed immunization experiments. L.B. and L.R.K. engineered and isolated recombinant Env proteins and isolated plasmids for pseudoviruses preparation. M.R., P.M. and J.T. wrote the manuscript with input from the other authors. M.R. and P.M. supervised the project.

### Declaration of Competing Interest

Nothing to declare.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.07.015>.

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