HIV-1 vaccine design: Learning from natural infection
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Chapter 9

An HIV elite neutralizer as a blueprint for the induction of neutralizing antibodies


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Abstract

The induction of broadly HIV-1 neutralizing antibodies (bNAb)s by vaccination is challenging, but HIV-1 infected individuals that develop such bNAb)s might serve as examples how to achieve this. We used BG505 SOSIP.664 gp140 trimers to isolate monoclonal antibodies from an HIV-1 infected elite neutralizer. One Ab family showed potent neutralization with Ab ACS202 having a breadth of 45%. ACS202 is trimer-specific, cleavage-dependent, and glycan-dependent, and binds to a novel epitope at the gp120-gp41 interface that includes the fusion peptide and the glycan at position 88. Using the early viruses isolated from the same elite neutralizer we designed a native-like Env protein, termed AMC011 SOSIP, which elicited NAb responses against the autologous Tier-2 virus as well as against some heterologous Tier-2 viruses in rabbits. The sera from AMC011 SOSIP immunized animals cross-competed with ACS202, suggesting that overlapping gp120-gp41 interface epitopes were targeted and illustrating that elite neutralizers might serve as blueprints for HIV-1 vaccine design.
Introduction

An HIV-1 vaccine should ideally elicit broadly reactive neutralizing antibodies (bNAbs) that can protect against acquisition of a wide variety of circulating virus. The isolation of such Abs from HIV-1 infected individuals has revealed that the human immune system is capable of eliciting them and their protective effect has been confirmed in macaque challenge studies [1-7]. Approximately one-third of HIV-1 infected individuals develop NAb s against various heterologous viruses from different subtypes [8-13], and about one percent develops unusually potent bNAb responses against the majority of HIV-1 subtypes. These individuals are termed “elite neutralizers” [12, 14] and they exemplify that the human B cell repertoire is in principle able to generate bNAbs against the envelope glycoprotein (Env) on the surface of virions. Understanding the target epitopes of bNAb responses in elite neutralizers should guide the design of Env vaccine immunogens.

bNAb epitopes are located within the conserved domains on the surface of the Env trimer, such as the CD4 binding site, conserved regions in the V1V2 and V3, the gp120-gp41 interface, the membrane proximal external region (MPER) of gp41 and outer domain glycans (OD-glycans) (reviewed in [15]). The fact that the majority of primary HIV-1 variants are neutralized by one or more of the currently known bNAbs implies that their epitopes are accessible on most circulating viruses. However, to this date, no immunogen has been developed that can elicit bNAbs needed for protection. Restricted epitope accessibility, because of the glycan shield and the high degree of variability in Env, are among the reasons to explain why eliciting protective Abs has been so difficult [16-21]. During natural infection bNAb development usually takes 2-3 years and requires high levels of affinity maturation. Furthermore, bNAbs often have unusual characteristics such as longer heavy chain Complementarity Determining Region 3 (CDRH3) loops and higher polyreactivity compared to Abs against other viral infections [22-24]. These features underline the importance of understanding the development of bNAbs in natural infection in order to mimic this with vaccine immunogens.

Soluble antigenic mimics of the native HIV-1 Env represent suitable starting points for immunogen design [25, 26]. Such proteins, termed SOSIP proteins, can be made by introducing a disulfide bond between the gp120 and gp41 [27], supplemented with a protein stabilizing mutation in gp41 (I559P; IP; [28]), and a truncation at position 664 to prevent MPER aggregation (.664; [29, 30]). SOSIP proteins derived from multiple virus isolates from different HIV-1 subtypes have been described [25, 31-37]. These proteins closely resemble the native viral spike, presenting multiple epitopes for bNAbs, but usually not non-bNAbs. Immunization studies in rabbits and macaques have shown that SOSIP proteins are superior to other subunit protein immunogens in eliciting NAb responses against the autologous neutralization-resistant (Tier-2) viruses [37, 38]. However, no bNAbs were elicited indicating that improvements are necessary to generate neutralization breadth. The early Env sequences from elite neutralizers could be exploited for native-like
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Protein vaccine design to mimic the bNAb development in these individuals. Here, we isolated a novel bNAb from an elite neutralizer, ACS202, targeting a new epitope on the gp120-gp41 interface of Env. ACS202 is trimer-specific, cleavage-dependent and requires the glycan at position 88 for neutralization, as well as residues 83-87 and 229 in gp120 and residues 516-520 in the fusion peptide of gp41. Using early viruses isolated from the same elite neutralizer we designed a SOSIP protein, which closely resembles the structure and antigenicity of the native viral spike. Remarkably, rabbits immunized with this protein elicited NAb responses against the autologous Tier-2 virus, as well as sporadic low titer neutralization against heterologous Tier-2 viruses. Furthermore, the rabbit sera competed with ACS202 for protein-binding, suggesting that the rabbit responses targeted gp120-gp41 interface epitopes that overlapped with that of ACS202. These findings show that elite neutralizers can serve as blueprints for the design of vaccine immunogens capable of eliciting protective Ab responses.

Methods

Human specimens
Peripheral blood mononuclear cells (PBMCs) were obtained from donor D12950, an HIV-1 infected male participant from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) [39] [van den Kerkhof et al. manuscript in preparation]. The ACS are conducted in accordance with the ethical principles set out in the declaration of Helsinki, and written consent was obtained prior to data collection. The study was approved by the Academic Medical Center’s Institutional Medical Ethics Committee. Individual D12950 was infected with HIV-1 subtype B via injecting drug use (IDU). He entered the ACS HIV-1 negative and seroconverted during active follow-up, and was initially described in studies that investigated the number of ACS individuals with broad neutralization [Van den Kerkhof et al. manuscript in preparation], and the longitudinal development of bNAbs in elite neutralizers ([39]; individual IDU2 in that study). Individual D12950 was under observation for >20 years. He had declining CD4+ T-cell numbers (average 390 (range 230-570) cells/μl) and detectable viral loads (average log4.4) until he started to receive HAART (at ~9 years post-seroconversion; post-SC), but no clinical signs of AIDS (Supplementary Fig. 1A). In addition, this individual had no protective HLA-type and was homozygous for the Δ32 deletion in the CCR5 gene. Serum and peripheral blood mononuclear cell (PBMC) samples were taken approximately every 4 months. The Env sequences used here were derived from clonal virus isolates from 8 months post-SC, while mAbs were isolated from a sample taken at 40 months post-SC (Supplementary Fig. 1B).
**Virus isolation from PBMCs**

PBMC samples from individual D12950 were collected at month 8 post-SC. Single clonal virus variants were isolated from selected PBMCs by direct limiting dilution of the cells. Cells were co-cultivated with PHA-stimulated PBMCs from ten healthy HIV-1 uninfected donors, as described previously [40, 41]. To prevent sequence changes during *in vitro* culture, the number of passages in PBMCs was kept to a minimum. An earlier study showed that the quasispecies of clonal viral variants isolated from PBMCs are highly similar to sequences from viral RNA in plasma samples from the same individual [42]. We were able to generate 3 clonal virus variants from the 8 month post-SC sample. Proviral env genes from PBMCs that were infected in vitro with a single clonal HIV-1 variant were PCR-amplified and sequenced [43-45]. Nucleotide sequences were aligned using ClustalW in the software package of BioEdit [46], and edited manually, excluding contamination.

**AMC011 SOSIP protein design**

The *env* genes from the 3 clonal viral isolates (D12950.8m.2D6, D12950.8m.2D7 and D12950.8m.2G9) isolated 8 months post-SC were used to obtain a consensus sequence, using a cut-off ≥60% for an amino acid at a specific position. We used early sequences for the construction of the autologous protein because contemporaneous viruses usually have escaped from the autologous NAbs and their Env proteins do not bind the contemporaneous NAbs anymore. Subsequently, the consensus sequence was used to generate a soluble SOSIP protein termed AMC011 SOSIP [25, 37]. In short, we introduced the A501C and T605C (HXB2 numbering system) substitutions, the protein-stabilizing mutations I559P, L543Q and Q567K, the hexa-arginine furin cleavage site at the C-terminus of gp120 to enhance cleavage, a tissue plasminogen activator (tPA) signal peptide replacing the natural Env signal peptide to improve secretion and a stop codon at position 664 to prevent aggregation [25, 27-31, 34, 37, 47]. The resulting gp140 SOSIP protein was designated AMC011, version 3.1 (AMC011 SOSIP.v3.1). During the course of this study the SOSIP design was further improved by the introduction of mutations E64K and A316W or H66R and A316W, resulting in AMC011 SOSIP.v4.1 or SOSIP.v4.2 proteins, respectively [37] and the introduction of a second disulfide bridge between gp120 and gp41 between residues A73C and A561C, resulting in AMC011 SOSIP.v5.2 [Torrents de la Peña et al. manuscript in preparation]. Consistent with previous results [37][Torrents de la Peña et al. manuscript in preparation], we found that the AMC011 SOSIP.v4.2 and SOSIP.v5.2 were superior to the AMC011 SOSIP.v3.1 protein (data not shown) and therefore we only describe the former two proteins here. For some analyses we introduced a D7324 epitope-tag or a His-tag at the C-terminus of gp41ECTO [25], referred to as SOSIP-D7324 and SOSIP-His, respectively.
Recombinant protein production
For this study we used untagged, D7324-tagged, Avi-tagged, or His-tagged BG505 SOSIP.664 gp140 and AMCO11 SOSIP.v4.2 and SOSIP.v5.2. In addition, we used Avi-tagged monomeric gp120 from isolates 94UG103, MGRM-C026 and BG505 (all with an L111A substitution to prevent dimerization [48], as well as mutant BG505 gp120 proteins. Recombinant Env proteins were expressed in HEK293F (Invitrogen) as described previously [25, 33]. Briefly, HEK293F cells were maintained in FreeStyle medium (Invitrogen). HEK293F cells were seeded at a density of 1x10^5/ml and transfected using 1 mg (1mg/ml) of 293Fectin (Invitrogen) or PEImax (1.0 mg/ml; 936 µg) with 300 µg of Env plasmid and 75 µg of furin plasmid (only for the SOSIP proteins) in OPTI-MEM according to the manufacturer’s protocol. Kifunensine-treated proteins were produced by adding kifunensine (TOCRIS) to HEK293F cells at a final concentration of 25 µM on the day of transfection. Culture supernatants were harvested 6-7 days after transfection. Recombinant gp140 proteins were purified by affinity chromatography using a 2G12 or PGT145 column as described previously [31, 37] and recombinant gp120 proteins were purified using a Galanthus nivalis lectin (Vector Labs) column [25]. The eluted Env proteins were concentrated using Vivaspin columns with a 100-kDa cut off (GE Healthcare). The 2G12 affinity-purified Env proteins were further purified to size homogeneity using size exclusion chromatography (SEC) on a Superose 6 10/300 GL column (GE Healthcare) in PBS. The desired fractions were collected and pooled and protein concentrations were determined using UV280 absorbance using theoretical extinction coefficients derived with Expasy (Protparam Tool). The Avi-tagged proteins were in vitro biotinylated using the BirA enzyme (Aviditiy) according to the manufacturer’s protocol. Biotinylated avitagged proteins are termed AviB proteins throughout the manuscript.

SDS-PAGE and Blue Native-PAGE were performed to verify protein purity and quality as described previously [28, 49]. Briefly, for SDS-PAGE the input material (2 µg protein) was mixed with loading dye (25 mM Tris, 192 mM Glycine, 20% v/v glycerol, 4% m/v SDS, 0.1% v/v bromophenol blue in water) and directly loaded onto a 8% Tris-Glycine gel (Invitrogen). The gels were run for 2 h at 125 V (0.07 A) using 50 mM MOPS, 50 mM Tris, pH 7.7 as the running buffer (Invitrogen). When reducing conditions were required, the proteins were mixed with loading dye and 0.1 M dithiotreitol (DTT) and boiled for 10 min prior to analysis on Tris-Glycine gels. For BN-PAGE the input material (1.1 µg protein) was mixed with loading dye (500µl 20x MOPS running buffer + 1000µl 100% Ultrapure glycerol (Invitrogen) + 50 µl 5% Coomassie Brilliant Blue G-250 + 600µl ddH2O) and directly loaded onto a 4-12% Bis-Tris NuPAGE gel. The gels were run for 65 min at 200 V at 4°C (0.07 A) using Anode-Buffer (20x NativePAGE Running Buffer (Invitrogen) in ddH2O) and Cathode-buffer (1% NativePAGE Catheter-Buffer Additive in Anode Buffer (Invitrogen).
Single B cell sorting by flow cytometry
Sorting was performed as described previously [50]. In brief, donor PBMCs were stained with primary fluorophore-conjugated antibodies to human CD3, CD8, CD14, CD19, CD20, CD27, IgG and IgM (BD Pharmigen) and 50nM of BG505 SOSIP.664-AviB and 50nM of 94UG103 and MGRM-C026 gp120-AviB coupled to Streptavidin-APC (Life Technologies), Streptavidin-BV785 (Biolegend) and Streptavidin-PE (Life Technologies), respectively, in equimolar ratios. Cells were stained for 1 h at 4°C in PBS supplemented with 1 mM EDTA and 1% FBS. In our gating strategy, we first excluded unwanted cell populations (CD3⁻/CD8⁻/CD14⁻) followed by selection of HIV Env-specific (positive for any of the 3 probes) memory B cells (CD19⁺/CD20⁺/IgG⁺/IgM⁻/HIV⁺). Cells of interest were single-cell sorted into 96 well plates containing lysis buffer on a BD FACSARia III sorter and immediately stored at -80°C.

Single B cell RT-PCR, gene amplification, and cloning
Reverse transcription and subsequent PCR amplification of IgG heavy and light chain variable genes (VH and VL, respectively) were performed according to previous protocols [51, 52]. All PCR reactions were performed in 25 µl volume with 2.5 µl of cDNA transcript using HotStar Taq DNA polymerase master mix (Qiagen). Primer sets used for gene amplification have been described elsewhere [52]. Amplified IgG VH and VL regions were sequenced and analysed using the IMGT V-quest webserver (www.IMGT.org) [53]. Wells for which VH and VL (kappa and lambda) sequences were deemed productive re-arrangements by IMGT analysis were selected for cloning into corresponding Igγ1, Igκ and Igλ expression vectors as previously described [51]. Abs were named according to ACS patient identifier (D12950: ACS2), clonal family (0-9) and additional number, example ACS201.

Antibody production
293F cells were co-transfected with heavy and light chain plasmids (1:1 ratio) in HEK293F cells using PEImax. Transfections were performed according to the manufacturer’s protocol and Ab supernatants were harvested four days following transfection. Abs were further purified over a protein A/G column as described previously [54].

Negative stain electron microscopy (NS-EM) and image processing
Purified SOSIP proteins were analysed by NS-EM. A 3 µl aliquot containing ~5.5 µg/ml of a protein was applied for 5 s onto a carbon-coated 400 Cu mesh grid that had been glow discharged at 20 mA for 30 s, then negatively stained with 2% (w/v) uranyl formate for 60 s. Images were collected on either a Tietz TemCam-F416 CMOS camera using a FEI Tecnai T12 electron microscope operating at 120 keV, with an electron dose of ~25 e-/Å² and a magnification of 52,000x that resulted in a pixel size of 2.05 Å at the specimen plane, or a FEI Ceta 16M camera using a FEI Talos electron microscope operating at 200 keV, with an electron dose of ~25 e-/Å² and a magnification of 73,000x that resulted in a pixel size of
1.98 Å at the specimen plane. The nominal defocus range for all data negative stain data sets was -1.5x10-6 to -2.0x10-6 m. Data processing methods are described elsewhere [26].

**Differential scanning calorimetry (DSC)**
Thermal denaturation was probed with a Nano DSC calorimeter (TA Instruments). Before carrying out the experiments, the SOSIP proteins were extensively dialyzed against phosphate-buffered saline (PBS; 150 mM NaCl, 50 mM sodium phosphate, pH 7.0). Protein concentration was adjusted to 0.16 mg/ml. After loading the protein sample (110 µg) into the cell, thermal denaturation was probed at a scan rate of 1°C/min. Buffer correction, normalization and baseline subtraction procedures were applied before the data were analysed using NanoAnalyze 3.1.2 software. We report the midpoint of thermal denaturation ($T_m$) value of the SOSIP proteins derived from a two-state model (Table 1), but because the asymmetry of some of the peaks suggested the presence of unfolding intermediates, the $T_m$ values based on an independent non-two-state model were also analysed (data not shown).

**Glycan profiling**
Glycan profiling was done as described previously [37]. Briefly, Env proteins (5.5 µg) were resolved by SDS-PAGE under non-reducing conditions, followed by Coomassie blue staining. Bands corresponding to gp140 were excised from the gels, washed and N-linked glycans were then released according to the manufacturer’s instructions (NEB). The released glycans were subsequently eluted from gel bands, then dried using a SpeedVac concentrator and labelled with 2-aminobenzoic acid (2-AA) as previously described [55]. Fluorescently labelled glycans were resolved by Hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC) using a 2.1 mm × 10 mm Acquity BEH Amide Column (1.7 µm particle size) (Waters, Elstree, UK). The following gradient was run: time = 0 min (t = 0): 22.0% A, 78.0% B (flow rate of 0.5 ml/min); t = 38.5: 44.1% A, 55.9% B (0.5 ml/min); t = 39.5: 100% A, 0% B (0.25 ml/min); t = 44.5: 100% A, 0% B (0.25 ml/min); t = 46.5: 22.0% A, 78.0% B (0.5 ml/min), t = 48: 22.0% A, 78.0% B (0.5 ml/min), where solvent A was 50 mM ammonium formate, pH 4.4, and solvent B was acetonitrile. Fluorescence was measured using an excitation wavelength of 250 nm and a detection wavelength of 428 nm. Data processing was performed using Empower 3 software. The percentage abundance of oligomannose-type glycans was calculated by integration of the relevant peak areas before and after Endoglycosidase H digestion, following normalization. Digestions were performed on free glycans at 37°C for 16 h. The digested glycans were purified using a PVDF protein-binding membrane plate (Millipore) prior to HILIC-UPLC analysis.
Surface Plasmon Resonance (SPR)

Ab binding to immobilized Env SOSIP proteins was performed as previously described [26, 56] with some modifications. All experiments were performed at 25°C (except as indicated for kinetic measurements, which were also carried out at 37°C). In all experiments HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.002% P20 surfactant) was used as running buffer (GE Healthcare) at maximum flow rate (50 µl/min). At the end of each cycle, the sensor surface was regenerated by a single pulse with 10 mM glycine (pH 2.0) for 120 s.

For the binding of a panel of Abs to AMC011 SOSIP.v4.2 protein, His-tagged the proteins were captured at RL ~500-530 RU on anti-His CM5 sensor chips. IgGs of VRC01, 3BNC60, F105, PGT121, 19b, PG16, PGT145, PGT151, ACS202, 35022, and 3BC315 were injected individually at 500 nM for 300 s and allowed to dissociate for 600 s. For a comparison of ACS202 IgG binding to BG505 SOSIP.664 protein, BG505 gp120-gp41ecto protomer, and gp120 monomer, D7324-epitope tagged proteins were immobilized on D7324-CM5 sensor surfaces to RL ~500-530 RU as previously described [56]. Binding of ACS202 IgG (500 nM) was monitored for 300 s of association and 600 s of dissociation. Kinetic analysis of ACS202 Fab binding to His-tagged BG505 SOSIP.664, and AMC011 SOSIP.v4.2 proteins was performed at two different temperatures, 25°C and 37°C. ACS202 Fab was injected at 1 µM as the highest concentration and titrated down to 15.6 nM in two-fold dilution steps. In each cycle, association was monitored for 300 s and dissociation for 600 s. For each cycle fresh Env was immobilized on an anti-His Ab surface to 500 ± 10 RU. The Langmuir and conformational-change models were compared and the fits of the latter found to be uniformly superior.

Competition of Ab binding to the proteins was measured as previously described [26]. The His-tagged BG505 SOSIP.664 protein was immobilized to anti-His CM5 sensor chips at ~500-520 RU. Abs were sequentially injected for binding to protein in the same cycle. The concentrations of the Abs were approximated so as to give near complete self-competition (ACS202 at 1 µM, VRC01 at 1 µM, 8ANC195 at 1.5 µM, PGT151 at 500 nM, 35022 at 1 µM, and 3BC315 at 1 µM). The first Ab (Competitor) was injected for 200 s immediately followed by the second Ab (Analyte), both at a flow rate of 30 µl/min. Dissociation was followed for 300s at the end of the second injection. The residual binding was calculated as (Response difference at 200 s for a second Ab)/(Response difference at 200 s for the same Ab as a single Ab) * 100 (%).

D7324-capture ELISA for gp140 SOSIP.664-D7324 proteins

ELISAs were performed as described previously [25, 26] with minor modifications. Microlon 96-well plates (Corning) were coated overnight with anti-D7324 (Alto Bioreagents, Dublin, Ireland), or Streptavidin (Thermo Scientific) at 2.5 µg/ml in PBS (50 µl/well) at 4°C. After washing and blocking with 3% BSA or 2% Milk for 1h at RT, AviB or D7324 proteins were added at 0.55 µg/ml in PBS/1% BSA or TBS/2% milk for 2h at RT. Unbound Env proteins were washed away, and serially diluted mAbs in PBS/1% BSA or TBS/2% milk were then added for
2h at RT. Unbound mAbs were washed away, and alkaline phosphatase-labeled goat-anti-human immunoglobulin G (IgG) (Jackson Immunoresearch, Suffolk, England) or HRP-labeled goat-anti-human immunoglobulin G (IgG) (Jackson Immunoresearch, Suffolk, England) was added for 1h at a 1:1000 or 1:5000 dilution, respectively, in PBS/1% BSA or TBS/2% milk at RT. After washing, absorption was measured at 405 nm using alkaline phosphatase or at 450 nm using TMB (Sigma-Aldrich, Zwijndrecht, The Netherlands) for HRP-labeled Ab. For competition ELISA experiments, sera of immunized rabbits were added as competitor and bNAbs as analyte, detected using HRP-labeled Donkey anti-Human IgG conjugate that was minimally cross-reactive with rabbit IgG (Jackson Immunoresearch, Westgrove, PA). Competition ELISAs were performed as described previously [38]. bNAbs were added at a concentration providing ~70% of their maximum binding signal and rabbit sera at a 1:100 dilution.

**Pseudovirus production**

To produce pseudoviruses, plasmids encoding Env were co-transfected with an Env-deficient genomic backbone plasmid (pSG3ΔEnv) in a 1:2 ratio with the transfection reagent Lipofectamin (ThermoFisher Scientific). Env encoding plasmids were obtained through the National Institutes of Health AIDS Research and Reference Reagent program. Pseudoviruses were harvested 72 h post transfection for use in TZM-bl based neutralization assays. Kifunensine-treated pseudoviruses were produced by treating 293T cells with 25 μM kifunensine (TOSCO) on the day of transfection.

**AMC011 infectious virus**

A non-codon optimized consensus sequence of the month 8 env genes from individual D12950, matching the SOSIP sequence at the amino acid level (with the exceptions of the substitutions to create the SOSIP protein; see above), was cloned into the full-length molecular clone (pLAI) as previously described [57][Van den Kerkhof et al. manuscript in preparation]. To produce the AMC011 virus, HEK293T cells (2×10^5) were seeded in a 6-well tissue culture plate (Corning) in 3 ml DMEM (Gibco) containing 10% FCS, penicillin (Sigma) and streptomycin (Sulphate-Gibco) (both at 100 U/ml) per well. After 1 day, when the cells reached a confluence of 90-95%, they were used for transfection. 4 μg plasmid DNA of the pLAI-AMC011 hybrid molecular clone in 250 μl of OPTI-MEM (Gibco) was mixed with 10 μl Lipofectamine 2000 in 240 μl of OPTI-MEM (Invitrogen), followed by an incubation for 20 min at RT. Subsequently, the transfection mixture was added to the cells, and the culture supernatants were harvested after 48 h and stored at -80°C until use in infection and neutralization experiments. When the neutralization sensitivity of the AMC011 virus was assessed using a panel of human sera and bNAbs at the Duke University Medical Center, it was classified as Tier-2 phenotype (Supplementary Table 1).
TZM-b1 based neutralization assays

The TZM-b1 reporter cell line stably expresses high levels of CD4-receptor and the HIV-1 coreceptors CCR5 and CXCR4 and contains the luciferase and β-galactosidase genes under control of the HIV-1 LTR promoter [58, 59]. The line was obtained through the National Institutes of Health AIDS Research and Reference Reagent (Program from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc., Durham, NC). TZM-b1 cells were maintained in DMEM containing 10% FCS, MEM nonessential amino acids, and penicillin/streptomycin (both at 100 U/ml). The experiments were carried out as described previously [38]. In summary, one day prior to infection, TZM-b1 cells were plated on a 96-well plate in DMEM containing 10% FCS, 1x MEM nonessential amino acids, penicillin and streptomycin (both at 100 U/ml), and incubated at 37°C in an atmosphere containing 5% CO₂ for 48 h. Virus (500 pg) was incubated for 1 hour at room temperature with 3-fold serial dilutions of Ab, starting at 25 µg/ml. This mixture was added to the cells and 40 µg/ml DEAE, in a total volume of 200 µl. Two days later, the medium was removed. The cells were washed once with PBS (150 mM NaCl, 50 mM sodium phosphate, pH 7.0) and lysed in Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured using a Luciferase Assay kit (Promega, Madison, WI) and a Glomax Luminometer according to the manufacturer's instructions (Turner BioSystems, Sunnyvale, CA). Uninfected cells were used to correct for background luciferase activity. Nonlinear regression curves were determined and 50% inhibitory concentrations (IC₅₀) were calculated using a sigmoid function in Graphpad Prism v5.01.

Antibody polyreactivity assay

Abs were screened for reactivity with a diverse set of antigens on protein microarrays following the manufacturer's instructions and as described previously [60, 61]. Briefly, ProtoArray microarrays (Invitrogen) were blocked and incubated on ice with 2 µg/ml of MAb ACS201 and ACS202 or isotype control Ab 151K for 90 min. Ab binding to array proteins was detected with 1 µg/ml of Alexa Fluor 647-labeled anti-human IgG (Invitrogen) secondary Ab. The ProtoArray microarrays were scanned using a GenePix 4000B scanner (Molecular Devices) at a wavelength of 635 nm, with 10 µm resolution, using 100% power and 650 gain. Fluorescence intensities were quantified with GenePix Pro 5.0 program (Molecular Devices) using lot-specific protein location information provided by the microarray manufacturer.

The fluorescence intensity of ACS201 and ACS202 binding to each protein on the microarray was graphed against that of control Ab 151K. The distance of each data point to the reference line, y = x (i.e., R = 1 [the diagonal line]), was determined using the distance formula: d=(x-y)/V2 on the log scale. To assess HIV-1 Ab polyreactivity, the distance of each data point to a reference line, y = x, was determined and graphed as a frequency histogram, with a mean fluorescence intensity (MFI) bin size of 0.02 (i.e. the resolution threshold of
Rabbit immunizations

Rabbit immunizations with the AMC011 SOSIP proteins and blood sampling were carried out under subcontract at Covance (Denver, PA), according to the immunization schedule shown in Fig. 4A, and as described previously [37, 38]. Female New Zealand White rabbits (n=5), were immunized intramuscularly with 22 µg of the SOSIP proteins, formulated in 75 Units of ISCOMATRIX™. The rabbits were immunized three times (at week 0, 4, and 20) with the SOSIP.v4.2 protein, and the fourth immunization was performed with the SOSIP.v5.2 protein (at week 38).

Results

Isolation of bNAb ACS202 from an elite neutralizer

We sorted Env-specific memory B cells from PBMCs taken at 40 months post-SC from an elite neutralizer enrolled in the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) who was infected with a clade B virus through injecting drug use (IDU; Supplementary Fig. 1B) [Van den Kerkhof et al. manuscript in preparation]). Negative selection (CD3- /CD8-/CD14-) was followed by positive selection for IgG memory B cells (CD19+ /CD20+/IgG+/IgM-/IgD-) [50]. Single Env-specific memory B cells were then sorted using three Env probes: BG505 SOSIP.664-AviB protein, 94UG103 gp120-AviB, and MGRM-C026 gp120-AviB. The BG505, 94UG103 and MGRM-C026 were all neutralized very potently by sera from this elite neutralizer (ID_{50} values of 484, 453 and 967, respectively, at 40 months post-SC) [39]. Approximately 3.7% of the memory B cells recognized one or more of the HIV proteins. 1.25% were positive for gp120 but not for the trimer and 1.34% were positive for the trimer only, while 0.45% of the memory B cells recognized all three proteins (Fig. 1A). We were able to sort 240 HIV-specific memory B cells that recognized at least one of the Env probes.

The individual 240 Env-specific memory B cells were sorted into lysis buffer and their mRNA was reverse transcribed and subsequently amplified and sequenced to generate immunoglobulin G (IgG) heavy (IgH) and light (IgL) chain variable (V) genes [52]. We were able to amplify 70 heavy chain, 38 light chain kappa and 84 light chain lambda BCR variable region sequences. A neighbor-joining tree constructed from the IgHV sequences showed multiple clonal families (Supplementary Fig. 2). Ab sequencing data revealed a normal distribution of the heavy and light chain V gene usage (Fig. 1B). Furthermore, the relative sequence identity of the heavy chain V genes with germline showed a normal distribution with an average of 90.8% (Fig. 1C) and an average CDRH3 length of 18 amino acids (Fig. 1D).
An elite neutralizer as blueprint for the induction of neutralizing antibodies

Figure 1: Isolation and characterization of HIV-specific memory B cells from the PBMCs of the elite neutralizer. (A) PBMCs were sorted using BG505 SOSIP.664-AviB (green) and 94UG103 gp120.AviB (purple), and MGRM-C026 gp120-AviB (red). (B, left) A total of 70 productive heavy-chain sequences were obtained, with a large variation in IgH V gene usage. (middle) A total of 38 productive light-chain kappa sequences and (right) 84 light-chain lambda sequences were obtained. (C) The heavy-chain sequences that were obtained are heavily mutated, with the majority having an average mutation frequency of 90% from the germline and (D) a range of CDRH3 amino acid lengths with an average of 18 amino acids.
From each of the different clonal families one or multiple IgH-IgL pairs were selected and cloned into expression vectors to study the mAbs in more detail. We successfully cloned 18 mAbs of which 13 showed strong binding to BG505 SOSIP.664 (Supplementary Fig. 3A). Assessing the binding of MAbs to the BG505 SOSIP.664 protein and BG505 gp120 in ELISAs showed that two families (ACS201-206 and ACS251-252) bound to protein but not gp120, while other mAbs (ACS211-212 and ACS241-242) recognized both. ELISAs with BG505 gp120 mutants further showed that mAbs ACS211-212 were dependent on the V3, while mAbs ACS241-242 were dependent on D368 (data not shown), suggesting that they were directed against the CD4bs (Supplementary Fig. 3A). A selection of mAbs per clonal family were further tested for their neutralizing capacity. Multiple Ab families were able to neutralize Tier-1 viruses, however the members of only one family, ACS201-206, were able to neutralize multiple Tier-2 viruses, but not Tier-1 viruses (Supplementary Fig. 3A). mAb ACS202 was the most potent and broadly neutralizing mAb and was therefore tested against 76 viruses of the Seaman panel [62]. ACS202 showed a breadth of 45% with a geometric mean IC_{50} (GM IC_{50}) value of 0.407 μg/ml on this virus panel with the ability to neutralize viruses from all different clades (Fig. 2A). Surprisingly, ACS202, isolated from a donor infected with a clade B virus, was less potent against clade B viruses than other viruses. ACS202 showed a similar level of neutralization breadth as bNAb PGT143 and VRC26.08, although it was somewhat less potent compared to VRC26.08 (Fig. 2B) [63, 64]. ACS202 uses the IgH V gene 3-30*03, J gene 6*02 and D gene 2-8*02 with V and J gene identities of 84% and 82%, respectively, comparable with other bNAb (IgHV range 68-88%). It uses the IgLx V gene 1-33*01 and J gene 3*01 with gene identities of 79% and 97%, respectively. In addition, it has a long CDRH3 region consisting of 24 amino acids with an uncommon repeat of 4 hydrophobic tyrosines (tetra-tyrosine (YYYY) motif) (Supplementary Fig. 3B). Tyrosines within the antigen-recognition site of Abs are often associated with polyreactivity and/or autoreactivity [61, 65, 66]. Indeed, ACS202 showed a very high level of polyreactivity, while the family member ACS201, which has a YHYY motif, was less polyreactive (data not shown).

Target epitope of bNAb ACS202

To determine the epitope of bNAb ACS202 we performed ELISAs and SPR assays with modified BG505 proteins (Supplementary Fig. 4B, 4C and 4D). ACS202 did not bind or bound poorly to gp120, gp41, SOSIP.664 monomer or uncleaved gp140, suggesting that it requires a cleaved, native-like trimer structure. ACS202 binding was not influenced by the removal of glycans at position 156, 160, 241, 332, 611, 625 and 637 (data not shown), however the binding was critically dependent on the glycan at position 88. In contrast, removal of the N234 glycan enhanced ACS202 binding. When BG505 SOSIP.664 was produced in the presence of kifunensine, a α-mannosidase I inhibitor, ACS202 binding was substantially reduced, suggesting that the Ab prefers a complex glycan which is consistent with the observation that N88 is occupied by a complex glycan (Crispin et al. submitted).
and 448 neutralization by ACS202 neutralization sensitivity to To further define the target epitope the plasma virus panel were tested for their sensitivity to ACS202. The JR-CSF N88A and T90A mutant viruses were completely resistant to ACS202 neutralization confirming that the glycan at position 88 is critical for binding and neutralization by ACS202 (Fig. 2C). In contrast, the deletion of the glycans at position 241 and 448 had no effect on neutralization, despite them being in close proximity of the N88 glycan. The neighbouring E83, V85, E87, and N229 residues were also critical for ACS202 neutralization. Surprisingly, ACS202 neutralization was likewise abrogated or severely
reduced by mutated residues S16, S17 and S20 in the fusion peptide of gp41, which was thought to be hidden in the trimer interior [32, 67]. An interaction with the fusion peptide might explain the presence of the tetratyrosine (YYYY) motif in the CDRH3 of ACS, as well as its polyreactivity. Similar to PGT151, ACS202 is cleavage-dependent, but ACS202 does not bind to the glycans N611 and N637 [68]. Similar to bNabs 35O22 and 8ANC195, ACS202 is dependent on glycan N88, but the former are not cleavage-dependent [69, 70].

Comparison of the sequences in the Seaman virus panel that were or were not neutralized did not yield further information on the target epitope of ACS202. Together these results point towards a target epitope at the gp120-gp41 interface but one different from the known gp120-gp41 interface bNabs PGT151, 35O22, 3BC315 and 8ANC195 [68-71].

**Construction of a native-like protein from early patient Env sequences**

Based on the consensus of the early env genes (8 months post-SC) derived from the elite neutralizer that developed ACS202 [39, 72][Van den Kerkhof et al. manuscript in preparation] we constructed a soluble recombinant SOSIP gp140 protein using a number of sequence modifications that have been described in detail elsewhere (see Methods) [25, 27-31, 34, 37, 47][Torrents de la Peña et al. manuscript in preparation]. The proteins were designated AMC011.v4.2 and SOSIP.v5.2.

AMC011 SOSIP.v4.2 and SOSIP.v5.2 proteins were purified by PGT145-affinity chromatography. An SDS-PAGE analysis performed under reducing and non-reducing conditions and stained with Coomassie blue showed that the purified SOSIP proteins were efficiently cleaved into their gp120 and gp41ECTO subunits (+DTT vs -DTT) (Supplementary Fig. 5A and 5B). The single gp140 bands observed under non-reducing conditions indicate that there were no interprotomer disulfide bonds, results that are in concordance with other SOSIP proteins [25, 31-35, 37]. A Coomassie blue stained BN-PAGE showed that SOSIP.v4.2 and SOSIP.v5.2 proteins were predominantly trimeric although trace amounts of monomers, dimers or higher aggregates were observed (Supplementary Fig. 5C).

---

**Table 1. Biophysical properties of AMC011 SOSIP proteins.**

<table>
<thead>
<tr>
<th>SOSIP trimer</th>
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Table 2. Antigenicity of AMC011 SOSIP proteins.

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#Half maximal binding concentration (EC50, in µg/ml)

b: Results are obtained with D7324-tagged proteins

\(-\): not tested

We used NS-EM to study the morphology of the PGT145-purified AMC011 SOSIP.v4.2 protein. The reference-free 2D class averages revealed that all the proteins adopted a native-like morphology with the three protomers closely associated with each other (Supplementary Fig. 5D and Table 1). DSC was used to measure the thermal stability of the PGT145-purified AMC011 SOSIP proteins. The DSC profile, obtained with a two-state model, showed a thermal denaturation midpoint (Tm) of 63.0°C and 67.3°C, for AMC011 SOSIP.v4.2 and v5.2, respectively (Supplementary Fig. 5H and I and Table 1). The Tm values of AMC011 SOSIP proteins is in the range of what we have typically seen with other SOSIP proteins [25, 29, 31, 35, 37][Torrents de la Peña et al. manuscript in preparation]. To acquire information on the glycosylation profile of the AMC011 SOSIP.v4.2 and v5.2 proteins we made use of HILIC-UPLC. The percentages of oligomannose glycans, Man5-8GlcNAc2, was 75% and 78%, for AMC011 SOSIP.v4.2 and v5.2, respectively (Supplementary Fig. 5J and 5K, Table 1). Man8GlcNAc2 and Man9GlcNAc2 structures accounted for about half of the glycan population. These results show that these proteins have a glycan profile that is comparable with virion-derived Envs [73].

**Antigenic analysis of the AMC011 protein**

To study the antigenicity of the AMC011 SOSIP proteins, we conducted ELISA experiments with a panel of bNAbs and non-NAbs (listed in Table 2). In addition, we performed binding experiments with AMC0011 SOSIP.v4.2 and a subset of bNAbs and non-NAbs using SPR (Fig.
Figure 3: bNAb binding to AMC011 SOSIP.v4.2. The binding of bNAb and non-bNAb (A) and ACS202 (B) to BG505 SOSIP.664 (blue) and AMC011 SOSIP.v4.2 (green) using SPR. All Abs were run at 500nM, only ACS202 was run at different concentrations, ranging from 15.6 – 100 nM, for 300 seconds. Binding kinetics (C) for ACS202 to BG505 SOSIP.664 and AMC011 SOSIP.v4.2 are calculated from the binding curves. (D) NS-EM class averages of AMC011 SOSIP.v4.2 with Fabs of ACS202 are used to model the different bNAb onto the trimer (E).
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3A). The results showed that the epitopes of most of the bNAb were present on the AMC011.v4.2 and v5.2 protein, and confirm that these proteins have a native-like conformation. When directly comparing AMC011 SOSIP.v4.2 and BG505 SOSIP.664 in SPR some differences were apparent. BG505 SOSIP.664 was more reactive with VRC01, 3BNC60, PG16 and 3BC315, while AMC011 SOSIP.v4.2 bound stronger to 35O22 as well as the autologous bNAb ACS202. PGT121, PGT145 and PGT151 interacted similarly with both proteins.

Binding of non-NAbs against CD4bs and V3 to SOSIP proteins is readily detectable in ELISA (although the binding is reduced in SOSIP.v4 and SOSIP.v5 compared to SOSIP.664 but not in SPR [25, 26, 37]. Indeed, we found that AMC011 SOSIP bound to non-NAb b6 and F105 (CD4bs) and 14e and 19b (V3) by ELISA, but binding of F105 and 19b was not observed in the SPR analyses. AMC011 SOSIP.v4.2 and SOSIP.v5.2 interacted very inefficiently with 17b and 412d, directed against the CD4-induced (CD4i) conformation, but their epitopes were induced by soluble CD4 (sCD4), indicating the CD4-induced conformational changes can occur. Overall, we show that the AMC011 SOSIP proteins have a similar antigenic profile as other native-like SOSIP proteins [25, 31, 34, 35, 37].

Complex of AMC011 SOSIP protein with the autologous bNAb ACS202

The AMC011 native-like protein provides an unique tool to study the interaction of bNAb ACS202 with the autologous Env. In SPR analyses, ACS202 bound stronger to the autologous AMC011 SOSIP.v4.2 protein compared to BG505 SOSIP.664 protein with a $K_D$ of 6.0 nM and 14.0 nM for AMC011 and BG505, respectively, with similar association and a slightly lower dissociation kinetics for AMC011 SOSIP.v4.2 (Fig. 3B and 3C). A low resolution NS-EM reconstruction of the AMC011 SOSIP.v4.2 in complex with ACS202 (as Fab) confirmed that the Ab targets a novel site at the gp120-gp41 interface (Fig. 3D and 3E). The EM reconstruction shows that ACS202 binds with a stoichiometry of three (Fig. 3C) and targets the gp120-gp41 interface surrounding the N88 glycan on gp120 and the fusion peptide of gp41. When the binding sites of other gp120-gp41 interface bNAb are compared to ACS202, some overlap is visible with the epitopes of PGT151 and 35O22 and also with 3BC315, but not with 8ANC195 (Fig. 3E). While bNAb 35O22 binds glycans at N88, N241, N230 and N625, ACS202 does not interact with the latter two. In addition, 35O22 is not dependent on Env cleavage, further confirming that bNAb ACS202 targets the gp120-gp41 interface in a different way than the known gp120-gp41 interface targeted antibodies. 3BC315 is also dependent on N88 and N241 for binding, similar to ACS202, but the angle of approach is different and ACS202 contacts more surface area on gp120, while 3BC315 contacts more surface area on gp41.

Neutralizing antibody responses elicited by AMC011 SOSIP proteins in rabbits

We performed were quantified at week 22 and week 38 (Fig. 4A; Supplementary Table 2). To be an immunization study with the AMC011 SOSIP proteins in rabbits (n=5). The rabbits
received four immunizations, at week 0, 4, 20 (SOSIP.v4.2) and 36 (SOSIP.v5.2) and the NAb responses able to assess the elicitation of autologous NAb responses, we constructed an infectious molecular clone containing the sequence-matched AMC011 Env. The AMC011 virus was classified as a relatively neutralization resistant (Tier-2) virus (Supplementary Table 1).

Autologous NAb responses were elicited in four out five animals at week 38, albeit at considerably lower titers compared to what we have previously observed using BG505 and B41 SOSIP proteins (Fig. 4B) [[37, 38]. The autologous Ab responses could not be depleted with V3 peptides, consistent with the Tier-2 phenotype of the AMC011 virus (Fig. 4D). Heterologous NAb responses were observed against the clade B Tier-1A virus SF162 (median ID$_{50}$ value of 2176) as well as three Tier-1B viruses, namely BaL (clade B), AMC008 (clade B) and ZM109F (clade C; median ID$_{50}$ values of 460, 102 and 37, respectively).
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We observed low but consistent and moderate consistent neutralization of the Tier-2 subtype B viruses WITO and REJO (median ID$_{50}$ values of 23 and 59, respectively), with one animal that developed moderate neutralization against WITO (UA0069; ID$_{50}$ value of 115) and one animal that developed unusually potent neutralization against the heterologous Tier-2 virus REJO (UA0068; ID$_{50}$ value of 1173). Heterologous NAb responses against two other Tier-2 subtype B viruses SHIV$_{162p3}$ and 92BR020 were also observed in one animal (UA0069; ID$_{50}$ values of 82 and 81, respectively). No NAb responses against the Tier-2 viruses B41 and BG505 were observed. Although these low heterologous NAb responses are weak they are more frequent and more potent than those observed in vaccination studies using BG505 and B41 SOSIP proteins. For example, the NAb responses against REJO were significantly higher than REJO NAb responses in BG505 or B41 SOSIP vaccinated animals (Fig. 4C [37, 38]).

To assess whether gp120-gp41 interface targeting antibodies were elicited by AMC011 SOSIP, we tested the rabbit sera for their ability to compete with bNAbs for protein binding in ELISA. We observed substantial competition of the sera with bNAb ACS202, as well as PGT151 and 35O22, suggesting that the AMC011 SOSIP protein induced Abs against the gp120-gp41 interface (Fig. 4E). The sera also competed with VRC01, PGT145 and PGT121 binding, indicating that multiple epitopes on AMC011 SOSIP were immunogenic (Fig. 4E). These results are in agreement with what we have previously observed for BG505 SOSIP.664

Discussion

HIV continues to cause significant morbidity and mortality around the world, emphasizing the need for an effective preventive HIV vaccine. Most existing viral vaccines, usually developed empirically, protect by inducing potent humoral immunity. However, vaccines against HIV have not been able to induce such protective responses. Elite neutralizers might provide blueprints for rational HIV vaccine design as they developed unusually potent and broad NAb responses, and did so very rapidly [13, 39]. Here we describe the isolation of a novel bNAb from an elite neutralizer, as well as the construction of a soluble recombinant native-like SOSIP trimer derived from env genes of early viruses of the same elite neutralizer.

Because we did not have prior knowledge about the bNAb specificity in the serum, we used a heterologous native-like trimer to isolate novel bNAbs instead of a specific epitope scaffold, yielding multiple mAbs targeting different regions. A member of one family, ACS202, neutralized 45% of viruses, including viruses from all clades. Although ACS202 is not the broadest and most potent bNAb described, it highlights a new target of vulnerability on HIV-1 Env: the fusion peptide of gp41. This might be unexpected as the fusion peptide was thought to be buried in the interior of the trimer [32, 67].
The lack of activity against 55% of viruses might be explained by the fact that some of the important binding residues (83-90, 229, and 516-520) are not among the most conserved ones in env. In particular residues 85, 87, 229 and 518, which are necessary for ACS202 neutralization, are only 41%, 54%, 78% and 44% conserved between viruses of different clades, respectively [Los Alamos Database]. On the other hand, the N88 glycan that is also part of the epitope is highly conserved. When the breadth and potency of ACS202 is compared with that of the serum of the elite neutralizer [13, 39], it is clear that the ACS202, even when combined with the other Abs we isolated, does not fully recapitulate the serum bNAb activity. This indicates that either more potent and broad ACS202 family members are present in the serum, or that other yet to be defined bNAb specificities are present, which could, together with the ACS202 family or independently, attribute to the serum breadth [9, 74, 75].

ACS202 uses the IgHV 3-30 gene, which is used also by HIV-1 bNAb HJ16 directed against the CD4bs [76], and, interestingly, PGT151 which binds to an overlapping epitope at the gp120-gp41 interface [68]. Additionally, ACS202 and PGT151 also use the same IgH J gene, but different IgL genes. The level of somatic hypermutation is 16% which is similar to other HIV-1 bNAbs [77]. The ACS202 CDRH3 region has a length of 24 amino acids, substantially longer than the average length of human Abs, but in the same range as many HIV-1 bNAbs [77]. The long CDRH3 loop typical of many HIV-1 bNAbs probably facilitates penetration of the glycan shield [22]. The one distinguishing feature of ACS202 compared to other bNAbs is its polyreactivity. Although many HIV-1 bNAbs show polyreactivity [24, 77], the level of polyreactivity of ACS202 is rather unusual. We hypothesize that the hydrophobic tetratyrosine (YYYY) motif in the CDRH3 domain contributes to this phenotype as ACS201, which has a YHYY motif, is less polyreactive. Thus, while ACS202 might require these hydrophobic residues to interact with the hydrophobic fusion peptide, it also causes polyreactivity, probably through “hydrophobic stickiness” [78]. Since autoreactive and polyreactive B cells are usually deleted in tolerance checkpoints [79], it might be difficult to induce similar responses by vaccination. Nevertheless, the rabbits that were immunized with AMC011 SOSIP did develop Abs to epitopes overlapping with that of ACS202.

The ACS202 Ab showed similar binding and neutralization characteristics as already described gp120-gp41 interface targeting bNAbs PGT151, 3BC315, 8ANC195 and 35O22 [68-71]. The low resolution NS-EM reconstruction of the AMC011 SOSIP in complex with ACS202 does not provide detailed interactions of the Ab and Env residues, however, together with the neutralization data of the different mutants, it discloses a novel target epitope at the gp120-gp41 interface of Env. In SPR we observed the strongest competition between ACS202 and PGT151 and slightly less with 35O22 and 3BC315 for the binding to BG505 SOSIP in SPR, especially when ACS202 was added as competitor (Fig. 2D). Yet, in the NS-EM reconstruction there appears to be a clear overlap with 3BC315, but only partial at best with PGT151 and 35O22 and no overlap with 8ANC195. Nevertheless, from the EM reconstruction it could be speculated that there can be a steric clash between ACS202 and
antibodies PGT151 and 35O22, which could explain the observed competition. In addition, it has been shown previously that the binding of an Ab can influence the orientation of glycans, thereby changing neighbouring epitopes [26, 80]. Thus, competition effects through conformational changes and/or changes in glycan orientation cannot be excluded. We constructed novel recombinant native-like proteins based on the consensus sequence of the three early viruses that were available from the individual from which ACS202 was isolated. The AMC011 SOSIP proteins were well-folded, stable and native-like trimers that bound many bNAbs, including ones against quaternary structure dependent epitopes at the V1V2 apex and the gp120-gp41 interface among which we can now include ACS202. By all means the AMC011 SOSIP proteins were similar to other SOSIP proteins [25, 31, 34, 35, 37][Van den Kerkhof et al. manuscript in preparation].

When immunizing rabbits, we observed that the AMC011 proteins induced an autologous Tier-2 NAb response that was substantially less potent than the those induces by BG505 and B41 proteins [37, 38]. We speculate that BG505 and B41 proteins expose immunodominant strain-specific epitopes on locations where glycans are missing (“glycan holes” [81]), while AMC011 does not. Indeed, the AMC011 contains all the conserved canonical potential N-linked glycosylation sites, while the BG505 sequence lacks the conserved glycan at N241, and the B41 sequence lacks the N130 and N289 glycans. On the other hand, the AMC011 proteins induce statistically significantly higher NAb responses against some heterologous Tier-2 viruses, particularly REJO, compared to BG505 and B41 SOSIP proteins. In fact, to our knowledge, this is the first study to show such consistent heterologous Tier-2 neutralization responses, even though only against a very limited number of viruses. Possibly, the reduced immunodominance of strain-specific NAb epitopes favours the induction of responses against more cross-reactive NAb epitopes. Our task now is to broaden and strengthen these weak and sporadic heterologous Tier-2 NAb responses further.

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Chapter 9

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Reference List


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Supplementary Table 1. Tier categorization of the autologous AMC011 virus.

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\(^a\)Reciprocal geometric mean (GM) ID_{50} against 6 serum pools. The reciprocal geometric mean ID_{50} is the 50% inhibitory concentration.

\(^b\)Values are the reciprocal plasma dilution at which infection was reduced 50% compared to virus control wells (no serum).

\(^c\)Values are the antibody concentration (µg/ml) at which infection was reduced 50% compared to virus control wells (no antibody).
An elite neutralizer as blueprint for the induction of neutralizing antibodies.

### Supplementary Table 2. Midpoint ID50 values for week 22 and week 38 sera from rabbits immunized with AMC011 SOSIP panels, tested against a panel of HIV-1 viruses.

#### week 22

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*Reciprocal serum dilution at which infection was reduced with 50% compared to virus control wells (no serum) and coloured according to their neutralization potency. No neutralization in white; ID50 < 20; very weak neutralization in light orange; ID50 20-40; weak neutralization in yellow; ID50 40-100; decent neutralization in dark orange; ID50 100-1000 and strong neutralization in red; ID50 > 1000.

*bCombinations tested in duplicate or triplicate are indicated with * or **, respectively.
Supplementary Figures

Supplementary Figure 1: CD4⁺ T-cell count, viral load and heterologous neutralization response during the course of infection. (A) The viral load is indicated in black with the legend on the left axis, while the CD4⁺ T-cell count is shown in red with the legend on the right y-axis. Antiretroviral treatment was given during the period indicated in grey. (B) The geometric mean IC₅₀ titer across a six-virus panel over time with the time points of virus isolation and mAb isolation indicated.
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Supplementary Figure 2: Phylogenetic tree of the IgH V genes. A maximum-likelihood tree of all the productive IgH V gene sequences was constructed using PhyML. Germine IgH V1-69 was added as an outlier and all the mAbs that were cloned and tested are indicated by name. The ACS201-206 clonal family, which uses the IgH V3-30 gene, is emphasized.
Supplementary Figure 3: Overview of the isolated mAbs. (A) A table of the successfully cloned and produced mAbs from the elite neutralizer with their ability to bind BG505 SOSIP or BG505 gp120 (green is binding; yellow: intermediate binding; red is no binding) in ELISA and to neutralize a panel of Tier-1 and Tier-2 viruses (red, no neutralization; yellow and green, intermediate to strong neutralization). ND is not done. Last column indicates the protein that the memory B cell bound in the FACS sort. (B) The IgH and Igkx gene sequences of ACS202 with the most closely related germline and V gene regions are indicated below on top, respectively. Differences with germline are indicated both at nucleotide and amino acid level.
Supplementary Figure 4: Phenotypic characteristics of the bNAb ACS202. (A) Protein microarray binding by bNAb was tested to determine polyreactivity and autoreactivity. Representative ProtoArray summary for protein arrays blotted with ACS202 (left) and ACS202 (right), or 151K control. Axis values are relative fluorescence signal intensity in the 151K array (y axis) or test Ab array (x axis). Each dot represents the average of duplicate array proteins. A diagonal line indicates equal binding by test Ab and 151K. Internal controls for loading of Ab and secondary detection reagent were equally bound by Ab pairs (boxes). Dashed lines indicate the 500-fold signal/background ratio defined as the cutoff for autoreactivity. The Gaussian mean of all array protein displacements is termed the polyreactivity index (PI). A PI value of 0.21 suggests 2-fold-stronger overall binding by test Ab than control 151K, and it was defined as the threshold of polyreactivity. (B) ACS202 binding to BG505 SOSIP, BG505 gp120, and BG505 SOSIP kifunensine treated, uncleaved (RRRRRR cleavage site replace by SEKS), non SOS to obtain gp41 and BG505 SOSIP mutants N88Q, N234S, N276S and N88Q+N234Q in ELISA. (C) The binding of ACS202 to BG505 SOSIP.664 (green), BG505 gp120-gp41ECTO (blue) and BG505 gp120 (pink) using SPR. ACS202 was run at 500nM, only ACS202 was run for 300 seconds. (D) Fold reduction of virus JR-CSF neutralization by ACS202 caused by a single amino acid substitution. Deletion of a PNGS site is indicated with an *.

Maximum concentration ACS202 tested was 10 µg/ml with an IC_{50} value of 0.59 µg/ml.
Supplementary Figure 5. Biochemical and biophysical properties of the PGT145-purified AMC011 SOSIP.v4.2 and v5.2 proteins. (A,B) The proteins were analyzed by SDS-PAGE under reducing (+DTT) and non-reducing (-DTT) conditions, and gp120 (cleaved) and gp140 (uncleaved) bands are indicated. (C) The same proteins were analyzed by BN-PAGE and the bands corresponding to the proteins are indicated. (D-G) Negative-stain EM analyses showing the 2D class averages: (D) SOSIP.v4.2 trimers; (E-G) D7324-tagged SOSIP.v3.1, v4.1 and v4.2 trimers, respectively. The percentage of closed and open native-like trimers are quantified below each panel and were calculated by dividing the total number of each subcategory by the total number of trimeric particles. (H,I) DSC analysis: (H) AMC011 SOSIP.v4.2. (I) AMC011 SOSIP.v5.2. The thermal denaturation midpoint temperature (T_m values) of the peaks were calculated by the two-state model and are listed in Table 1. (J,K) Glycan profile: (J) AMC011 SOSIP.v4.2 (K) AMC011 SOSIP.v5.2. The peaks correspond to the oligomannose glycans (Man_5,6GlcNAc_2) indicated with M5 to
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M9, respectively, and their peak areas (as a percentage of total glycans) are illustrated in pie charts, colored in shades of blue. The remaining peaks correspond to complex and hybrid-type glycans, and their peak areas are indicated in grey in the pie charts. The percentages of Man$_{5}$GlcNAc$_{2}$ and Man$_{9}$GlcNAc$_{2}$ glycans are listed in Table 1.