Immunization with HIV-1 trimeric SOSIP.664 BG505 or founder virus C (FVC_{Env}) covalently complexed to two-domain CD4^{560C} elicits cross-clade neutralizing antibodies in New Zealand white rabbits

Nancy L. Tumba, Gavin R. Owen, Mark A. Killick, Maria A. Papathanasopoulos

HIV Pathogenesis Research Unit, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

A R T I C L E   I N F O

Article history:
Received 7 March 2022
Received in revised form 21 September 2022
Accepted 28 September 2022
Available online 30 September 2022

Keywords:
HIV-1 vaccine immunogens
Envelope glycoprotein trimers
Covalent complexes
Broadly neutralizing antibodies
New Zealand White rabbits
Immunogen design

A B S T R A C T

Background: An ongoing challenge in HIV-1 vaccine research is finding a novel HIV-1 envelope glycoprotein (Env)-based immunogen that elicits broadly cross-neutralizing antibodies (bnAbs) without requiring complex sequential immunization regimens to drive the required antibody affinity maturation. Previous vaccination studies have shown monomeric Env and Env trimers which contain the GCN4 leucine zipper trimerization domain and are covalently bound to the first two domains of CD4 (2dCD4^{560C}) generate potent bnAbs in small animals. Since SOSIP.664 trimers are considered the most accurate, conformationally intact representation of HIV-1 Env generated to date, this study further evaluated the immunogenicity of SOSIP.664 HIV Env trimers (the well characterized BG505 and FVC_{Env}) covalently complexed to 2dCD4^{560C}.

Methods: Recombinant BG505 SOSIP.664 and FVC_{Env} SOSIP.664 were expressed in mammalian cells, purified, covalently coupled to 2dCD4^{560C} and antigenically characterized for their interaction with HIV-1 bnAbs. The immunogenicity of BG505 SOSIP.664-2dCD4^{560C} and FVC_{Env} SOSIP.664-2dCD4^{560C} was investigated in New Zealand white rabbits and compared to unliganded FVC_{Env} and 2dCD4^{560C}.

Rabbit sera were tested for the presence of neutralizing antibodies against a panel of 17 pseudoviruses.

Results: Both BG505 SOSIP.664-2dCD4^{560C} and FVC_{Env} SOSIP.664-2dCD4^{560C} elicited a potent HIV-specific response in rabbits with antibodies having considerable potency and breadth (70.5% and 76%, respectively) when tested against a global panel of 17 pseudoviruses mainly composed of harder-to-neutralize multiple clade tier-2 pseudoviruses.

Conclusion: BG505 SOSIP.664-2dCD4^{560C} and FVC_{Env} SOSIP.664-2dCD4^{560C} are highly immunogenic and elicit potent, broadly neutralizing antibodies, the extent of which has never been reported previously for SOSIP.664 trimers. Adding to our previous results, the ability to consistently elicit these types of potent, cross-neutralizing antibody responses is dependent on novel epitopes exposed following the covalent binding of Env (independent of sequence and conformation) to 2dCD4^{560C}. These findings justify further investment into research exploring modified open, CD4-bound Env conformations as novel vaccine immunogens.

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Introduction

The HIV/AIDS pandemic has now continued for four decades, with approximately 37.7 million individuals living with HIV globally, and 1.5 million new infections reported in 2020 [1]. While these numbers have vastly decreased from 1997 when the new infections peaked at 2.9 million [1], a decisive halt to this spread can only be achieved with an effective prophylactic vaccine. The broad diversity of HIV-1 has contributed to the difficulty in identifying an Env-based vaccine immunogen capable of inducing immunity against all circulating HIV-1 groups and clades. To address viral diversity, researchers have tried to design immunogens that direct the immune responses towards regions of the viral envelope glycoprotein (Env) that are highly conserved, such as the CD4 receptor binding site (CD4bs), the gp120-gp41 interface, the V1V2 trimer apex, and the membrane proximal external region (MPER) [2]. A number of broadly neutralizing antibodies (bnAbs) that target these conserved sites have been isolated from HIV-infected individuals thereby showing the possibility of their induction in humans. However, if HIV-infected individuals develop bnAbs, it is too late to impact on disease progression. By contrast,
their neutralizing potential, and their ability to protect against infection in animal challenge models has also been studied [3–5], providing evidence of the importance of preexisting humoral immune responses in preventing transmission.

In an effort to improve immunogens, the HIV-1 vaccine research field has made use of multiple techniques such as mutations, cleavage-site disruption and the introduction of trimerization domains to create stabilized, recombinant HIV-1 Env trimers [6,7]. This gave rise to SOSIP trimers, the first Env-based immunogens with antigenic and topological structures that best represent the native HIV-1 virion trimeric spike [7–9]. When assessed in rabbits, the stabilized SOSIP trimers induce autologous neutralizing antibodies as well as neutralization of the more sensitive Tier-1 viruses [10]. Env trimers can be stabilized in the closed, prefusion conformation or the open, post–CD4 engaged state. These different Env conformations expose diverse epitopes and ultimately impact the elicitation of neutralizing antibodies, relevant for HIV-1 vaccine design [10,11]. Some of the broadest and most potent antibodies characterized from HIV-infected individuals target epitopes presented in the closed, prefusion conformation, when the trimers are in its quaternary fold. As a result, the majority of trimer and immunogen design efforts have been directed towards fastening the Env in the closed conformation, with studies on SOSIP trimers being the most advanced. However, not much research has been directed towards the use of SOSIP trimers in the CD4-bound, open conformation.

It is believed that sites of vulnerability of HIV-1 may be contextual and that the different Env conformations (i.e., prefusion, CD4-bound intermediates, pre-hairpin intermediate, and postfusion) structurally expose different epitopes for antibodies to target [2]. The CD4-bound conformation seems to contain less neutralization epitopes than the closed, prefusion conformation of Env, evidenced by the fewer number of antibodies identified from natural infection that specifically target this state [2]. The CD4-bound Env conformation exists in two distinct states: when a single gp120 monomer is engaged by CD4 (asymmetric trimmer) or when all three gp120 monomers are bound to CD4 (open trimmer) [12]. The transient nature of these CD4-bound conformations may contribute to the lack of antibodies to these epitopes in natural infection [13].

Transmitted/founder (TF) HIV-1 viruses are defined as the viruses that establish productive infection following mucosal transmission [14,15]. They exhibit certain characteristics such as shorter variable loops, less putative N-linked glycans, enhanced interactions with dendritic cells, better infectivity as cell-free virions, and a preference for the chemokine receptor 5 (CCR5) [14]. Of interest, the shorter variable loops and reduced number of glycans have been linked to a modest increase in susceptibility to neutralization or the lack of antibodies to these epitopes in natural infection [13].

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Previous work using an Env immunogen composed of the consensus sequence obtained from over 1,800 subtype C TF envelopes [19]; namely, the Founder Virus C Env (FVCENV), covalently complexed to the first two domains of CD4 (2dCD4) via cysteine-linkage through targeted intermolecular binding of gp120 to CD4 by introduction of a serine to cysteine substitution in CD4 [20] (2dCD4S60C) gave rise to exceptional immunogens [21]. The former study made use of various Env conformations, from monomeric to leucine-zipper linked gp140 trimers, with the same outcome: all consistently elicited potent, cross-neutralizing antibody activity against clinically-relevant HIV-1 isolates in New Zealand white rabbits [21] and rhesus macaques (Pereira et al. unpublished data).

Here we describe the SOSIP trimerization of FVCENV as compared to the well characterized BG505. SOSIP trimers constitute the best antigenic mimics of HIV-1 in its native state and, therefore, provide an excellent immunogen model. Moreover, by locking the BG505 and FVCENV SOSIP.664 trimer in the CD4-bound, open state by covalently complexing our SOSIP Env trimer to 2dCD4S60C, we interrogated whether the CD4-bound conformation of SOSIP trimers is antigenically compromised or would replicate and possibly improve on results seen with other SOSIP.664 and Env-2dCD4S60C conformations. Consequently, the immunogenicity of BG505 SOSIP.664–2dCD4S60C and FVCENV SOSIP.664–2dCD4S60C was evaluated in New Zealand white rabbits and the antisera response characterized and compared for neutralization potential.

Materials and methods

Ethics statement. Twenty female New Zealand white rabbits were maintained at the Wits Research Animal Facility (WRAF) in a pathogen-free environment, where all animal work was conducted in accordance with the Animal Research Ethics Committee (AREC) directives and National/International recommendations. Animal housing, care and the immunization and bleed protocols were all conducted in accordance with the institutional requirements set by AREC and WRAF. All AREC comply with the South African National Standard on the care and use of animals for scientific research. Clearance was obtained from the Animal Ethics Screening Committee, University of the Witwatersrand (certification #: 2018/02/108).

Recombinant plasmids description. The BG505 SOSIP.664 clone in the VRC3831 plasmid was kindly provided by Dr. Peter Kwong. The subtype C derived FVC Env gene constructed from the consensus sequence of TF viruses [19] was used to incorporate the SOSIP.664 mutations: I559P, A501C, T605C,508REKR511 to R6, and MPER truncation at residue 664 [7,8]. The codon-optimized FVCENV SOSIP.664 clone was synthesized by GeneArt (Life Technologies, Regensburg, Germany) and cloned into the pCDNA3.3 expression vector (Invitrogen, Grand Island, NY). The plasmid containing the coding-sequence of the furin protease, i.e., pcDNA3.1-Furin, was obtained from Dr. J.P. Moore [8]. Two-domain CD4 (2dCD4) plasmids– pET15b–2dCD4WT (WT/S60C) were used for the expression of wild-type (WT) and S60C mutant versions of 2dCD4. The pET15b (Novagen, Germany) plasmids contain a 6 x polyhistidine (His) tag at the 3′ terminus in frame with the 2dCD4 sequence to allow for Nickel affinity purification.

Mammalian cell lines and antibodies. The CD4- and CCR5-positive TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (ARRRP), (Contribution of Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.). The HEK293T cells were obtained from the American Type Culture Collection, also through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. TZM-bl and 293 T cells were cultured in Dulbecco’s minimal essential medium (DMEM: Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM Glutamax (Gibco/Invitrogen, Waltham, MA, USA), and 100 U/mL penicillin–streptomycin (Gibco/Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to prepare the complete growth medium. Cells were incubated at 37°C in 5% CO2 and 80% relative humidity. Purified mAbs PG9 (Dr. D. Burton), VRC01 & VRC03 (Xueling Wu, Zhi-Yong Yang, Yuxing Li, Gary Nabel, John Mascola) [22], HJ16 (Dr. Antonio Lanuz Vecchia), and 10E8 (Dr. Mark Connors) were obtained from ARRRP.
Expression plasmids encoding heavy- and light-chain sequences of the monoclonal antibodies 17b and PGT145 were a generous gift from Dr. Pascal Paignard.

**Subcloning of FVC<sub>Env</sub> into the VRC3831 backbone.** To improve the expression of FVC<sub>Env</sub> recombinant protein, the FVC<sub>Env</sub> coding region from the pCDNA3.3 plasmid was subcloned into the VRC3831 plasmid backbone using the Gibson Assembly cloning method (New England Biolabs, USA), according to manufacturer's instructions. The integrity of the resultant clones was verified by sequencing along the 5′- and 3′-end portions of the inserted gene using vector-specific primers (SeqinF: 5′TGTATCAGATATTCGGG3′ and SeqinR: 5′AAGGCACTGGGGGAGGGCGG3′) with the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using an automated ABI 3100 genetic analyzer. Chemically competent E. coli DH5α bacterial cells were transformed with the final DNA constructs as previously described [23]. Transformants were selected on Luria Bertani agar plates supplemented with 50 µg/mL kanamycin. Plasmid DNA was isolated and purified using QIAEAGENTM plasmid purification kits (Qiagen, Maryland, USA) according to the manufacturer's instructions.

**Expression and purification of Env SOSIP.664 trimers.** HIV-1 Env were expressed in HEK293T cells following transient co-transfection with the Env plasmids and pCDNA3.1-Furin as described previously [22]. Supernatants containing the secreted Env recombinant proteins were clarified by centrifugation at low speed and filtration through a 0.22 µm filter. Enzyme-linked Immunosorbent Assay (ELISA). The monoclonal antibodies 17b and PGT145 were a generous gift from N.L. Tumba, G.R. Owen, M.A. Killick et al. Vaccine: X 12 (2022) 100222

**Results**

**FVC<sub>Env</sub> SOSIP.664 amino acid sequence comparison to BG505 and other Env sequences**

The BG505 SOSIP.664 modifications [9] were introduced in the original FVC<sub>Env</sub> sequence [19] to produce the FVC<sub>Env</sub> SOSIP.664 gp140 protein. The changes included the A501C and T605C mutations to create the interprotomer SOS disulphide link, the I559P mutation to stabilize the trimer, the replacement of the R517, E518, K519, R520 to to 6 Arg (K520R521), the removal of the CNE55, CH119.10, BJOX2000, CE1176, X1632, and the residues 17b, 20, 35, 64 with bleeds on days 0 (prior to immunization), 14, 42, 70, and 98.

**Pseudovirion production.** The plasmid encoding the HIV-1 env gene of strains QH0692.42, 398_F1, 246F3, 25710, C176, 398, 246F3, 398_F1, 25710, C176, and 10E8, and 17b. The presence of bound antibodies was detected with a horseradish peroxidase-conjugated anti-human IgG antibody (GE Healthcare).

**Immunizations and serum collection.** New Zealand white rabbits were divided into four groups with five animals in each group. All experimental animals weighed between 3 and 5 kg at the commencement of the study. The rabbits were given intramuscular injections of 50 µg total protein/animal of either FVC<sub>Env</sub> SOSIP.664 (group A), 2dCD4<sup>WT</sup> (group B), BG505 SOSIP.664-2dCD4<sup>S60C</sup> complex (group C), or FVC<sub>Env</sub>-2dCD4<sup>S60C</sup> complex (group D). Each injection was adjuvanted with Adjuplex Adjuvant (Advanced BioAdjuvants, Omaha, Nebraska) comprising of detergent-free lecithin and the carbomer homopolymer. The immunization schedule consisted of multiple doses with injections on days 0, 28, 56, and 84 with bleeds on days 0 (prior to immunization), 14, 42, 70, and 98.

**TZM-bl neutralization assay.** Neutralization of pseudovirions was measured with the TZM-bl cells-based luciferase assay as previously described [29]. Neutralization was determined as the difference in RLU between the test wells and the cells-only control wells divided by the difference in RLU values between the virus-only control and cells-only control wells [30]. Each serum was tested against the VSV-G pseudovirions to confirm the HIV-1 Env specificity of neutralization. Positive controls included human monoclonal antibodies (mAbs) PG9 and VRC03 at concentrations ranging between 0.005 and 10 µg/mL. Neutralization was reported as the 50% inhibitory dilution (ID<sub>50</sub>) for sera, or 50% inhibitory concentration (IC<sub>50</sub>) for mAbs which represent the dilution of serum (or mAbs) resulting in a 50% reduction in RLU.

Results

**Expression and purification of 2dCD4.** Competent BL21<sup>E</sup> coli (DE3; Invitrogen) cells (New England Biolabs, Ipswitch, MA, USA) were transformed with pET15b-2dCD4<sup>WT</sup>/S60C plasmids using a standard protocol (Novagen, USA). Bacterial cells were propagated at 37°C under ampicillin selection pressure in LB medium (1% w/v tryptone, 0.5% w/v yeast, 1% w/v NaCl, pH 7.3). Recombinant 2dCD4<sup>WT</sup>/S60C proteins were expressed and purified as described previously [20]. Briefly, the 2dCD4 insoluble fraction was purified by denaturing the proteins using chaotropic agents, capturing the asparagine beads-conjugated <i>Galanthus nivalis</i> lectin (Sigma-Aldrich) column chromatography with affinity for mannose glycans, as previously described [24]. A second purification by gel filtration chromatography (HiPrep Sephacryl S-300; GE Healthcare, Piscataway, NJ) with an AKTA FPLC (GE Healthcare) was performed to isolate the trimers from aggregates, dimers, and monomers, followed by concentration using centrifugal filtration with a 50 kDa MWCO Amicon Ultra filter (Millipore, Billerica, MA, USA). The final protein concentration was determined by bicinechonic acid protein assay (Pierce, Rockford, IL). The purity of expressed proteins was evaluated by reducing and non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting to confirm size and identity, as well as by blue native (BN)-PAGE to confirm the trimeric state.

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664 to delete the MPER region of gp41 and to enhance the solubility of the protein (Supplementary Fig. S1). An Asn at position 332 and a Ser at position 334, the potential N-linked glycosylation site on which certain V3-directed antibodies are highly dependent, was already present in the FVCENV sequence. Comparatively, the FVCENV sequence, which is a consensus envelope of subtype C founder viruses [19], contained shorter V1V2 and V4 loops (Supplementary Fig. S1). The FVCENV SOSIP.664 sequence contains 17 N-linked glycosylation sequons (Supplementary Fig. S1).

**FVCENV SOSIP.664 and BG505 SOSIP.664 express native-like trimers and form protein complexes with 2dCD4S60C**

The FVCENV SOSIP.664 and BG505 SOSIP.664 trimers were successfully expressed in HEK293T cells and purified via lectin-affinity chromatography (SEC) (Fig. 1). While the trimmer fraction constituted the largest peak, there was a proportion of dimers and monomers formed for both SOSP.664 Envs (Fig. 1A). Reducing and non-reducing SDS-PAGE confirmed successful cleavage of the FVCENV and BG505 trimers as observed by the transition of gp140 to gp120 in the presence of DTT (Fig. 1B). This indicates that the optimal 6-arginine cleavage site and co-transfection with furin provided efficient cleavage. Purified FVCENV and BG505 SOSIP.664 trimers were complexed to 2dCD4S60C in the presence of a low concentration of reducing agent, to facilitate reduction of the native disulphide bond and subsequent formation of the intermolecular covalent bond between Env and 2dCD4 locking the FVCENV and BG505 SOSIP.664 Envs in the open, CD4-bound conformation. SEC purification of the FVCENV-2dCD4S60C and BG505-2dCD4S60C complexes showed elution of the higher molecular weight complexes (Fig. 1A) whose increased molecular mass was confirmed by BN-PAGE (Fig. 1C).

**FVCENV SOSIP.664 produces native-like trimers with preserved antigenic qualities, similar to BG505 SOSIP.664**

We used ELISAs to quantify the binding of various HIV-1 neutralizing antibodies to the SOSIP.664 gp140 trimers in the unliganded and 2dCD4S60C complexed forms. The SEC-purified SOSIP.664 trimers (FVCENV and BG505) were immobilized onto ELISA plates and we monitored the binding of serially-diluted mAbs that recognize various regions of the Env, namely, the quaternary epitope V1V2 apex (PG9 & PGT145), a V3 glycan epitope (10–1074), the CD4 binding site (VRC01 & IgG1b12), the first and second constant region C1-C2 (A32), the gp120/gp41 interface (35O22), and the MPER (10E8) (Fig. 2A). Antibodies whose epitopes are only formed when the trimmer is in the quaternary native fold such as PG9, PG16 and PGT145- are used to confirm trimers have adopted the correctly folded, quaternary conformation. All three of these antibodies recognize the V1V2 crown of the trimmer when the protomers are in close proximity to each other in the closed, prefusion trimeric conformation (Fig. 2B). This epitope is compromised in the open, CD4-bound conformation although the PG9 and PG16 somatically-related antibodies indiscriminately bind to the monomeric versions of Env albeit with lower affinity [31]. These antibodies are also dependent on N160 or N156 glycans which are present in the FVCENV and BG505 trimers based on the amino acid sequence analysis (Supplementary Fig. S1). We observed strong binding of the VRC01, IgG1b12, 10–1074 and PG9 antibodies and weaker binding of the A32, 35O22 and PGT45 antibodies for the FVCENV SOSIP.664 trimer (Fig. 2A). Similarly, VRC01, 10–1074 and PG9 bound well to the BG505 SOSIP.664 trimer, however,
the binding of IgG1b12 - while detectable - was significantly lower than its recognition of FVCENV (Fig. 2A). In contrast, antibodies A32 and 35O22 displayed more pronounced binding to the BG505 SOSIP.664 Env compared to the FVCENV SOSIP.664 trimer (Fig. 2A). 10E8 was included as a negative control as its epitope is absent in the SOSIP.664 trimers and it showed no binding to the FVCENV SOSIP.664 trimer (Fig. 2A).

We then tested the effect of complexing FVCENV SOSIP.664 to 2dCD4S60C on the antibodies which bound well to the trimer. We observed that the binding of the CD4bs antibody IgG1b12 was substantial for the FVCENV SOSIP.664 trimer but was significantly reduced in FVCENV SOSIP.664-2dCD4S60C (Fig. 2C). The binding of the other CD4bs mAb VRC01 remained unchanged between FVCENV SOSIP.664 and FVCENV SOSIP.664-2dCD4S60C (Fig. 2C). Recognition of the V1V2 apex mAb PG16 was also similar for the unliganded FVCENV SOSIP.664 and the 2dCD4S60C-complexed trimer (Fig. 2C). Another monoclonal antibody that lost most of its binding capacity in FVCENV SOSIP.664-2dCD4S60C was the V3 glycan binding mAb 10–1074 (Fig. 2C). In addition, we were able to confirm CD4 binding through preferential recognition of the CD4-induced epitope by the 17b mAb in the FVCENV SOSIP.664-2dCD4S60C complex (Fig. 2C).

The presence of neutralizing antibodies in the immunized rabbit sera was determined using the standard TZM-bl neutralization assay against 17 Tier 2 pseudoviruses comprising clades A, AC, B, C, AE, BC, E, and G (Fig. 3B). These Tier 2 pseudoviruses are considered as excellent determinants of antibodies neutralizing potential as they provide an exceptional representation of biologically relevant strains that have Env conformations of most circulating viruses [32]. We used sera collected two weeks after the last immunization as the antibody responses are shown to be at the highest titres following the fourth longitudinal immunization and neutralizing antibodies PG9 and VRC03 were used as controls (Fig. 3B). Results demonstrate that the covalently complexed Env SOSIP trimers (both FVCENV and BG505) were able to elicit antibodies that displayed robust neutralization of 12 (BG505 complex) and 13 (FVCENV complex) of the seventeen pseudoviruses tested (Fig. 3B). Comparatively, sera from the rabbits immunized with the unliganded FVCENV (Env control) were only able to weakly neutralize two of the pseudoviruses (398_F1 and CH119.10) and the sera from animals who received the 2dCD4S60C showed negligible neutralization with only two rabbits able to neutralize one pseudovirus (QH0692.42) at titers below ID$_{50}$ values of 240 (Fig. 3B). Of interest, all five animals who received FVCENV SOSIP.664-2dCD4S60C generated a cross-neutralizing antibody response against twelve of the thirteen pseudoviruses neutralized with two antisera - from rabbits 0289 and 0330 - failing to neutralize the Du422.12 pseudovirus (Fig. 3B). Similarly, all the animals who received BG505 SOSIP.664-2dCD4S60C elicited cross-neutralizing antibodies against ten of the twelve neutralized pseudoviruses, with two rabbits (0300 and 0329) whose sera could not neutralize two of the clade C pseudoviruses (CAP210.2.00.E8 and Du422.12) (Fig. 3B). Overall, the cross-neutralizing response was consistently elicited in all the rabbits who received the covalent complexes with a few exceptions for one or two pseudoviruses. As expected, PG9 and VRC03 displayed different neutralization profiles against the panel of 17 pseudoviruses.

**Immunization with BG505 SOSIP.664 and FVCENV SOSIP.664-2dCD4S60C elicits potent, cross-neutralizing antibodies in rabbits.**

To evaluate the ability of the covalently complexed BG505 SOSIP.664-2dCD4S60C and FVCENV SOSIP.664-2dCD4S60C to induce humoral immune responses, we conducted immunogenicity studies with the purified recombinant proteins (expressed in mammalian cells with natural glycosylation preserved) using rabbits as the pre-clinical hosts. Each group of immunized animals was given either the 2dCD4S60C protein, unliganded FVCENV SOSIP.664, BG505 SOSIP.664-2dCD4S60C or FVCENV SOSIP.664-2dCD4S60C, as outlined in the immunization schedule in Fig. 3A.
Fig. 3. Rabbit sera neutralization of a Tier 2 pseudovirus panel. (A) Schematic of rabbit immunization schedule with FVCRN/SOSIP.664, 2dCD4S60C, BG505 SOSIP.664-2dCD4S60C and FVCRN/SOSIP.664-2dCD4S60C. The rabbits were immunized four times, monthly, with 50 μg of the soluble proteins adjuvanted with Adjuplex. Two weeks prior to the first immunization and two weeks following each immunization, blood samples were collected. Immunizations and bleeds time-points are indicated in weeks. (B) Neutralization titers of the rabbit sera in each of the four immunization groups. Sera obtained prior to immunizations (pre-bleed) and at week 14, following the final immunization, were tested in a TZM-bl reporter cell assay against the VSV-G specificity control pseudovirus and HIV-1 pseudoviruses QH0692.42, 398F1, 246F3, TRO.11, X2278, CAP210.2.00.E8, Du422.12, 246F, 25710, CE0217, CH19.10, BJ02X000, CE1176, and X1632. Monoclonal antibodies (mAbs) PG9 and VRC03 were included as neutralization controls for each pseudovirus except the non-HIV VSV-G pseudovirus. Numerical values represent ID₅₀ (for sera) and IC₅₀ (for mAbs) neutralization titers. ID₅₀ < 40 and IC₅₀ > 10 indicate no detectable neutralization for the rabbit sera or mAbs, respectively.
Discussion

An important milestone in HIV-1 vaccine development is the ability to elicit potent, bNAbs following vaccination. Our results demonstrate the ability to induce antibodies that neutralize heterologous HIV-1 tier 2 pseudoviruses in rabbits, with a single immunogen complex composed of trimeric SOSIP.664 envelopes covalently coupled to 2dCD4<sup>60C</sup>. Sera from rabbits immunized with the FVC<sub>ENV</sub>SOSIP.664-2dCD4<sup>60C</sup> displayed neutralization against 13/17 (76.5%) of tier 2 HIV-1 isolates from clades A, AC, B, C, AE, BC, and E, at notably high titers. Similarly, sera from rabbits immunized with the BG505 SOSIP.664-2dCD4<sup>60C</sup> complex were also able to neutralize the same pseudoviruses, with the exception of the clade A QH0962.92 pseudovirus, with comparatively high titers. The foundation of this work was accomplished in a previous study where various HIV-1 Env conformational reagents (monomeric gp120 and GCN4 trimeric Env) covalently complexed to 2dCD4<sup>60C</sup> elicited potent, broadly neutralizing antibodies against 100% of twelve subtype B and C isolates made up of tier-1, tier-2 and a single tier-3 pseudoviruses [21]. This study further investigated the impact of SOSIP.664 (the archetype HIV-1 trimer mimic) on the immunogenicity of the covalently bound complexes locked in an open, 2dCD4-bound conformation in small animal models, comparing the well characterized BG505 sequence to FVC<sub>ENV</sub>. Overall, sera from rabbits immunized with BG505 SOSIP.664-2dCD4<sup>60C</sup> and FVC<sub>ENV</sub>SOSIP.664-2dCD4<sup>60C</sup> neutralized over 70% of the Tier-2 pseudoviruses tested, at exceptionally high potencies. While these results are significant, the spectrum of neutralization cross-reactivity of the SOSIP.664-2dCD4<sup>60C</sup> complexes is narrower compared to the Env panels in the previously reported study. This could be attributed to the difference in Env conformation or to the inclusion of pseudoviruses from clades A, AC, AE, BC, E and G which were not tested in the previous study [21]. Of note, one clade B (X2272) and two clade C (CE1176 and ZM53.12) pseudoviruses were resistant to neutralization in this study while all clade B and C pseudoviruses tested were neutralized in the Killick et al. study [21]. However, no direct comparison can be made as the pseudovirus panels used in the antibody neutralization assay varied between the two studies.

As expected, the rabbit sera from the groups immunized with either FVC<sub>ENV</sub>SOSIP.664 or 2dCD4<sup>60C</sup> exhibited low potency, antibody neutralization potential, with narrow breadth. A limitation of this study is that we did not include immunization with unliganded BG505 SOSIP.664 as a control. However, multiple groups have previously tested unliganded BG505 SOSIP.664 in rabbit immunizations, and shown its immunogenicity is limited to eliciting antibodies capable of neutralizing the autologous, immunogen-matched pseudovirus with the occasional cross-neutralization but of very limited breadth and potency [11,33]. Thus, in an effort to reduce the number of animals used in experimentation, the ethical decision was taken not to include an unliganded BG505 SOSIP.664 group. Particularly since an unliganded FVC<sub>ENV</sub>SOSIP.664 group was included. In summary, this is the first report of covalently bound SOSIP.664-2dCD4<sup>60C</sup> complexes capable of eliciting potent, broadly neutralizing antibodies in small animals, and further expands our knowledge on SOSIP.664 immunogenicity.

The observation that neither the Env sequence (BG505 or FVC<sub>ENV</sub>) nor the Env conformation (SOSIP trimer in this study versus monomeric or GCN4-linked Env trimers described in Killick et al., 2015) used influences the ability to elicit the potent, heterologous neutralizing response is suggestive that covalently complexing the Env with 2dCD4<sup>60C</sup> creates the auspicious conformational change responsible for the elicitation of the potent bNAbs displayed here.

The CD4-bound conformation has not been widely explored, particularly in terms of immunogen development, as prefusion (ligand-free) states are known to favour neutralizing antibodies epitopes [34,35], including conformational ones [9,36] while non-neutralizing antibodies sometimes target epitopes exposed upon CD4 binding such as the CD4-induced (CD4i) and the crown of the third hypervariable (V3) loop [37,38] epitopes. As such, immunogen development strategies have sought to maximize prevention of immunodominant (neutralization-hypo-sensitivity) regions, targets of non-neutralizing antibodies, by engineering Env with increasing mutations for structural stability of the trimer in its native state with much reduced CD4 affinity [39,40]. While it cannot be argued that the preferential antigenic properties of these improved trimers have been finely mapped and their improved immunogenicity in preclinical studies well demonstrated [11,41], the limitations of focusing on the closed, ligand-free trimer as an immunogen have been highlighted here and in other studies [21].

The CD4-bound conformation of Env has been shown to exhibit some conformational plasticity [42] as intermediate transitional conformations occur when the various loops engage with CD4 [43]. We have shown that the CD4-bound FVC<sub>ENV</sub> displays the open trimer conformation as evidenced by the improved binding of CD4-induced mAb 17b. We have also observed the loss of some bNAbs epitopes in the CD4-bound conformation, most of which could be explained by either steric hindrance or epitope loss due to the conformational changes involved in binding to CD4 (as may have been the case for PGT145; however, the ELISA binding was not substantial enough to perform a comparative assessment between CD4-ligated and -free conformations). In the first instance, we observed the contrast between two CD4 binding site antibodies whereby IgG1b12 seemed to compete with the bound CD4 molecule evidenced by the reduced binding of the mAb in the CD4-bound conformation and VRC01 whose binding remained unaffected in the CD4-ligated and -unliganded conformations. This is in accord with other studies that have shown VRC01 to have less steric hindrance than IgG1b12 in the way they approach their cognate epitopes, with IgG1b12 having a loop-proximal angle (similar to soluble CD4) and being more sensitive to loop packing, whereas VRC01 has a different angle of approach and its interaction with the functional virus spike is amenable to some alteration of the spike configuration [44]. The loss of recognition of the V3-targeting antibody 10–1074 was unexpected as the V3 loop exposure should be heightened post-CD4 attachment with the removal of the V2 glycan (N197) which normally occludes V3 antibodies from accessing their epitopes [9]; however, a study looking at cell surface expressed Env conformational states with increasing CD4 engagement has shown that antibodies targeting the V3 high man-nose patch can drastically differ in their reactivity to unbound- and CD4 induced triggered conformations [45]. Specifically, of the four V3-directed bNAbs they tested, PGT128 and PGT135 bound better to the CD4 engaged, open trimer compared to the closed trimer, the binding of 2G12 was largely unchanged for all Env conformations, while PGT121 preferentially bound to the ligand-free, closed trimer with a noticeable decrease in binding potential for the CD4-bound Env [45]- similar to our result for 10–1074. Future studies to validate the different structural conformations between our FVC<sub>ENV</sub>SOSIP.664-2dCD4<sup>60C</sup> complexes can be conducted using cryoelectron microscopy.

Notably, the conformational difference between closed and open states could expose additional epitopes and may explain the potent cross-neutralizing response seen here and similar studies [21]. Depletion of the antisera response in the Killick et al. study showed that the neutralizing response could be broadly mapped to the CD4 portion of the immunogen [21]. Exposure of previously shielded residues could explain the improved immunogenicity
seen with Env covalently complexed to 2dCD4S60C as the epitopes may be presented in the Env and CD4 components of the complex. The potential concern in the vaccine field with using CD4-bound HIV-1 Env immunogens is that following vaccination, they could induce “self” antibodies that target the host CD4 T-lymphocytes. This concern was fueled by research conducted in the 1990’s showing the presence of CD4-targeting autoantibodies in HIV-1 infected individuals which presumably arise from the presence of naturally occurring gp120-CD4 complexes during disease progression [46–55]. In a variety of clinical settings, anti-CD4 antibodies result in a decrease in CD4 expressing T cells [56–58]. However, in HIV-1 preclinical studies, anti-CD4 autoantibodies are rarely elicited by vaccination as evidenced by immunotoxicologic studies conducted in immunized cynomolgus macaques [59].

Of interest, antibodies which target host receptors have been discovered and engineered- Ibalizumab is an example of such a monoclonal antibody, which targets host CD4, and has shown efficiency in reducing viral load when administered as an antiviral therapeutic [60,61]. Furthermore, Ibalizumab has been FDA approved for use in combination antiretroviral treatment against multi-drug resistant HIV-1 in treatment experienced patients [62,63]. While Ibalizumab is a humanized monoclonal antibody, its approved use in a clinical setting sets a precedent for looking at modifying the current Env-2dCD4S60C immunogen using rational design to ensure we only elicit Ibalizumab-like antibodies. Such an immunogen would be capable of consistently eliciting potent, broadly neutralizing antibodies against a range of clinically relevant HIV-1 strains, without the requirement of complex, sequential Env immunization regimens currently being tested for driving appropriate neutralizing antibody affinity maturation.

Funding

This study was supported in part by grants from the department of Molecular Medicine and Haematology, HIV Pathogenesis Research Unit (HPRU) and the South African National Research Foundation (Thuthuka 113998 & 129859).

CRediT authorship contribution statement

Nancy L. Tumba: Conceptualization, Funding acquisition, Validation, Writing – original draft. Gavin R. Owen: Conceptualization, Supervision, Validation. Mark A. Killick: Conceptualization, Supervision, Validation. Maria A. Papathanasopoulos: Conceptualization, Funding acquisition, Supervision, Validation.

Data availability

Data will be made available on request.

Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2022.100222.
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