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Isolation and Characterization of Cross-Neutralizing Human Anti-V3 Single-Chain Variable Fragments (scFvs) Against HIV-1 from an Antigen Preselected Phage Library

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Abstract
Recently conducted human phase- I trials showed protective effect of anti-HIV-1 broadly neutralizing antibodies (bnAbs). The V3 region of the HIV-1 envelope is highly conserved as it is the co-receptor binding site, and is highly immunogenic. Recombinant single-chain antibody fragments (scFvs) can serve as potential tools for construction of chimeric/bispecific antibodies that can target different epitopes on the HIV-1 envelope. Previously, we have constructed a V3 specific human scFv phage recombinant library by a combinational approach of Epstein–Barr virus (EBV) transformation and antigen (V3) preselection, using peripheral blood mononuclear cells (PBMCs), from a subtype C HIV-1 infected antiretroviral naive donor. In the present study, by biopanning this recombinant scFv phage library with V3B (subtype B) and V3C (subtype C) peptides, we identified unique cross reactive anti-V3 scFv monoclonals. These scFvs demonstrated cross-neutralizing activity when tested against subtype A, subtype B, and subtype C viruses. Further, molecular modeling of the anti-V3 scFvs with V3C and V3B peptides predicted their sites of interaction with the scFvs, providing insights for future immunogen design studies. A large collection of such monoclonal antibody fragments with diverse epitope specificities can be useful immunotherapeutic reagents along with antiretroviral drugs to prevent HIV-1 infection and disease progression.

Keywords HIV-1 · scFv · V3B · V3C · Phage library

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Abbreviations

HIV-1	Human immune deficiency virus-1
scFv	Single-chain variable fragments
V3B	V3 region of subtype B HIV-1 envelope
V3C	V3 region of subtype C HIV-1 envelope

Introduction

HIV-1 interacts with the host cell CD4 and chemokine receptors via a trimeric envelope glycoprotein complex (gp160). This initial binding induces conformational changes in the viral envelope that exposes the V3 loop, which is involved in binding to one of the chemokine receptors CCR5 or CXCR4. The V3 loop is a 35-amino acid long region of the HIV-1 envelope glycoprotein and harbors a conserved motif (i.e., GPGQ/R) at its tip which is responsible for its interaction with chemokine co-receptors CCR5 and CXCR4 for viral entry. The V3 epitope is highly immunogenic and is known as a major target for anti-HIV-1 neutralizing antibodies [1, 2].

A large panel of monoclonal antibodies has been isolated against the V3 region and other regions demonstrated cross-neutralizing potential against HIV-1 of different subtypes [3–8]. The recently isolated anti-V3 monoclonal antibodies of the PGT series (PGT, 121,126, 128, 135) are found to be broadly neutralizing [9]. A recent phase I clinical trial showed that passive infusion of a single V3 glycan directed bnAb (10-1074) in humans reduced the viraemia in HIV-1 infected donors efficiently [10]. All these properties make the V3 loop an attractive target for immunogen design.

In studies conducted on HIV-1 infected adult and pediatric patients in India, we have observed the presence of high titres of anti-V3 antibodies in the plasma [11, 12]. In addition, the plasma of some of these patients effectively neutralized both subtype C and B viruses [13, 14]. Further, we have generated anti-V3 human mAbs and scFv monoclonals from patients whose plasma exhibited good viral neutralization potential that were effective in neutralization of viruses of different subtypes [7, 13, 15].

Anti-V3 cross-neutralizing scFvs were identified from a V3 specific human scFv phage recombinant library that we constructed from the PBMCs of a drug naive HIV-1 subtype C infected Indian donor [15]. The plasma antibodies of this donor exhibited neutralizing potential against a panel of different subtype viruses and also displayed cross-reactive anti-V3 antibodies. This phage library was constructed using a combinational approach of EBV transformation, antigen (V3) preselection followed by phage library construction. The anti-V3 scFvs generated from this library demonstrated cross-neutralizing activity against subtype C and non-subtype C viruses [15].

In the present study, we have further screened/biopanned this V3-specific library against HIV-1 envelope variable region 3 (V3) peptides of subtype C and subtype B to select out unique cross reactive scFv clones. We successfully isolated two cross reactive anti-V3 scFv clones (1E7B and 1F2C) after four rounds of biopanning.

Methods

Biopanning of scFv Phage Library

Clones displaying scFv fragments were selected by four rounds of biopanning over antigen coated plates (V3C and V3B). Both V3C (CTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC) and

V3B (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) peptides were mixed and coated onto the same well in ELISA plates at 100 ng/well in 0.1 M bicarbonate buffer (pH 9.6) and kept overnight at 4 °C. Next day, plates were washed three times with PBS. Rescue of the phage was performed by infection with M13-KO7 helper phage, precipitated with PEG/NaCl solution. The precipitated phage was resuspended in sterile PBS. Phage number was calculated and approximately 10^{12} phage were used in biopanning using Maxisorp ELISA plates (Nunc). Phage was diluted in 3% Milk PBS and were initially allowed to bind to the empty ELISA plate for 1 h. After 1 hour, unbound phage was transferred onto milk-coated wells; followed by BSA (bovine serum albumin), MPER (membrane proximal external region), ID loop-coated wells respectively for 1 h to remove nonspecific phage binders and then transferred to antigen-coated plates (V3C/V3B) for 1 h. After thorough washing, 10–15 times with PBS and then 20 times with PBST (0.2%) to remove the nonspecific phage binders, the bound phage was eluted by incubating with 0.2 M glycine, pH 2.2 for 10 mins. The eluted phages were immediately neutralized with 1 M Tris pH 9.2 and infected with *E. coli* TG1 (OD ~ 0.4 to 0.5) for 30 mins without shaking followed by 30 mins with shaking (200 rpm) at 37 °C. The cells were spun down at 3000×*g* for 10 mins. The pellet was resuspended in 200 μl of 2YT (Yeast and Tryptone) medium and spread on 2XYT agar plates containing chloramphenicol and 2% glucose. Individual clones were picked and grown in 96-well culture plate and glycerol stocks were made and plates were stored at − 80 °C until further use.

Phage Rescue

Individual colonies were grown in 2 ml of 2XYT media containing chloramphenicol (70 mg/ml) and 2% glucose overnight with shaking at 37 °C at 180 rpm. A small inoculum of this overnight grown culture was transferred to 1 ml of 2XYT media containing chloramphenicol (70 mg/ml) and 2% glucose at 37 °C at 180 rpm until the OD reaches 0.4 to 0.5. The helper phage M13-KO7 was added and plate was incubated at 37 °C for 30 mins without shaking and 30 mins with shaking at 220 rpm. The cells were spun down at 2000×*g*, supernatant was discarded and pellet washed with 2XYT broth. The pellet was then suspended in 1 ml of 2XYT broth containing chloramphenicol (70 mg/ml) and kanamycin (100 mg/ml) with shaking at 150–160 rpm for 16–20 h at 30 °C. The cells were pelleted down at 6000×*g* and supernatant was collected and stored at 4 °C.

Phage ELISA

ELISA plates were coated with 100 μl of V3C/V3B antigens (1 μg/ml) in coating buffer overnight at 4 °C. Plates were washed three times with 1X PBS. Plates were blocked with 200 μl of 5% MPBS for 1 h 30 mins at 37 °C. ELISA plates were then washed three times with PBS followed by addition of 100 μl of phage supernatant to each well. The plates were then incubated for 50 mins at RT. The ELISA plates were then washed six times with PBST (0.2%). The bound phages were detected by incubation with 1:2000 dilution of anti-M13 rabbit antibody for 1 h at RT, followed by 1h incubation with 1:3000 dilution anti-rabbit HRP. The ELISA reaction was developed by adding 100 μl of ortho-phenylenediamine (OPD) substrate and incubated at RT until the color developed. The reaction was stopped by adding 50 μl of stop solution 8 N H_2SO_4. Absorbance was measured at 490 nm.

PCR Amplification of Selected scFv Clones

Plasmids were isolated from the antigen binding clones (1E7B and 1F2C) using Qiagen Mini prep and quantified. A PCR reaction was performed to check the presence of complete scFv. The scFv was amplified using forward primer PTfw 5′CAG TCA TTC TAT GCG GCC CAG CCG GCC ATG GCC 3′ and reverse primer pAK sfi 5′ TCA GCA T***GG CCC CCG AGG CC***G CAC GTT TRA T 3′ (94 °C for 1 min, annealing at 61 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 mins). The amplified PCR product was resolved on a 1% agarose gel.

Soluble scFv Expression and Purification of Antigen Binding Clones

The antigen binding clones 1E7B and 1F2C showing binding/positive reactivity in phage ELISA were further processed for soluble expression. The scFv clones were digested from pAK100 vector and sub-cloned into pAK400 vector and transformed into *E. coli* strain HB2151. Transformed clones were grown in 10 ml of 2XYT media (Chloramphenicol) overnight at 37 °C with shaking at 200 rpm and next day 1/100th volume of overnight culture was inoculated in 1 L of 2XYT media supplemented with Chloramphenicol. The culture was grown at 37 °C with shaking at 220 rpm till the OD reaches 0.6 to 0.8. The soluble scFv expression was induced with 1 mM IPTG for 4 h at 24 °C. The cells were harvested at 4000 x g for 10 mins at 4 °C. The scFvs were purified from the periplasmic fraction. The pellet was resuspended in 30 mM Tris-Cl, 20% sucrose, pH 8.0. The cells were placed on ice and 500 mM EDTA was slowly added (dropwise) to final concentration of 1 mM and finally incubated on ice for 30 min with gentle agitation. Cell suspension was centrifuged at 8000×*g* for 25 mins at 4 °C. The supernatant was collected and pellet was resuspended in 5 mM $MgSO_4$. The cells were kept on ice for 30 mins with gentle agitation. Cells were centrifuged at 8000×*g* for 20 mins at 4 °C and the supernatant was collected. Both the fractions (sucrose and $MgSO_4$) were extensively dialyzed against PBS (pH 7.4) and finally dialyzed against lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The periplasmic extract was filtered through a 0.22-μm filter to remove any cell debris. The scFv clones were purified by metal affinity chromatography. The column was prepared using Ni-NTA resin (Qiagen) as per the manufacturer's protocol. In brief, the column containing Ni-NTA resin was equilibrated with 10 column volume of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The equilibrated Ni-NTA resin was mixed with the filtered periplasmic extract and placed on a rotatory shaker for binding at 4 °C for 30 mins. Further, the periplasmic extract was allowed to pass through the column with a flow rate of 3 ml/min. The column was washed with 150 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole). The bound scFv clones were eluted out in 1.5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8.0). The eluted scFv protein was extensively dialyzed against filtered sterile PBS and concentrated by Centriprep 3K filter. The concentration of purified scFv clones were determined using bradford assay using different dilutions of bovine serum albumin as a standard.

Soluble scFv ELISA

Soluble scFv ELISA was performed exactly as described in phage ELISA, except herein 100 μl of soluble scFv periplasmic extract/purified scFv (10 μg/ml) was added to the ELISA plates instead of phage. A 1:1000 dilution of anti-His tag antibody (Sigma-Aldrich) in 2% Milk phosphate buffer saline was used as detecting antibody for the His-tagged scFvs and

1:3000 dilution of anti-rabbit HRP (Thermo-Fischer scientific) antibody was used as secondary antibody.

DNA Fingerprinting Analysis

Uniqueness of the sequences of the two scFv clones was checked by DNA fingerprinting analysis. The scFv sequences from the two clones were amplified using primers PTfw 5′ CCT TTC TAT GCG GCC CAGCCG GCC ATG GCC 3′ and pAK sfi 5′ TCA GCA T***GG CCC CCG AGG CC***G CAC GTT TRA T 3′ (94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 mins). The amplified PCR products were digested with a frequent cutter restriction enzyme B*stN*1 (New England Biolabs) for 4–6 hrs at 60 °C and resolved on a 2% agarose gel.

Blue Native PAGE, SDS-PAGE, and Western Blot

Purified scFvs were analyzed in a gradient 4–15% blue native polyacrylamide gel electrophoresis (BN-PAGE) (Mini-PROTEAN TGX ™ Bio-Rad, Inc.) without denaturing the protein as described previously [16]. For SDS-PAGE, scFvs were denatured in presence of SDS and beta-mercaptoethanol by boiling the samples for 5 mins. Next, scFvs were separated on a 12% resolving gel and 5% stacking gel. The resolved gel for both BN-PAGE and SDS-PAGE was stained with Coomassie Brilliant Blue (R-250) for 1 h, de-stained and visualized. For Western blotting, gel was transferred onto nitrocellulose membrane using electroblotting (100 V for 1 h), probed with anti-His tag (Sigma-Aldrich) primary antibody and detected with anti-rabbit HRP (Thermo-Fischer scientific) conjugate secondary antibody. The blot was developed using diaminobenzidine (DAB-Sigma-Aldrich) as substrate.

Nucleic Acid Analysis of Anti-V3 scFv Antibody Genes

Plasmids were isolated from the antigen binding clones (Qiagen Mini prep) and sequenced by Macrogen (South Korea). The sequences were analyzed using IMGT/V-BASE software (http://imgt.cines.fr) and homology searches were done using immunoglobulin BLAST. Nucleotide sequences of the two scFvs (1E7B and 1F2C) were submitted to GenBank database having given accession numbers: BankIt20614201E7B[homoMG471438] BankIt20614201F2C[homoMG471439].

Neutralization Potential of the Anti-V3 scFvs

The neutralization efficiency of the purified scFvs was tested against a standard panel of pseudoviruses of subtypes A, B, and C obtained from the NIH AIDS Research and Reference Reagent Program, by TZM-bl assay [15]. The standard panel of pseudoviruses have been categorized from tier 1 to tier 3, based on the decreasing order of susceptibility to neutralization sensitivity by the known monoclonal antibodies. The neutralization assay was carried out in 96-well tissue culture plates. Briefly, 50 μl of the purified scFvs (100 to 6.25 μg/ml) was added to 200 TCID (tissue culture infective dose) of the virus and incubated for 1 h at 37 °C incubator. A cell control well containing culture media and cells only and a virus control well containing both the virus and the cells were tested in parallel. The scFv HepB, a scFv against the hepatitis B surface antigen [17] and monoclonal antibody 1418 (antibody to parvovirus),

were both used as negative controls. The percentage neutralization was calculated based on the relative luminescence units (RLU) in the presence of scFv divided by that of the virus control. The cell control value was subtracted from the scFv-RLU value as the background cutoff.

Molecular Modeling and Docking Studies of Interactions between scFvs and V3B/V3C Peptide

The antibodies were numbered as per Kabat rule and the Complementarity Determining Regions (CDRs) and Framework regions (FRs) were analyzed. Molecular modeling and docking studies were carried out as described earlier [18]. Briefly, the molecular models of the scFvs were generated by web-based antibody modeling server, Prediction of Immunoglobulin Structure (PIGS) using default parameters (http://arianna.bio.uniroma1.it/pigs) [19]. The molecular model of V3B and V3C peptides were predicted using I-TASSER of threading method (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [20]. The server generated five models and the best one was selected based on C-score. All the predicted models were viewed and analyzed in Pymol viewer. Web-based server "PatchDock" was used for the peptide-scFv docking studies (http://bioinfo3d.cs.tau.ac.il/PatchDock/) [21], where molecular models of scFv and V3B/V3C peptide were fed as input in the antigen-antibody algorithm of the docking server. The model ranked highest by the server based on geometric score, desolvation energy, interface area size, and the actual rigid transformation of the solution was selected for analysis.

Results

Selection of Cross Reactive Anti-V3 scFv Monoclonals

In this study, we identified two unique anti-V3 scFvs, 1E7B and 1F2C, by performing four rounds of biopanning of a previously constructed V3-specific scFv phage library using V3C and V3B as antigens [15]. In brief, the V3 specific phage library of 7000 scFv clones was earlier constructed from a drug naïve HIV-1 subtype C infected Indian donor whose plasma exhibited cross-neutralizing antibodies against a panel of viruses. The binding specificity of the anti-V3 scFvs identified here was checked using phage from a 24 well plate. A total of 30 clones were randomly picked up and specificity of these clones was checked by indirect ELISA. Among the clones tested, two scFv clones 1E7B and 1F2C demonstrated V3C and V3B binding reactivity in phage ELISA and did not show any reactivity against other unrelated peptides; however, the other reactive clones showed very weak binding (Fig. 1). Hence, the 1E7B and 1F2C scFvs were further analyzed.

On DNA fingerprinting analysis, both scFvs clones demonstrated different patterns on the agarose gel, confirming their distinct sequences (Fig. 2). Homology searches using the immunoglobulin BLAST revealed VH4-31 heavy chain gene usage by both the 1E7B and 1F2C scFvs; 1E7B showed VK3-20 light chain gene usage while for 1F2C it was VK2-28 (Table 1).

Expression, Purification, and Specificity of the scFv Monoclonals Were Confirmed by SDS-PAGE, Western Blotting, and ELISA

The scFv clones (1E7B and 1F2C) that showed effective binding in phage ELISA were solubilized. The yield of the purified 1E7B & 1F2C using Ni-NTA resin was 0.5 and

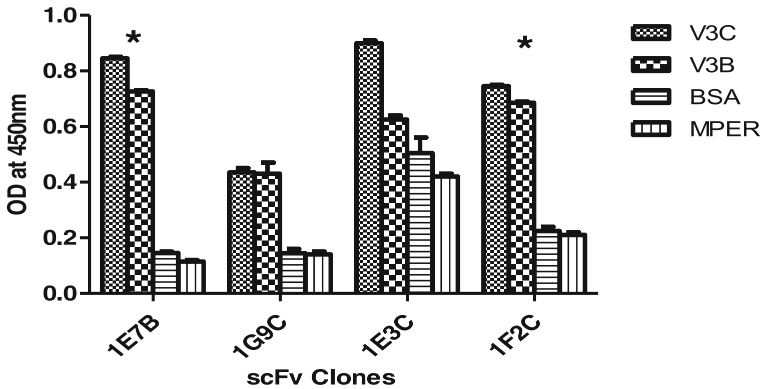

Fig. 1 Phage ELISA of scFv clones showing high binding to V3B and V3C. BSA and MPER were taken as negative controls. The experiment was repeated at least twice and the mean OD values are shown. An OD at least two times higher than the negative control was taken as significant

0.3 mg scFv/l culture respectively. On BN-PAGE and SDS-PAGE analysis, bands of expected size of 32 kDa of both scFv clones were observed, confirmed by Western blotting (Fig. 3).

The functional activity of the purified scFvs was demonstrated by the specific binding reactivity of the scFvs to V3C and V3B peptides in indirect ELISA and no binding to other unrelated peptides like ID loop, MPER of HIV-1, and BSA (Fig. 4).

Further, we assessed the relative binding efficiency of the previously described [15] scFv clones 3E6B and 3E7B compared to the scFv clones 1E7B and 1F2C identified in this study with gp120 consensus C and gp120 consensus B proteins at a concentration range of 10 to 0.625 μg/ml (Fig. 5). The 1E7B scFv showed highest binding to both subtype C and subtype B

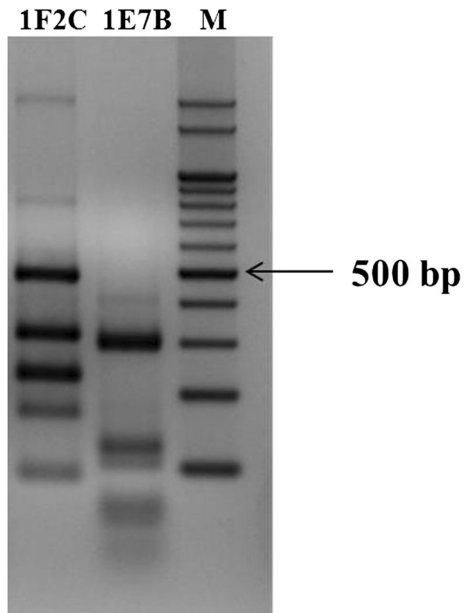

Fig. 2 DNA fingerprinting of scFv clones. scFvs were amplified by PCR and digested with BstN1 at 60 °C for 3 h. Restriction pattern of 1F2C and 1E7B analyzed on 2% agarose gel is shown. Lane M is the molecular weight marker

Table 1 Gene usage of the two scFvs

Clone ID	VH (heavy chain)			VL (light chain)	
	V	D	J	V	J
1E7B	IGHV4-31*03	IGHD5-24*01	IGHJ6*03	IGKV3-20*01	IGKJ2*02
1F2C	IGHV4-31*02	IGHD5-5*01	IGHJ6*03	IGKV2-28*01	IGKJ4*01

Heavy and light chain gene usage of two scFvs. The scFvs 1E7B and 1F2C showed distinct gene usage

proteins whereas binding efficiency of 1F2C and 3E6B were comparable. The scFv HEP, a scFv against the hepatitis B surface antigen [17] was used as negative control.

Molecular Modeling of the Anti-V3 scFvs with V3C and V3B Peptides Predicted the Epitopes of scFvs

Advances in molecular modeling based on homology with known structures in the absence of solved crystal structures has made it possible to decipher the near-native structures and interactions of antibodies and antigens. Prediction of Immunoglobulin Structure, PIGS (http://arianna.bio.uniroma1.it/pigs), server was used for building the molecular model for the 1E7B and 1F2C scFvs, and two previously reported 3E6B and 3E7B scFvs using default parameters. The predicted structures showed β-pleated structures characteristic of the immunoglobulins and the CDRs of all scFvs attained the characteristic loop structures, forming distinct antigen binding pockets with unique surface topology. The structural models for the HIV V3B and V3C peptides were predicted by the web-based molecular modeling server, *i-Tasser* (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Model for both the peptides was the best predicted structural model and assigned the highest confidence score (C-score) by the server. The molecular models for both peptides were analyzed by Pymol and it was predicted that the V3B peptide attained a loop conformation in solution and with the N-terminus attaining a secondary beta-sheet conformation. The V3C peptide also attained a similar conformation but differed structurally in the C-terminus where amino acids 29-32 attained a helical structure.

A cartoon representation of the docking of the modeled V3C and V3B peptides and molecular interactions with the 1E7B, 1F2C, 3E6B, and 3E7B scFvs is shown in Fig. 6 (left panel). The putative docking models for 1E7B and 3E6B scFvs with both the peptides show that the residues from both heavy chain and light chain contribute to their binding to the

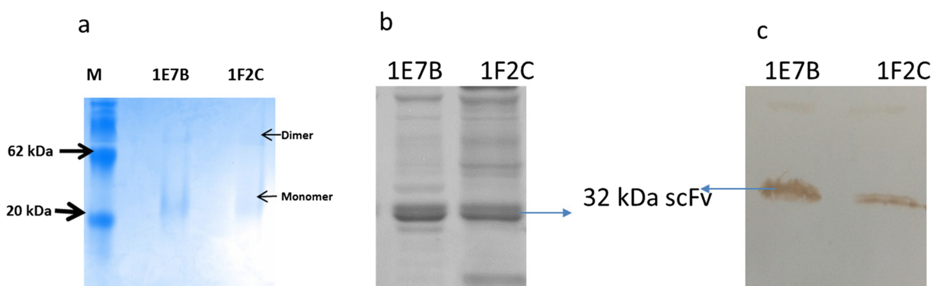

Fig. 3 BN-PAGE, SDS-PAGE, and Western blot: BN-PAGE (**a**), SDS-PAGE (**b**), and Western blot (**c**) of purified 1E7B and 1F2C scFvs. Arrow indicates the 32-kDa band of purified scFvs

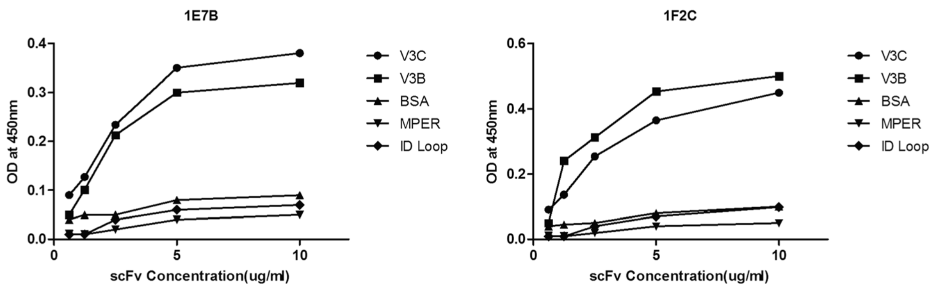

Fig. 4 ELISA to confirm functional specificities of purified scFvs. ELISA binding reactivity of 1E7B and 1F2C scFvs (10 to 0.625 μg/ml), with 35 mer V3C and V3B peptide. BSA, MPER, and ID loop were taken as negative controls

peptides. On the other hand, the predicted docking structure of the 1F2C-scFv to the two peptides shows a preferential usage of the heavy chain residues with no contributions from the light chain towards peptide binding. Also, the docking prediction for 3E7B-scFv shows preferential usage of light chain for binding to V3C whereas both chains are involved in binding to the V3B peptide. The right panel of Fig. 6 shows the putative residues of the two peptides interacting with the heavy and light chain residues of the scFvs. The 1E7B-V3B interaction comprises of just two 1E7B heavy chain residues, ThrH57 from CDRH2 and SerH57 from FRH3 forming polar contacts with Asn5 and His13 respectively, of the V3B peptide (Fig. 6a). On the contrary, 1E7B forms multiple interactions with the V3C peptide wherein the interacting residues are predominantly light chain residues with a lone heavy-chain contribution towards binding coming from GlyH42 from the FRH2 which interacts with the Asp29 of the V3C peptide. Four light chain residues predicted to be contributing to the binding of 1E7B to V3C were surprisingly not CDR residues but were framework residues from FRL1, FRL2, and FRL4. The predicted epitope was found to be discontinuous extending from position 17 to 32 encompassing the short helix forming residues between amino acid 29-32. SerL12 from FRL1 and GlyL41 from FRL2 were found to form polar contacts with the two V3C Gln residues at position 18 and 32 respectively while the two FRL4 residues, LysL103, and LysL107 interact with Tyr21 and Gly17 of the V3C peptide respectively.

Similar to 1E7B, the 1F2C-scFv interacts with the V3B peptide through its FRH1 and FRH3 residues. SerH7 and AlaH8 residues of FRH1 form polar contacts respectively with the Ala19 and Lys10 of the V3B peptide (Fig. 6b). Other contributions to the binding come from the FRH3 residues SerH70, AspH72, and SerH74 which form interactions with Glu32, Arg31,

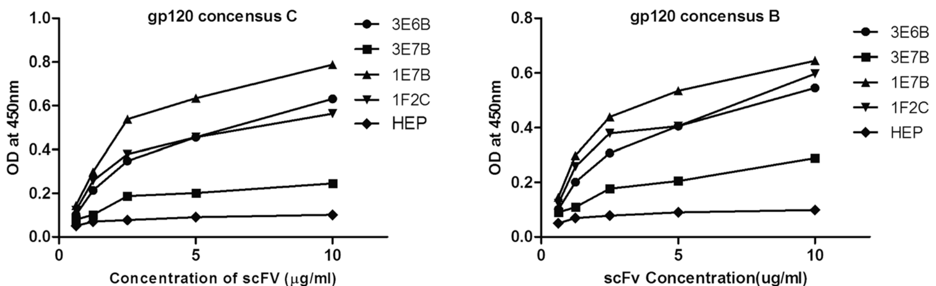

Fig. 5 Relative binding efficiencies of purified scFvs. ELISA binding reactivity of scFvs (10 to 0.625 μg/ml), with gp120 conc C and gp120 conc B. HEP scFv (a scFv against the hepatitis B surface antigen) was taken as negative control

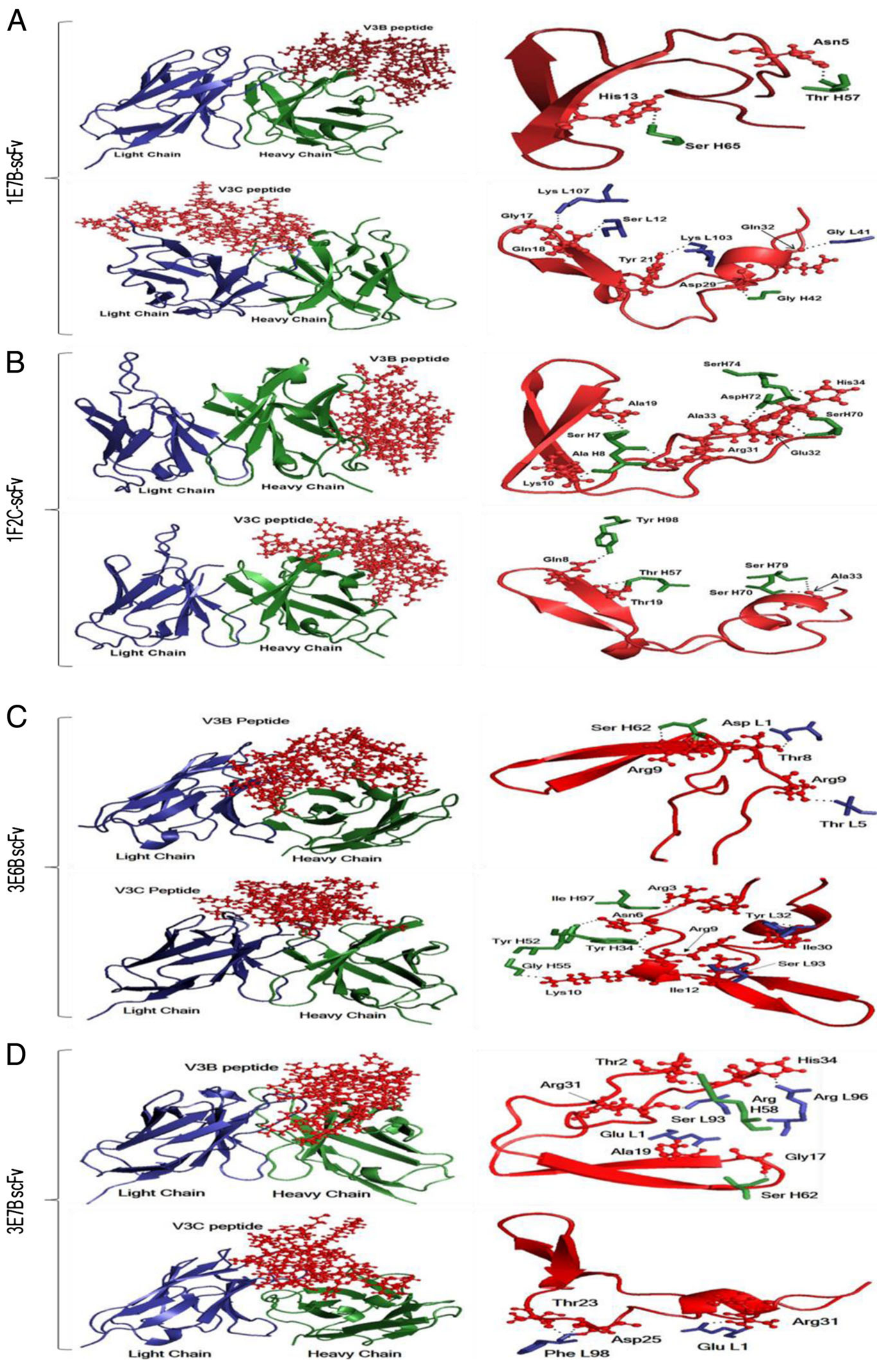

Fig. 6 Molecular modeling. Structural prediction of 1E7B, 1F2C, 3E6B, and 3E7B scFvs with V3C and V3B peptides were done using the antigen-antibody interface of the web-based docking server "Patchdock." Left panel shows the docking of V3B and V3C peptides to the respective scFvs (cartoon) while the right panel shows the predicted interacting residues of the peptide (red) with the heavy chain (green) and light chain (blue) residues of the scFvs

Ala33, and His34 respectively. AspH72 provides a dual interaction with V3B-peptide residues Arg31 and Ala33. 1F2C-scFv interacts with the V3C peptide through its CDRH2, CDRH3, and FRH3 residues wherein the ThrH57 of CDRH2 and TyrH98 of CDRH3 interact respectively with Thr19 and Gln8 of the V3C peptide while Ala33 of V3C forms a dual interaction with two FRH3 residues, SerH70 and SerH79.

Molecular docking of the previously reported scFvs, 3E6B, and 3E7B (Fig. 6c, d) suggests that they have a different mode of antigen recognition as compared to the 1E7B and 1F2C scFvs and interact with the V3B and V3C peptides using both heavy and light chain residues; 3E7B scFv-V3C being an exception as only light chain residues are involved in antigen binding. Both the scFvs interact with distinct putative interacting residues of the V3B and V3C peptides using distinct paratopic residues. Interestingly, the predicted 3E6B-scFv residues involved in interaction with the V3B peptide are framework region residues of heavy and light chain. Similarly, it is predicted that the 3E7B residues that interact with the V3C peptide are also framework residues but only from the light chain. Of the two residues involved in binding, one residue, L98Phe is a vernier residue which belongs to the FRL4. On the other hand, 3E6B scFv-V3C and 3E7B scFv-V3B interaction is predominantly through the heavy and light chain CDRs of scFvs and the number of scFv residues involved in antigen recognition are 6 and 5 respectively. However, the set of V3B and V3C residues recognized by the four scFvs are completely distinct. The residues recognized by the four scFvs and the paratopic residues interacting with them are enlisted in Table 2.

Neutralization Efficiency of 1E7B and 1F2C scFvs

The neutralization efficiency of 1E7B and 1F2C scFvs were checked against a standard panel of 16 pseudoviruses (obtained from the NIH ARRRP) and primary isolates [11, 22, 23] from different subtypes (7 subtype B, 7 subtype C, and 2 subtype A). The scFv monoclonals were tested at a maximum concentration of 100 μg/ml. Both the scFv clones were able to neutralize the tier 1 viruses. The 1E7B scFv clone showed a better neutralization profile as compared to 1F2C against tier 2 viruses (Fig. 7).

The relative neutralization efficiency of the scFv clones 1E7B and 1F2C identified in this study [15] was evaluated with respect to that observed for the 3E6B and 3E7B, against four representative pseudoviruses (2 each from subtype B and from subtype C). The scFv monoclonals were tested at a concentration range of 100 to 6.25 μg/ml. The 1E7B scFv was most effective against 6535.3, 25710.2.43, and SF162 viruses whereas 1F2C was most effective against 25710.2.43 and 6535.3 viruses. (Fig. 8).

Discussion

The single-chain variable fragments (scFvs) hold several advantages over the conventional full-length monoclonal antibodies. Being smaller in size, scFvs can easily penetrate interior masked epitopes, can be easily engineered and modified to enhance their properties like greater thermal and pH stability, better biocompatibility and tissue permeability, lower cost and ease of production as aqueous and lyophilized formulations [24]. Currently, scFvs are widely used as therapeutic agents for angiogenesis dependent diseases [25] and for targeting metastatic cancer [26].

The phage displayed scFv libraries are found to be efficient for identification of antigen specific scFvs [27]. Using biosensor-based high-throughput screening system, high affinity

Table 2 Interacting residues of the V3B/V3C peptide with heavy and light chains of scFvs

1E7B scFv-V3B interaction			1F2C scFv-V3B interaction			1E7B scFv-V3C interaction			1F2C scFv-V3C interaction		
V3B residue	1E7B scFv residue	Location	V3B residue	1F2C scFv residue	Location	V3C residue	1E7B scFv residue	Location	V3C residue	1F2C scFv residue	Location
Asn5	H57Thr	CDRH2	Ala19	H7Ser	FRH1	Asp29	H42Gly	FRH2	Thr19	H57Thr	CDRH2
His13	H57Ser	FRH3	Lys10	H8Ala	FRH1	Gln18	L12Ser	FRL1	Gln8	H98Tyr	CDRH3
			Glu32	H70Ser	FRH3	Gln32	L41Gly	FRL2	Ala33	SerH7 SerH79	FRH3
			Arg31, Ala33	H72Asp	FRH3	Tyr21	L103Lys	FRL4			
			His34	SerH74	FRH3	Gly17	L107Lys	FRL4			
3E6B scFv-V3B interaction			3E7B scFv-V3B interaction			3E6B scFv-V3C interaction			3E7B scFv-V3C interaction		
V3B residue	3E6B scFv residue	Location	V3B residue	3E7B scFv residue	Location	V3C residue	3E6B scFv residue	Location	V3C residue	3E7B scFv residue	Location
Thr8	L1Asp	FRL1	Thr2	H58Arg	CDRH2	Lys10	H55Gly	CDRH2	Thr23 Asp25	L98Phe	FRL4
Asn6	L1Thr	FRL1	His34	L96Arg	CDRL3	Asn6	H52Tyr	CDRH2	Arg31	L1Glu	FRL1
Arg9	H62Ser	FRH3	Arg31	L93Ser	CDRL3	Asn6 Arg9	H34Tyr	CDRH1			
			Ala19	L1Glu	FRL1	Arg3	H97Ile	CDRH3			
			Gly17	H62Ser	FRH3	Ile12	L93Ser	CDRL3			
						Ile30	L32Tyr	CDRL1			

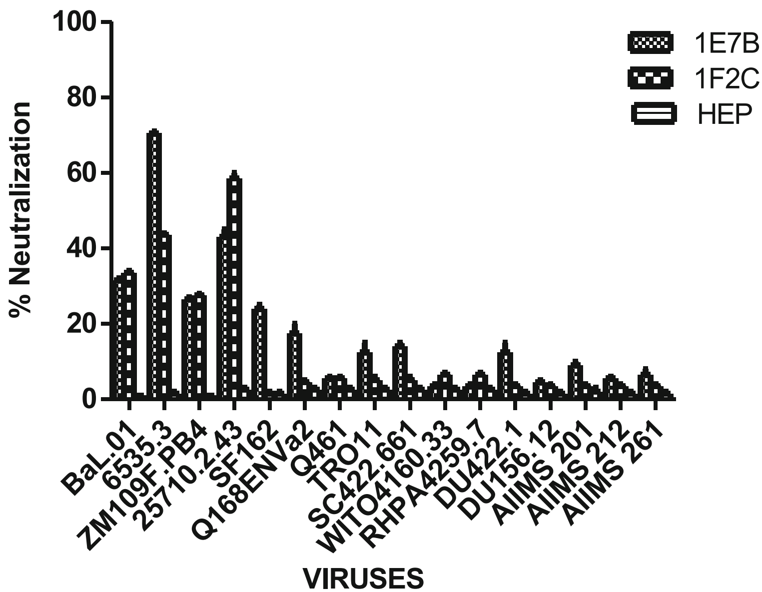

Fig. 7 Neutralization efficiency of 1E7B and 1F2C scFvs: scFvs were checked against a standard panel of subtype A (Q168ENVa2, Q461), subtype B (BaL01, 6535.3, SF162, TRO11, SC422.661, WITO4160.33, and RHPA4259.7), and subtype C (ZM109F.PB4, 25710.2.43, DU422.1, DU156.12, AIIMS201, AIIMS212, AIIMS261) pseudoviruses and primary isolates

scFvs have been identified from such libraries [28]. We had earlier reported the construction of antigen-specific phage library using a modified strategy of EBV transformation followed by recombinant phage library construction. This strategy was found to be an efficient way to isolate antigen-specific scFv clones from a small phage library. In this study, we have further demonstrated that antigen preselected phage libraries are suitable for rapid isolation of unique scFv clones and assessed the potential of the library as a source of potent V3 specific scFvs, of similar, yet diverse epitope specificities within the V3 region. Use of both V3C & V3B peptides during biopanning of the phage library increases the likelihood of identifying cross reactive clones, as the population of cross reactive phages increase after every round of biopanning. Both the 1E7B & 1F2C scFv clones were derived from same parental germline clone (VH4), as was inferred from their gene sequences.

The scFv antibodies have been reported by many research groups to form dimers, trimers, and multimers, when short linker peptides are used [29, 30]. Dimerization of scFv antibodies has been reported, even when long linker peptides were used [31, 32]. The explanation for this phenomenon may be that the linker is presumably constraining the folding, and hindering appropriate association of VH and VL fragments located on the same polypeptide. Then, VH-VL interface exposes hydrophobic residues, thus increasing the likelihood of intermolecular associations, and resulting in multimerization. In this study, Western blot analysis indicated the predominance of the monomeric scFv purified. The yield of the purified scFvs ranged from 0.2 to 0.5 mg/l culture. These were in the same range as reported by the other groups for recombinant antibodies [33–35].

It has been shown in mice, that an extensive V (both V_H and V_L) gene repertoire is not necessary for the production of specific antibodies to most antigens, and that antigen-specific IgM molecules isolated from primary response of mice constrained to use a single VH gene but full CDR3 diversity to generate their B cell repertoire, and can differ only in the CDR3 of the

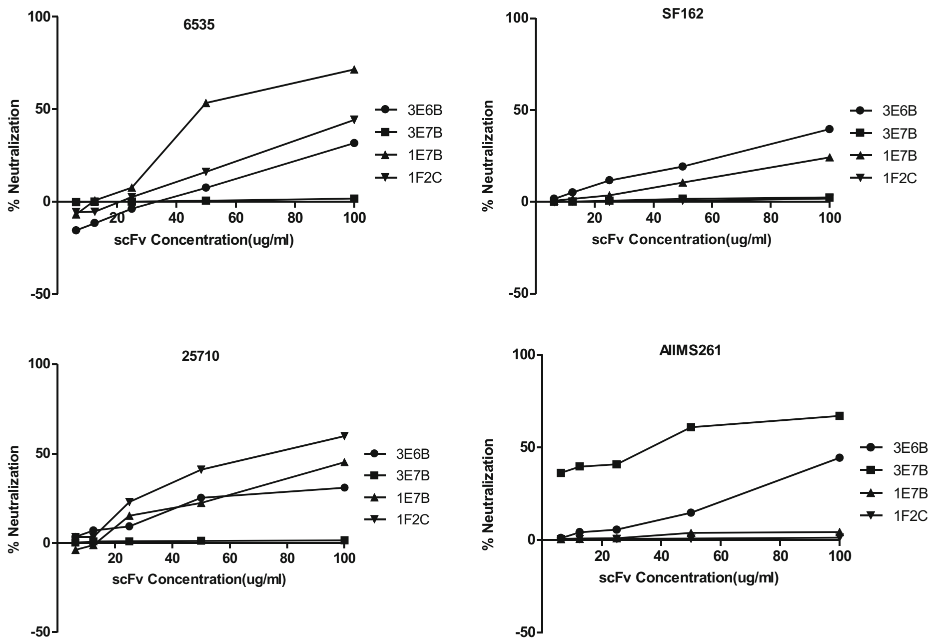

Fig. 8 Comparative neutralization efficiency of scFvs: Percent neutralization of scFvs was evaluated with representative subtype B (6535.3, SF162) and subtype C (25710.2.43, AIIMS261) pseudoviruses and primary isolates. The scFv monoclonals were tested at a concentration range of 100 to 6.25 μg/ml

VH domain [36]. Also, the FR residues are known to contribute to antigen recognition and binding through direct antigen contact or being in close proximity to it in the 3D structure. The heavy chain FRH3 loop in particular which is also enriched (in human Abs) in somatic hypermutations other than the hypervariable CDRs also accounts for some human Ab-Ag contacts [37, 38]. Taken together, it can be safely inferred that the heavy chain has a greater role in antigen binding. Our molecular docking results suggest that the 1F2C scFv preferentially utilizes the heavy chain residues (mainly FRH3 residues) for binding to both V3B and V3C peptides while 1E7B scFv putatively utilizes residues from both heavy and light chains.

The comparative analysis of the neutralization efficiency of 1E7B and 1F2C scFvs isolated in this study with the 3E6B and 3E7B scFv isolated in the previous study [15] from the same library showed varied neutralizing activity of the individual scFvs against different viruses. The four antibodies have distinct sequence and structures and the docking studies predict different modes of recognition of the HIV peptides by each scFv, which would be the possible explanation for the varied neutralization abilities of the four scFvs. A cocktail of antibody fragments with varying specificities have been shown to provide better coverage of the antigenic epitopes and may also provide broader neutralization [18]. Similarly, the diverse specificities and cross-neutralization potentials of these four scFvs can be better harnessed in combination for the development of novel therapeutic agents for blocking HIV-1 infection. However, the need for use of scFvs (being monovalent) at a high concentration to achieve the desirable viral neutralization breadth is a limitation. This might be overcome by improving their affinity through antibody engineering or by using a combination of scFvs of similar or unique specificities. Taken together, a large collection of recombinant, neutralizing antibodies can be used as immunotherapeutic agents to prevent HIV-1 infection and disease progression.

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Authors' Contributions KL conceptualized and planned the study, edited and finalized the manuscript; RK1 constructed phage library. RK1 and RK2 biopanned the library, selected and expressed scFv clones. LK and HAP helped RK1 and RK2 in neutralizations assays of scFvs. RK1 and LK analyzed the data and co-wrote the manuscript with KL; AS helped RK1 with analysis of molecular modeling and docking studies. NW provided the HIV-1 infected donor samples. AT and SS provided scientific inputs in phage library construction. SS also provided valuable inputs in the manuscript write-up.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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