

THE ROAD TO A CURE

Characterization of a New HIV Antibody

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A PHAGE DISPLAY LIBRARY WAS PREVIOUSLY CONSTRUCTED FROM AN SIV-INFECTED RHESUS MAQUAE. SEVERAL SINGLE CHAIN FV (SCFV) WERE SELECTED USING PHAGE DISPLAY TECHNOLOGY. SEQUENCES CORRESPONDING TO SU24, SU343 AND LL25X WERE OPTIMIZED FOR EXPRESSION IN A MAMMALIAN SYSTEM AND COMMERCIALY SYNTHESIZED. IN THIS STUDY, WE AIMED TO CHARACTERIZE THE SPECIFICITY OF SU24, SU343, AND LL25X. THE CODON-OPTIMIZED VERSION OF THE SCFV LL25X GENE SEQUENCE WAS CLONED INTO A MAMMALIAN EXPRESSION VECTOR (PCEP4). LL25X DNA WAS AMPLIFIED BY PCR, AND THE PCR PRODUCT AND MAMMALIAN EXPRESSION VECTOR WERE BOTH DIGESTED WITH KPNI/SAPI RESTRICTION ENZYMES. THE FRAGMENTS WERE LIGATED USING T4DNA LIGASE. E. COLI CELLS WERE TRANSFORMED WITH THE LIGATION REACTION. SINGLE COLONIES WERE SELECTED ON LB AGAR PLATES CONTAINING THE SELECTIVE ANTIBIOTIC (AMPICILLIN). POSITIVE COLONIES WERE IDENTIFIED WITH KPNI AND SAPI. SANGER SEQUENCING CONFIRMED CLONING RESULTS AND DNA SEQUENCE ACCURACY. FOLLOWING TRANSFECTION OF MAMMALIAN CELLS (293T), LL25X-FC CELLS, AND PURIFYING OUR PROTEIN, THE BINDING OF LL25X-FC TO THE SIV GP140 ENVELOPE PROTEIN WAS CONFIRMED.

INTRODUCTION

Since the discovery of HIV/AIDS in 1984, an effective vaccine has remained an elusive goal, despite the conjugation of numerous efforts. Progress made in the RV144 Thai vaccine trial has hinted that HIV envelope glycoprotein variable loop V1-V2 has a role in the prevention of infection.¹ The isolation of anti-SIV monoclonal antibodies (mAb) will allow a pre-clinical evaluation of such antibodies in the SIV-rhesus macaque model of HIV/AIDS pathogenesis and vaccine.

A phage display library was previously constructed from a SIV-infected rhesus macaque, and several single chain variable fragments (scFv) such as SU24, SU343, and LL25X were selected using phage display technology.² Sequences corresponding to SU24, SU343, and LL25X were optimized for expression in a mammalian system and commercially synthesized (IDT DNA). SU24 and SU343 had previously been cloned in a mammalian expression vector. In this study, we aimed to characterize the specificity of SU24, SU343, and LL25X.

MATERIALS AND METHODS

PCR (Polymerase Chain Reaction) Amplification

The codon-optimized version of the LL25X gene was to be amplified by PCR. A PCR mixture was prepared in a PCR tube. The PCR mixture consisted of 1 μ L of template DNA (100 ng - 1 μ g), 2 μ L of forward primer (FP), 2 μ L of reverse primer (RP), and 45 μ L of Platinum PCR Supermix High Fidelity from Thermo Fisher Scientific. The forward primer was LL25-co-F (GGCCGGTACCGGCGGCCAC-CATGG) and included the restriction site for KpnI. The reverse primer was LL25-co-R (ATTGTACTGCTCTTCCC-GTGGCTTGGTTTTTCGC) and included the restriction site for SapI. The PCR tube was closed tightly, mixed via vortex, and spun down using a small bench-top rotor.

The PCR tube was placed in a thermocycler for 25 cycles. The initial denaturation step was performed at 94°C for 5 minutes and was followed by 25 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 30 seconds), and extension (68°C for 1 minute). A final extension step was performed at 62°C for 10 minutes.

DNA gel electrophoresis of the PCR products was performed on 1% agarose gel. The DNA vector was observed

around 1092 bp under a UV transilluminator and was extracted using a gel extraction kit (QIAGEN).

Extraction of PCR Products

The PCR products were extracted from the 1% agarose gel following the QIAquick Gel Extraction Kit by QIAGEN. Instead of the recommended 50 μ L of Buffer EB, 30 μ L of double distilled water was added to the QIAquick membrane column to elute DNA and centrifuged immediately. Loading Dye was not added to purified DNA.

Double Digestion of PCR Products with KpnI and SapI

After gel extraction, the DNA vector from PCR was double digested with KpnI and SapI. The double digest reaction mixture consisted of 30 μ L of the DNA vector, 4 μ L of CutSmart Buffer from New England Biolabs, 1 μ L of KpnI, 1 μ L of SapI, and 4 μ L of double distilled water for a total concentration of 40 μ L. DNA gel electrophoresis of the double digested LL25X PCR product was performed on 1% agarose gel for 1 hour.

Extraction of PCR Fragment Double Digested with KpnI and SapI

The DNA of the insert vector resulting from the PCR fragment double digested with KpnI and SapI was extracted from the 1% agarose gel using the QIAquick Gel Extraction Kit protocol by QIAGEN. Instead of the recommended 50 μ L of Buffer EB, 30 μ L of double distilled water was added to the QIAquick membrane column to elute DNA and centrifuged immediately. Loading Dye was not added to purified DNA.

Double Digestion of Mammalian Expression Vector pCEP4 with KpnI and SapI

Three samples were prepared for the double digest with KpnI and SapI was performed on the mammalian expression vector pCEP4. One sample was the reaction mix for the double digest consisted of 1 μ L of cloning vector pCEP4, 2 μ L of CutSmart Buffer from New England Biolabs, 1 μ L of KpnI, 1 μ L of SapI, and 15 μ L of double distilled water. The other two samples served as controls. Control 1, the reaction mix for the single digest using KpnI, consisted of 1 μ L of cloning vector pCEP4, 2 μ L of CutSmart Buffer from New England Biolabs, 1 μ L of KpnI, 0 μ L of SapI, and 16 μ L of double distilled water. Control 2, the reaction mix for the single digest using SapI, consisted of 1 μ L of cloning vector pCEP4, 2 μ L of CutSmart Buffer from New England Biolabs, 0 μ L of KpnI, 1 μ L of

SapI, and 16 μL of double distilled water. DNA gel electrophoresis was performed on the sample reaction mix and the reaction mixes of the two controls on 1% agarose gel for 1 hour.

Extraction of Mammalian Expression Vector pCEP4 Double Digested with KpnI and SapI

The DNA backbone vector of mammalian expression vector pCEP4 double digested with KpnI and SapI was extracted from the 1% agarose gel using the QIAquick Gel Extraction Kit protocol by QIAGEN. Instead of the recommended 50 μL of Buffer EB, 30 μL of double distilled water was added to the QIAquick membrane column to elute DNA and centrifuged immediately. Loading Dye was not added to purified DNA.

Ligation of LL25X into Backbone Vector pCEP4

DNA gel electrophoresis of 2 μL of the double digested backbone vector pCEP4 and 2 μL of the double digested LL25X PCR product was performed on 1% agarose gel for 1 hour. Based on the band intensities on the gel, the volumes for the backbone and insert vectors could be estimated to achieve a backbone-to-insert ratio of 1:2 or greater. Two tubes were prepared. One tube was for ligation, and the other tube served as a control. The reaction mixture for ligation of the double digested LL25X PCR product into backbone vector pCEP4 consisted of 2 μL of the backbone vector (KpnI/SapI-digested pCEP4), 4 μL of the insert vector (KpnI/SapI-digested LL25X), 2 μL of T3 DNA ligation buffer (10x), 1 μL of T4 DNA ligase, and 11 μL of sterile water. The reaction mixture for the control consisted of 2 μL of the backbone vector (KpnI/SapI-digested pCEP4), 0 μL of the insert vector (KpnI/SapI-digested LL25X), 2 μL of T3 DNA ligation buffer (10x), 1 μL of T4 DNA ligase, and 15 μL of sterile water. The ligation reaction products were incubated overnight at room temperature.

Transformation of Ligation Reaction Products into E. coli TOP10F'

Frozen electrocompetent *E. coli* TOP10F' was removed from a -80°C freezer and thawed on ice for 10 minutes. The *E. coli* was aliquoted to obtain 50 μL in a tube, and 7 μL of ligation reaction was added to it. The tip of the pipette was swirled in the tube to mix the solution, and the tube was incubated on ice for 30 minutes. The transformation tubes were heat shocked at 42°C for 1 minute and put on ice for 2 minutes. The heat shock allowed the bacterial

membranes to be permeable enough for vector pCEP4 containing the LL25X DNA to enter the cells. Afterwards, 1 ml of LB media without antibiotics was added to the tube and cultured at 37°C for 45 minutes in a shaking incubator at 250 rpm. The tube was centrifuged at 9,000 rpm for 1 minute, and all but 100 μL of the supernatant was pipetted out. The remaining 100 μL of supernatant was mixed with the bacterial pellet using a pipette tip. All of the bacterial mix was plated onto an LB agar plate containing Ampicillin. A background control of cloning vector pCEP4-Fc-HA, digested and ligated without the LL25X insert vector, was also plated on the selective media.

Colony Screening of Positive Clones on Selective Media

Growth of the bacterial cells transformed with the pCEP4/LL25X ligation product was observed on the LB agar plate containing Ampicillin. Two colonies were picked up from the LB agar plate containing Ampicillin and were inoculated with 3 ml of LB media containing 50 $\mu\text{g}/\text{ml}$ of Ampicillin in a 15 ml culture tube. The tube was cultured overnight at 37°C in a shaking incubator at 250 rpm. The day after, 2 ml of bacteria was aliquoted into a 2 ml Eppendorf tube and centrifuged at 13,000 rpm at room temperature to pellet down the bacteria. The supernatant was discarded, and the pellet was obtained.

Extracting the pCEP4/LL25X DNA from the E. coli Cells via Miniprep

The QIAprep Spin Miniprep Kit protocol by QIAGEN was followed to isolate the pCEP4/LL25X DNA from the *E. coli* cells. The recommended step involving washing the QIAprep spin column with 0.5 ml of Buffer PB was omitted because endA+ strains or other bacterial strains with high nuclease activity or carbohydrate content were not used. None of the optional steps were taken, such as adding LyseBlue reagent to Buffer P1. The DNA concentration, determined by using Nanodrop, was 120.2 ng/ μL .

Determination of a Positive Clone by Restriction Analysis

One reaction mix contained the DNA template, and one control tube contained the cloning vector pCEP4. The reaction tube consisted of 3 μL of mini-prep DNA, 2 μL of CutSmart Buffer from New England Biolabs, 1 μL of KpnI-HF/SapI, 1 μL of KpnI-HF/BamHI-HF, and 13 μL of sterile water. The control tube consisted of 3 μL of cloning vector pCEP4, 2 μL of CutSmart Buffer from New England Biolabs, 1 μL of KpnI-HF/SapI, 1 μL of KpnI-HF/BamHI-HF, and 13 μL of sterile water. Both tubes had a total volume of

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20 μ L. The tubes were incubated at 37°C for 1 hour, and the products of the double digest were analyzed on a 1% agarose gel.

Preparation of Samples for DNA Sequencing

After DNA mini-prep, samples were prepared for DNA sequencing. Recommendations for DNA sequencing from Eton Bioscience were followed. For successful DNA sequencing, the reaction mix consisted of 8 μ L of the LL25X PCR product, 8 μ L of plasmid that was less than 6 kb and had a concentration of 50-150 ng/ μ L (obtained by DNA mini-prep and determined by Nanodrop), and 5 μ L of primer that had a concentration of 5 μ M. The reaction mix was sent to Eton Bioscience for DNA sequencing.

Sequence Analysis of LL25X

Once the DNA sequence results were received from Eton Bioscience, the software Geneious was used to analyze the sequence of LL25X.

Bacteria Culture in Aqueous LB media with Ampicillin, followed by DNA Maxi-prep

A selected positive clone was cultured for 8 hours in a culture tube containing 3 ml of LB/Ampicillin media. From the tube, 100 μ L of the culture was transferred to a flask with 500 ml of LB/Ampicillin and cultured overnight. The culture was transferred into centrifugation tubes the next day. The centrifuge was set at 7,000 rpm for 15 minutes to pellet the cells. The supernatant was discarded, and the pellet was obtained.

The PureLink HiPure Plasmid DNA Purification Kit Maxi-prep procedure by Invitrogen was followed to extract DNA from the pellet. The supernatant was removed, and the pellet was air-dried for 10 minutes and resuspended in 100 μ L of double distilled water, rather than the recommended 500 μ L of TE Buffer (TE).

Initiation to Tissue Culture

Human Embryonic Kidney 293 cells (HEK293) of the 293T cell line were used. For storage, cells were trans-

ferred into a new T75 flask containing 500 ml of medium consisting of 440 ml of RPMI + 50 ml of 10% FBS, 5 ml of supplemental glutamine 200 mM L-glu, and 5 ml of 1x Penicillin and 1x Streptomycin.

Transfection of 293T Cells

The pCEP4/LL25X DNA that was isolated via maxi-prep was used to transfect 293T cells by following the Lipofectamine 2000 DNA Transfection Reagent Protocol.

One day before transfection, the 293T cells were trypsinized and resuspended into a 10 ml/T75 flask. Using a haemocytometer, the cell number was determined, and trypan blue was added to determine cell viability. The cells were seeded at 6×10^6 cells/T75 flask.

On the day of transfection, the culture media (DMEM consisting of 10% FBS, 1x PBS (Phosphate Buffer Saline)/Strep, 1x Glutamine) replaced the old media 1 hour before transfection. The transfection mixture was prepared using 500 μ L of Serum-free media (SFM ie. OPTI-MEM) + 10 μ g of maxi-prep DNA, 500 μ L of SFM + 10 μ L of Lipofectamine 2000 reagent, and DNA mix, which was incubated before at room temperature for 20 minutes. The transfection mixture was added to the cell culture and incubated overnight at 37°C, 5% CO₂, and 70% humidity. Cleavage of SIVmac239gp140 into monomeric surface subunit SIVmac239gp120 and ectodomain SIVmac239gp41 occurred once the proteins were expressed in the 293T cells.

One day after transfection, the culture media was replaced with 10 ml of SFM=DMEM (1x Pen/Strep, 1x Glutamine, and without FBS). Five days after transfection, the culture supernatant was transferred into 15 ml conical tubes. The tubes were centrifuged at 3,000 rpm for 10 min to pellet the debris in the tube. The supernatant was transferred to new 15 ml conical tubes.

Protein Purification

The Purification on Protein A-Agarose Protocol by Roche was followed to purify the protein. The resulting eluate,

containing the protein, was mixed by vortex and transferred into a dialysis cassette (10,000 Molecular Weight Cut-Off MWCO). Dialysis was performed overnight in 1xPBS at 4°C and was slowly stirred.

The next day, a centrifugal filter unit (Amicon Ultracel 10,000 MUCO) was used to concentrate the protein eluate until the remaining buffer was around 100-200 µL. The unit was centrifuged at 3,000 rpm for 30 minutes.

Protein/Antibody Characterization by ELISA (Enzyme-Linked Immuno-sorbant Assay)

Antigens (SIVmac239gp140, SIVmac239gp120, and control BSA) were prepared at 1 ng/µL with 1xPBS. A plate was divided so that 100 µL of each diluted antigen was added to each corresponding well. The coated ELISA plate was incubated for 1 hour at 37°C. After incubation, the plate was washed once with 1xPBS containing 0.5% Tween20. The tray was blocked by adding 500 µL of 5% skim milk in 1xPBS. The tray was sealed and incubated for 1 hour at 37°C.

A 1000x serial dilution of the primary antibody/protein was prepared, beginning with 10 µg/µL. The dilutions were 10 µg/µL, 1 µg/µL, and 0.1 µg/µL. The dilutions were prepared using 1xPBS/FBS 2%, and 50 µL of the antibody/protein dilution was added to each well. For each antigen, one of each dilution was added. Therefore, each antigen would have three different dilutions. The tray was incubated for 1 hour at 37°C. The plate was washed 10 times with 1xPBS containing 0.5% Tween20 and flipped over on a paper towel to dry for 1 minute. After drying the tray, 50 µL of secondary antibodies, HRP-conjugate anti-human IgG antibodies (1000x dilution in 5% skim milk), were added to all of the wells. The tray was sealed and incubated for 1 hour at 37°C. The plate was washed 5 times with 1xPBS containing 0.5% Tween20 and flipped over to dry on a paper towel for 1 minute, and 100 µL of TMB solution was added. The plate was incubated for 30 minutes at room temperature. Once blue color was observed, the reaction was stopped with 100 µL of TMB stop solution. The absorbances of the wells were read on Wallac (Victor plate reader) at 450 nm.

Protein Characterization by Coomassie Blue Staining

A 12% agarose gel was prepared. Samples to be loaded into the wells consisted of 5 µg of each envelope protein (gp120 and gp140) and 1xPBS was added to each sample until a total volume of 10 µL was achieved. To the 10 µL of each

sample, 10 µL of 2x Sample Loading Buffer was added for a total volume of 20 µL. The samples were boiled at 100°C for 5 minutes, spun down, and loaded onto the gel. Lane 1 contained the ladder, Lane 2 contained the Env glycoprotein, gp120, and Lane 3 contained the Env glycoprotein, gp140. After the gel was run, the gel was stained with Coomassie Blue, and the protein bands were observed.

Protein/Antibody Characterization by Western Blotting

A 12% agarose gel was prepared. Samples to be loaded into the wells consisted of 5 µg of each protein/antibody (SU24, SU343, and LL25X) and 1xPBS was added to each sample until a total volume of 10 µL was achieved. To the 10 µL of each sample, 10 µL of 2x Sample Loading Buffer was added for a total volume of 20 µL. The samples were boiled at 100°C for 5 minutes, spun down, and loaded onto the gel. Lane 1 contained the ladder, Lane 2 contained SU24-Fc, Lane 3 contained SU343-Fc, and Lane 4 contained LL25X-Fc. After the gel was run, the proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked by using 5% skim milk to prevent nonspecific binding and was treated with the primary antibody, Anti-IgG-HRP. The membrane was left for 1 hour in 5% skim milk that was prepared with 1xPBS. The gel was washed 3 times with 1xPBS for 10 minutes each. This membrane, Blotting Gel 1, consisted of SU24, SU343, and LL25X and used anti-human IgG-HRP as the primary antibody.

Three 12% agarose gels were prepared. Samples to be loaded into the wells consisted of 5 µg of each protein/antibody (SIVmac239gp120 and SIVmac239gp140) and 10 µL of 2x Sample Loading Buffer, and 1xPBS was added to each sample until a total volume of 10 µL was achieved. The samples were boiled at 100°C for 5 minutes, spun down, and loaded onto the gel. Lane 1 contained the ladder, Lane 2 contained the Env glycoprotein, gp120, and Lane 3 contained the Env glycoprotein, gp140. After the gel was run, the proteins in the gel were transferred to a nitrocellulose membrane.

For the first gel, Blotting Gel 2, the membrane was blocked by using 5% skim milk to prevent nonspecific binding and was treated with the primary antibody, SU24. The membrane was left for 1 hour in 5% skim milk that was prepared with 1xPBS. The gel was washed 3 times with 1xPBS for 10 minutes each. Anti-human IgG-HRP was added as the secondary antibody. The gel was washed 3 times with 1xPBS for 10 minutes each. The chemiluminescence reagent was added to the gel. The gel was observed under UV light, and pictures were taken

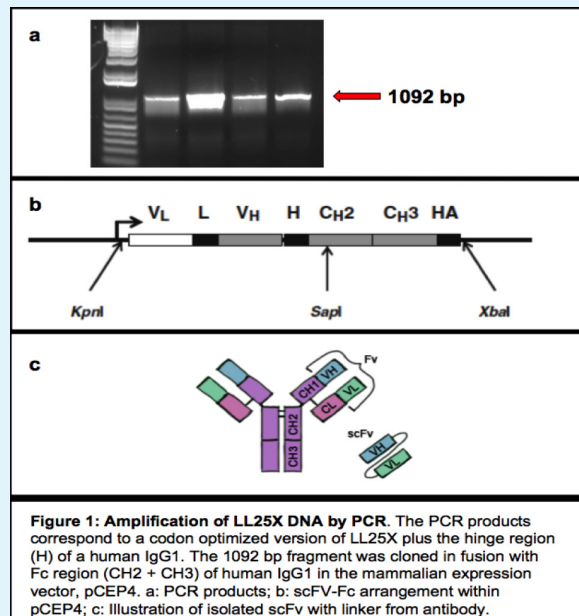
using the Chemiluminescence Dock. The first gel contained SIVmac239gp120 and SIVmac239gp140, used SU24 as the primary antibody, and used anti-human IgG-HRP as the secondary antibody.

For the second gel, Blotting Gel 2, the membrane was blocked by using 5% skim milk to prevent nonspecific binding and was treated with the primary antibody, SU343. The membrane was left for 1 hour in 5% skim milk that was prepared with 1xPBS. The gel was washed 3 times with 1xPBS for 10 minutes each. Anti-human IgG-HRP was added as the secondary antibody. The gel was washed 3 times with 1xPBS for 10 minutes each. The chemiluminescence reagent was added to the gel. The gel was observed under UV light, and pictures were taken using the Chemiluminescence Dock. The second gel contained SIVmac239gp120 and SIVmac239gp140, used SU343 as the primary antibody, and used anti-human IgG-HRP as the secondary antibody.

For the third gel, Blotting Gel 2, the membrane was blocked by using 5% skim milk to prevent nonspecific binding and was treated with the primary antibody, LL25X. The membrane was left for 1 hour in 5% skim milk that was prepared with 1xPBS. The gel was washed 3 times with 1xPBS for 10 minutes each. Anti-human IgG-HRP was added as the secondary antibody. The gel was washed 3 times with 1xPBS for 10 minutes each. The chemiluminescence reagent was added to the gel. The gel was observed under UV light, and pictures were taken using the Chemiluminescence Dock. The third gel contained SIVmac239gp120 and SIVmac239gp140, used LL25X as the primary antibody, and used anti-human IgG-HRP as the secondary antibody.

RESULTS

After PCR, the bright band was observed, as shown in Figure 1a. The location of the band is approximately at 1092 bp, which is the size of the LL25X fragment. This indicates that the LL25X fragment was successfully amplified. The PCR products, corresponding to a codon-optimized version of LL25X plus the hinge region (H) of a human IgG1, were extracted from the gel. The amplified LL25X fragments were double digested with KpnI and SapI, and the double digest products were run on 1% agarose gel. The resulting insert vector, an scFv, was extracted from the gel. The mammalian expression vector pCEP4 was also double digested with KpnI and SapI, and the double digest prod-



ucts were run on 1% agarose gel. The resulting backbone vector was extracted from the gel.

Using complementary “sticky ends,” which resulted from the double digest, the insert vector, LL25X, was effectively ligated into the backbone vector, pCEP4. TOP10F' *E. coli* were transformed with the ligation product; the 1092 bp fragment was cloned in fusion with the Fc region (CH2 + CH3) of human IgG1 in the mammalian expression vector pCEP4. These cells, containing the ligation product, were grown on LB agar plates containing Ampicillin, as shown in Figure 2b. A negative background control with *E. coli*

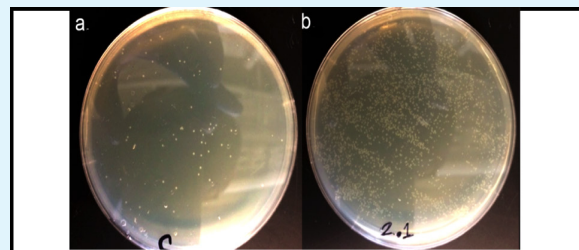


Figure 2: Identification of Positive Clones on Selective Media LL25X was ligated into pCEP4, and TOP10F' *E. coli* were transformed with the ligation product. Single colonies were selected and inoculated into culture tubes containing 3 mL LB media supplemented with 50µg/ml of Ampicillin. a: negative control with the digested vector only b: vector + insert ligation product. The greater amount of colonies obtained with “vector + insert” suggested a successful ligation reaction.

containing mammalian expression vector pCEP4-Fc-HA, digested and ligated without the LL25X insert vector, was also grown on LB agar plates containing Ampicillin, as shown in Figure 2a.

Theoretically, there should not be any growth on the negative control. The vector, digested and ligated without LL25X, would not be able to provide antibiotic resistance because the replication machinery of the bacteria would be affected, preventing the replication of the plasmid. Since the plasmid is not replicated, the Ampicillin resistance gene cannot be expressed. However, the growth of a few colonies is clearly visible. This incidence may be due to some of the pCEP4 not being completely digested, resulting in a functional Ampicillin resistance gene. There is an abundance of colonies on the plate with the bacteria that contain the ligation product. The ligation of LL25X to the backbone vector pCEP4 allows the cell to replicate, resulting in the expression of the Ampicillin resistance gene. The greater amount of colonies present on the plate with the bacteria containing the ligation product compared to the plate with the bacteria containing pCEP4, digested and ligated without LL25X, suggests a successful ligation reaction. A colony, corresponding to the bacteria transformed with the pCEP4/LL25X ligation product, was selected, and the pCEP4/LL25X DNA from the colony was extracted via DNA mini-prep.

The pCEP4/LL25X DNA and the pCEP4-Fc-HA DNA, used as a control, were double digested with KpnI-HF/SapI and KpnI/BamHI-HF. The products of the double digests were run on 1% agarose gel. The pCEP4/LL25X DNA double digested with KpnI-HF and SapI produced a band at approximately 1092 bp, and the pCEP4-Fc-HA DNA double digested with KpnI-HF and SapI also produced a band at approximately 1092 bp. The double digested DNA fragments were similar in size; therefore, it could not be verified that the ligation product contained the LL25X DNA based on this result alone. When double digested with KpnI and BamHI-HF, pCEP4/LL25X DNA and pCEP4-Fc-HA DNA resulted in bands with different sizes. This is due to the BamHI restriction site residing in different locations between the two vectors. The pCEP4/LL25X contains a BamHI restriction site that appears before SapI, and the pCEP4-Fc-HA DNA contains a BamHI restriction site that appears after SapI; therefore, the double digested pCEP4/LL25X DNA will result in a smaller fragment than the fragment produced by the double digested pCEP4-Fc-HA DNA. This result verified that the ligation product contained the LL25X DNA.

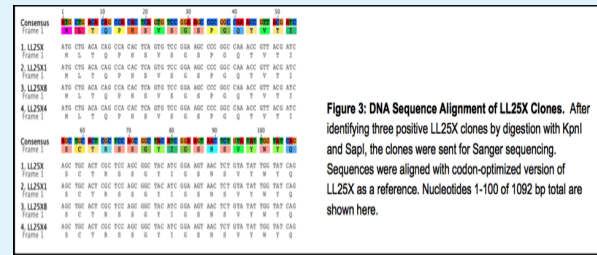


Figure 3: DNA Sequence Alignment of LL25X Clones. After identifying three positive LL25X clones by digestion with KpnI and SapI, the clones were sent for Sanger sequencing. Sequences were aligned with codon-optimized version of LL25X as a reference. Nucleotides 1-100 of 1092 bp total are shown here.

After identifying three positive LL25X clones via double digestion, the clones were sent to Eton Bioscience for Sanger sequencing. Once the DNA sequence results were received, the software Geneious was used to analyze the sequence of LL25X. The sequence analysis confirmed that the correct LL25X sequence had been cloned into the mammalian expression vector pCEP4. The sequences of all three LL25X clones were aligned identically to the original codon-optimized version of the LL25X sequence, as shown in Figure 3.

A positive LL25X clone was also selected to be cultured in a tube containing 3 ml of LB/Ampicillin media, and the resulting pCEP4/LL25X DNA from the bacteria was extracted via DNA maxi-prep. The extracted pCEP4/LL25X DNA was used to transfect 293T cells. Cleavage of SIVmac239gp140 into monomeric surface subunit SIVmac239gp120 and ectodomain SIVmac239gp141 occurred once the proteins were expressed in the 293T cells. Five days after the transfection, the culture supernatant was centrifuged to remove the cell debris, and the supernatant was transferred to new tubes. The proteins in the supernatant

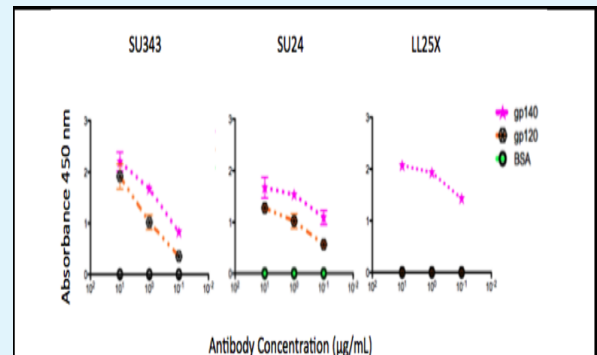


Figure 4: Analysis of mAb SU343, SU24, and LL25X binding by ELISA
 SU343, SU24 and LL25X binding specificity to gp120 and gp140 was analyzed. Each well was coated with 100ng of gp120 and gp140 antigen. BSA antigen was used as a negative control for background readout. Serial dilutions (0.01µg/mL, 0.1µg/mL, and 1.0 µg/mL) of SU343, SU24 and LL25X were used as primary antibodies. Anti-human IgG-HRP was used as a secondary antibody. TMB substrate was used for detection. The plate was read at 450nm. SU343 and SU24 bound envelope protein gp120 and gp140. LL25X bound gp140 but not gp120.

“ELISA determined that the protein, LL25X-Fc, binds to Env glycoprotein SIVmac239gp140 and not monomeric surface subunit SIVmac239gp120.”

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were isolated via Protein A-Agarose beads and dialysis, and ELISA was performed, as shown in Figure 4.

The binding specificity of antibodies, SU24, SU343, and LL25X, against antigens, SIVmac239gp140, SIVmac239gp120, and BSA (Bovine Serum Albumin – used as a control), was analyzed. SU24, SU343, and LL25X were used as primary antibodies, and anti-human IgG-HRP was used as a secondary antibody. TMB solution was used for detection, and the plate was read at 450 nm. SU24, SU343, and LL25X did not bind to BSA, which was expected. SU24 and SU343 bound to the envelope proteins gp140 and gp120; however, LL25X bound to gp140 but not gp120. ELISA determines the binding specificities of antibodies to antigens; however, ELISA does not reveal the specific location on the antigen to which the antibody binds. Further tests were required to determine the location of where the binding of LL25X occurred on gp140.

Coomassie Blue Staining was performed to determine the presence and quantity of proteins since the dyes bind directly to the proteins, as shown in Figure 5b. The Coomassie gel displayed equal loading amounts of both gp140

and gp120. Western blotting was performed to determine the locations on antigens where the binding of antibodies occurred, such as the location where the binding of LL25X occurred on gp140. Figure 5a displays the immunoblotting of SU24, SU343, and LL25X with Anti-IgG-HRP. The gel confirmed successful purification of antibodies since Anti-IgG-HRP was able to bind to all 3 antibodies.

Figure 5b displays the binding specificity of SU24, SU343, and LL25X, which were used as primary antibodies, and anti-human IgG-HRP was used as a secondary antibody. The results of the Western blots supports the data received from ELISA. SU24 and SU343 were revealed to be gp120-specific, and thus, bound to both gp120 and gp140, as illustrated by the dark bands. LL25X was revealed to be gp41-specific, and thus, bound to both gp41 and gp140, as illustrated by the dark bands. A band is present at the gp140 location because not all of the gp140 was cleaved. LL25X binds to ectodomain gp41 that that remained a part of Env glycoprotein gp140. The lack of a band at the gp120 location indicates that LL25X did not bind to the monomeric surface subunit gp120 of the Env glycoprotein gp140. The band at the gp41 location indicates that LL25X binds to the ectodomain gp41 of Env glycoprotein gp140.

CONCLUSION

The LL25X DNA fragment was successfully cloned into the mammalian expression vector pCEP4 by digesting the LL25X PCR products and the cloning vector pCEP4 with KpnI and SapI, ligating the resulting LL25X insert vector and the resulting pCEP4 backbone vector, and transforming *E. coli* TOP10F' cells with the ligated product. The LL25X scFv fragment that was inserted and fused to the Fc region (CH2 + CH3) of human IgG1 in the mammalian expression vector pCEP4 was confirmed and verified by DNA sequence analysis. ELISA determined that the protein, LL25X-Fc, binds to Env glycoprotein SIVmac239gp140 and not monomeric surface subunit SIVmac239gp120. Monoclonal SIVmac239 antibodies SU24 and SU343 were also revealed to bind to monomeric surface subunit SIVmac239gp120 and Env glycoprotein SIVmac-

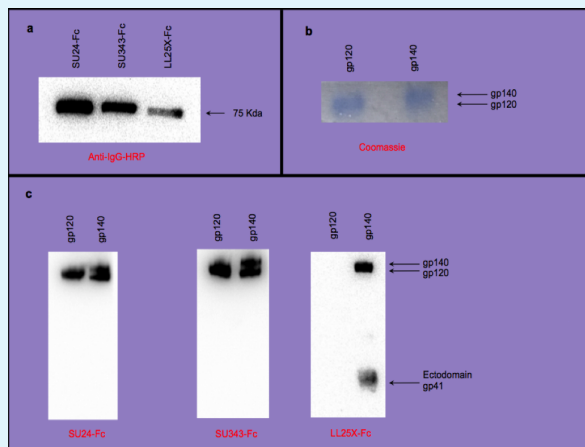


Figure 5: Analysis of SU24, SU343 and LL25X binding to SIV Env glycoprotein
Proteins were submitted to electrophoresis onto 12% SDS gel and transferred onto a nitrocellulose membrane.
a) Immunoblotting of SU24-Fc, SU343-Fc, and LL25X-Fc with Anti-IgG-HRP. Confirmed successful purification of antibodies.
b) and c) SIV239 gp120 in left lanes and SIV239 gp140 in right lanes. b) Coomassie staining showing equal loading of both gp120 and gp140. c) binding specificity of SU24, SU343 and LL25X. Corresponding antibody was used as a primary and anti-human IgG-HRP as a secondary antibody. Blots were visualized using a chemiluminescence reagent. SU24 and SU343 are gp120-specific and bind to gp120 and gp140. LL25X is gp41-specific and bind to the ectodomain of gp41, as well as gp140.

239gp140. Western blotting determined that monoclonal SIVmac239 antibodies SU24 and SU343 bind specifically to monomeric surface subunit SIVmac239gp120 of Env glycoprotein SIVmac239gp140. Western blotting also revealed that monoclonal SIVmac239 antibody LL25X binds specifically to ectodomain SIVmac239gp41 of Env glycoprotein SIVmac239gp140. Future work should be directed at testing the ability of SU24, SU343, and LL25X to neutralize diverse SIV strains, such as the sensitive strain SIVmac316 and the resistant strain SIVmac239.

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ENDNOTES

1. Yates, Nicole L. et al. "Vaccine-Induced Env V1-V2 IgG3 Correlates with Lower HIV-1 Infection Risk and Declines Soon After Vaccination." *Science translational medicine* 6.228 (2014): 228ra39. *PMC*.
2. Ita, Sergio et al. "Analysis of SIVmac-specific Antibodies Selected via Phage Display" *AIDS research and Human Retroviruses* (2017).

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- Ita, Sergio et al. "Analysis of SIVmac-specific Antibodies Selected via Phage Display" *AIDS research and Human Retroviruses* (2017). Accepted.