CHAPTER 2: MATERIALS AND METHODS

2.1 Source of PBMC for EBV-transformation

The PBMC were obtained from HIV-1 subtype C infected individuals previously identified as having high levels of neutralizing antibodies (Table 1). These individuals were identified as Du23, Du179, LTNP28 and TM8. Du23 and Du179 were sex-workers on the truck stops between Johannesburg and Durban, South Africa, identified prospectively for HIV-1 infection (Williamson et al., 2003). LTNP28 was selected from a cohort of patients defined as long-term non-progressors on the basis of being infected for more than 5 years, with a low viral load. TM8 was from a cohort of pediatric patients attending an out-patient clinic at the Chris Hani-Baragwanath Hospital. All the patients were clinically well and none received anti-retroviral (ARV) therapy. Serum from these patients showed high levels of neutralizing antibody activity (with an ID80 >1:40) against Du151, the South African prototype HIV-1 subtype C vaccine strain.

About 40 ml of heparinized blood was obtained from Du23 (on two occations), Du179 and LTNP28 while about 12 ml was obtained from TM8. Blood was centrifuged at 1, 000 rpm (Rotanta 46R, Hettich, Germany), for 10 minutes at 4^{o}C. CD4 count was done on whole blood and plasma was collected and HIV-1 viral load was performed (Table 1). The buffy coat containing white blood cells was collected and diluted at 1:1 with phosphate buffered saline (PBS) and mixed thoroughly. The diluted blood was layered onto a Ficoll histopaque gradient (Amersham Phamacia Biotech AB, Sweden) and centrifuged at 1,800 rpm for 30 minutes at room temperature. Cells from the interface of Ficoll (top layer) and red cells

(bottom layer) were collected and washed three times by centrifugation at 1,000 rpm at 4°C in PBS to remove platelets. Contaminating red cells were lysed with 10 ml of ammonium chloride (155 mM NH_4Cl, 9 mM $KHCO_3$, 0.1 mM EDTA at pH 7) for 3-5 minutes and washed in PBS. PBMC were resuspended in RPMI-1640 (Gibco) and counted using trypan blue stain (Sigma-Aldrich). 1 ml aliquots of 5-10 X 10^6 cells were stored in a freezing medium containing 10% dimethylsulfoxide (DMSO), 20% fetal bovine serum (FBS) (Gibco) and 70% RMPI-1640 medium at -70°C for use in transformation experiments.

Table 1. Patient information from whom PBMC samples used for EBV-transformations were derived. The data refers to the time when the blood samples were donated.

Sample	**Gender**	**Age**	**Duration of Infection (years)**	**CD4 Count (Cells/μl)**	**Viral load (RNA copies/ml)**	**ID80 Titres Against Du151**
Du23 (1)	F	35	>3	549	<50	1:40
Du23 (2)	F	36	>4	978	<50	1:40
Du179	F	36	>8	231	2 228	1:120
LTNP28	M	34	>7	303	4 053	1:80
TM8	F	8	>8	888	2 030	1:40

2.2 Monoclonal antibodies

2.2.1 MAbs used in screening

The anti-HIV-1 envelope human mAb 1.7B, A32, C11, and 7B2 were used to select the subtype C B-cell clones from EBV-transformed cultures (Table 2). Except for 7B2 which bind to gp41 all of these mAbs bind to discontinuous regions of the gp120 envelope. Cell lines expressing these mAbs were obtained from Dr James Robinson, and were used to generate large quantities of antibodies. These cell lines were generated from subtype B infected patients. Cells were grown up and supernatants were harvested from which mAbs were purified on protein A affinity columns. 1.7B is a CD4i mAb while A32 binds to C1-C4 regions of the gp120. The 7B2 mAb recognizes the immunodominant region of the gp41while C11 binds to C1-C5 regions of gp120.

2.2.2 Additional mAbs used in analysis

In addition to the above described mAbs, the remainder of the mAbs described in Table 2 were obtained in purified form (from Dr James Robinson's laboratory) and were used in the analysis experiments. The V3 antibodies 2.1E, KX16, 3.9F and 1.9B defining continuous epitopes on gp120 were used in various competition assays. EH21 binds to peptide representing amino acid sequences near the N-terminus while 2.3G recognizes a peptide representing sequences at the C-terminus regions of gp120. The 1.5E and F91 are CD4BS mAbs and require the proper conformation of the protein for their binding.

Table 2. Properties of mAbs used as tools in screening and analysis processes

Part	mAb	IgG Subclass	Epitope	Requirement for Epitope[a]
A[b]	A32	$IgG_{1\gamma}$	gp120C1-C4	Discontinuous
	1.7B	$IgG_{1\gamma}$	gp120CD4i	Discontinuous
	C11	$IgG_{1\gamma}$	gp120C1-C5	Discontinuous
	7B2	$IgG_{1\gamma}$	Immunodominant gp41 domain	Continuous
B[c]	EH21	$IgG_{1\gamma}$	N-terminus (gp120)	Continuous
	2.1E	$IgG_{1\gamma}$	V3 (gp120)	Continuous
	KX16	$IgG_{1\gamma}$	V3 (gp120)	Continuous
	3.9F	$IgG_{1\gamma}$	V3 (gp120)	Continuous
	1.9B	$IgG_{1\gamma}$	V3 (gp120)	Continuous
	1.5E	$IgG_{1\gamma}$	CD4BS (gp120)	Discontinuous
	F91	$IgG_{1\gamma}$	CD4BS (gp120)	Discontinuous
	2.3G	$IgG_{1\gamma}$	C-terminus (gp120)	Continuous

[a]Epitopes that require conformational structure of a protein are described as discontinuous and those that do not are described as continuous.
[b]Cell lines producing these mAbs were obtained from Dr James Robinson, and mAbs were purified from culture supernatant.
[c]These mAbs were obtained as purified preparations from Dr James Robinson.

2.2.3 Production of antibodies from cell lines

Cell lines producing A32, 1.7B, C11 and 7B2 antibodies were initially seeded in T75 culture flasks, and then transferred to Integra CL 1000 culture flasks for antibody production (Integra Biosciences). A 15 ml cell suspension containing 1 x 10^6 cells was prepared in RPMI-1640 supplemented with 15% FBS, 100 μg/ml Gentamycin and 20 μg/ml Ciprofloxycin antibiotics. A 5 ml portion of this suspension was used to wet the cell compartment (bottom half) of the flask. Then, 1L of RPMI-1640 was poured into the cell-free compartment (top half). The remainder of the cell suspension was put into the cell compartment. The flasks were then incubated for one week at 37^{o}C with 5% CO_2 (Forma Scientific CO_2 Water Jacketed Incubator). The flasks were maintained at cell densities of 3-9 x 10^6 cells/ml and culture supernatants were harvested after 3-4 days for purification of IgG. On reaching confluence the cells were thoroughly mixed and because of osmotic flux of water into the cell compartment about 20-25 ml of antibody supernatant was obtained. Approximately 70% (14-17.5 ml) of the cell suspension was harvested for antibody purification and centrifuged at 1,000 rpm for 10 minutes to pellet the cells. The final volume in the cell compartment (bottom half) was adjusted back to 15 ml volume by adding 6-7.5 ml of fresh RPMI supplemented with 10% FBS. The serum-free medium in the top half was changed when the medium turned yellowish. The culture flasks were maintained for up to 2-3 months.

2.2.4 Purification of mAbs

Batches of harvested antibody supernatants from the Integra flasks were pooled together (3-4 harvests) for antibody purification. The supernatant was filtered through 0.22 μm filter and purified using Protein A (Sigma) affinity chromatography. About 500 μl of Protein A slurry

was packed into appropriate columns through which 50-100 ml antibody supernatant was passed. This was done at least twice to ensure that all the antibodies were captured onto the protein A, and the column was washed with 100 ml of the PBS. The antibody was then eluted with 10 ml of Immunopure IgG Elution Buffer (Pierce, USA). The purified antibody was then concentrated to approximately 1 ml by centrifugation at 1800 rpm using concentrators (Vivaspin, 50 000 MWCO PEG) before determination of the IgG protein concentration.

2.2.5 Determination of protein concentration

The antibody protein (purified IgG) concentration was determined using the bicinchoninic Acid (BCA) Protein Assay Reagent Kit according to the manufacturer instructions (Pierce, USA). The principle of this assay is based on colorimetric detection and quantification of total protein that combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium, a process known as biuret reaction. It is a highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing bicinchoninic acid. The assay exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range of 20-2,000 μg/ml. Protein concentrations were determined and reported with reference to standard controls using horse IgG (Sigma). Serial dilutions of the standard controls IgG concentrations of between 2-5 mg/ml were prepared from the protein and assayed alongside the unknowns.

The BCA working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A (containing sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium

hydroxide) with 1 part BCA Reagent B (containing 4% cupric sulfate) (50:1 Reagent A:B). 50 µl of each standard and unknown antibody sample were prepared into appropriately labeled test tubes. To this, 1 ml of the WR was added to each tube and mixed well. The tubes were covered and incubated in a water-bath at 37^{o}C for 30 minutes. The samples were then allowed to cool to room temperature. The samples were prepared into triplicate on a microtiter plate for absorbance reading at 560 nm (VersaMax Microplate reader) and the results were analyzed using the SoftMaxPro software. The concentration of appropriate sample dilutions was determined from the standard curve.

2.2.6 Biotinylation of mAbs

Purified mAbs were biotinylated with Biotin-X-NHS (Calbiochem, USA) as previously described (Robinson et al., 1998). For this 2 mg of biotin was dissolved in 1 ml DMSO and then added to 1 mg of an antibody solution that was prepared and washed three times with 0.1 M sodium bicarbonate solution at pH 8. Then 100 µl of the biotin solution was added to the antibody preparation, and the reaction was developed at room temperature in the dark for a minimum of 4 hours. Following this, the biotinylated antibody was washed 4-5 times with 0.1 M sodium bicarbonate at pH 8, before being dialyzed in mini-dialysis slides (Slide-Alyzer 10 000MWCO, Pierce) in PBS for 12 hours. Once dialyzed, the biotinylated antibodies were stored in 5 ml glass vials. The antibodies were preserved and stabilized by adding 0.001% sodium azide and 1% bovine serum albumin (BSA) solutions and stocks were stored at 4^{o}C.

2.3 Preparation of macrophage feeders

2.3.1 Properties of macrophages

Macrophages produce a variety of growth factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF), that support the growth of transforming B cells. GM-CSF stimulates the proliferation of cells from both granulocyte and macrophage cell lineages. Research has shown that some antibody-secreting clones are lost if macrophage feeder layers are not used in transforming B cells (Buchacher et al., 1994).

2.3.2 Production of GM-CSF

The Giant Cell Tumor (GCT) (Liesveld et al., 1993) cell line was used for the production of GM-CSF which is secreted at high concentration by this cell line. This cell line was kindly provided to us by Dr James Robinson (American Type Tissue Culture Collection, ATCC, TIB-223). 1 x 10^6 cells were seeded in T-75 flasks containing 50 ml of McCoy's 5A medium (Gibco) supplemented with 10% FBS. The cells were grown to confluence at 37^{o}C in a CO_2 incubator. Supernatant containing GM-CSF was harvested by centrifugation at 1,000 rpm for 10 minutes and filtered through 0.22 μm filters. 1 ml aliquots of the filtrate were stored at -20^{o}C. The quality of the harvested GM-CSF was evaluated by observing the changes in the culture of the macrophage population in PBMC.

Once the supernatant was harvested, GCT cells were passaged by placing 5 ml of 0.25% Trypsin into the flask. The flask was incubated for not more than 3 minutes, after which the adherent cells were shaken off and 10 ml of FBS was added to inactivate the trypsin; the volume was brought to 50 ml with McCoy's 5A medium. The cells were centrifuged at 1,000

rpm for 10 minutes and the pellet re-suspended in McCoy's 5A medium supplemented with 10% FBS for re-seeding into the flask. Cell stocks were frozen at 5 x 10^6 cells/ml in 1 ml aliquots in medium containing 10% DMSO, 20% FBS and 70% McCoy's 5A medium.

2.3.3 Culturing and harvesting of macrophage feeders

PBMCs were isolated by Ficoll-gradient centrifugation (section 2.1) from HIV-1 negative and hepatitis B negative blood units obtained from the South African Blood Transfusion Services. The cells were cultured in RPMI medium supplemented with 10% FBS at 10^6 per T75 flask in the presence of two sources of GM-CSF to enrich for macrophages: purified GM-CSF obtained commercially (Leucomax, NOVARTIS, South Africa) was used at a low concentration of 20 μg/ml plus 1 ml of supernatant harvested from the GCT culture. Cultures were incubated for one week during which time the macrophages became adherent and differentiated. Macrophages increased in cell size and spread-out forming an adherent layer at the bottom of the flask. To harvest the macrophages, the cell medium from the flask was discarded and replaced with 20 ml of Hank's Balance Salt Solution (HBSS) (Gibco) and incubated at 37^{o}C for an hour. Macrophages were gently scraped off the surface of the flask using cell scrapers and transferred to 50 ml tubes for irradiation. The viability of the cells was evaluated using trypan-blue staining, with greater than 80% viability considered acceptable.

2.3.4 Irradiation and seeding of feeders

Harvested macrophages were irradiated at 3,000 rads for 10.5 minutes using gamma (γ) source irradiator (Steuerungstechnik & Strahlenschutz GmbH, Germany). This time and dose causes fragmentation of DNA and prevent cell proliferation but yet cells remain viable to produce growth factors to support proliferation of transformed B-cells. Once irradiated, the macrophages were cultured in RPMI supplemented with 10% FBS in the presence of 50 μg/ml Gentamycin. These macrophages were then plated at 1 x 10^4 cells per well in 96-well flat bottom tissue culture plates and incubated at 37^{o}C in CO_2 for at least 12 hours before use as feeders. During this time, macrophage cells enlarge and spread-out. Cultures that contained healthy and confluent cell layer were used in the experiments.

2.4 Making antigen preparations

2.4.1 Sources of subtype B antigens

Antigens obtained from several subtype B viruses were used to analyze isolated mAbs (Table 3). All of these antigen stocks were prepared and kindly provided by Dr James Robinson. In addition to these antigen preparations, we also obtained purified Yu-2 gp120 proteins and a construct of Yu-2 gp120 protein with deleted V1/V2 regions kindly provided by Dr Richard Wyatt at the NIH Vaccine Research Centre.

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Table 3. Subtype B viruses used to generate antigen preparations

Viruses[c]	Subtype	Preparation of antigens[a]	Phenotype[b]
ADA	B	Sup + lysate	R5
89.6	B	Sup + lysate	R5
SF162	B	Sup + lysate	R5

[a]Sup refers to supernatant; sup + lysate is cells harvested with supernatant and lysed in 1% Triton-X100 to release any antigen trapped within cytosol.
[b](Hung, Vander Heyden, and Ratner, 1999).
[c]89.6, SF162, and ADA Env were vaccinia expressed in 293T cells.

2.4.2 Generating antigen preparations from HIV-1 subtype C viruses

2.4.2.1 Viruses

Viral isolates were used to infect cell lines to generate antigen stocks used in the screening process. For this purpose we selected viruses isolated from the same patients from which PBMC samples for transformation were derived. We used the following viruses: Du23, Du179, LTNP28 and TM8. In addition, Du151 and Du421 viruses were used because they generated good quality antigen stocks using the STR5 cell line. Biological properties of these viruses are described in Table 4 below.

Table 4. Subtype C viral isolates used to infect various cell lines to make antigens used for screening transformation cultures.

Viruses	Source	Subtype env	MT-2 Assay	Phenotype
Du23	PBMC	C	NSI	R5
Du151	PBMC	C	NSI	R5
Du179	PBMC	C	SI	R5X4
Du421	PBMC	C	NSI	R5
LTNP28	PBMC	C	NSI	R5
TM8	PBMC	C	NSI	R5

2.4.2.2 Cell lines

STR5, D3R5 and C816645R5 cell lines were used to generate antigen stocks (Table 5). D3R5 was derived by expressing puroBABE-CCR5 retrovector (kindly provided by Dr David Dorsky) in PM1 cells, and the CCR5 expressing cells were selected in puromycin containing media (Dorsky and Harrington, 1999). Similarly, the STR5 and C8166R5 cell lines were derived by expressing the puroBABE-CCR5 in SupT1 (Smith et al., 1984) and C8166-45 (Salahuddin et al., 1983), respecitively (www.aidsreagent.org). The CCR5 expressing cells were selected and maintained in puromycin containing media. The STR5 and C8166R5 cells were grown in RPMI supplemented with 10% FBS and 1 μg/ml puromycin, and the cell lines were infected to raise antigen stock used in screening assays. Several other cell lines were used in various experiments and they are described in relevant sections: GCT cells for making GM-CSF (section 2.3.2), B-95/8 for production of EBV

(section 2.10), CHO.ST4.2 for production of soluble CD4 (section 2.11), 293T cells were used in transfection experiments (section 2.14) and MT-2 and JC53 cells were used in neutralization assays (sections 2.16.5.3 and 2.16.5.3 respectively).

Table 5. Summary of characteristic features of cell lines used in various experiments.

Cells	**Useful Surface Molecule Expressed**	**Presence of Reporter (indicator) Gene**	**Application (Assay)**
STR5 (SupT1 + puroBABE-CCR5)	CCR5 & CD4	None	Infection with T-tropic, macrophage-tropic, and primary HIV-1 isolates
D3R5 (PM1 + puroBABE-CCR5)	CCR5 & CD4	Green Fluorescence	Indicator cell assay for T-tropic and primary HIV-1 isolates
C816645 (C8166 + puroBABE-CCR5)	CCR5 & CD4	None	Infection with T-tropic, macrophage-tropic, and primary HIV-1 isolates
JC53 (TZM-bl or JC57BL-13)	CCR5 and CD4	Luciferase and β-galactosidase	Luciferase Neutralization Assay
MT-2	CXCR4	None	Virus neutralization assay

2.4.2.3 Infection of cell lines

The STR5, C8166R5 and D3R5 cell lines were first cultured at 1 x 10^4 cells per well in 24-well culture flasks (Corning) and incubated at 37^{o}C in CO_2 incubator for 12 hours. Once the cells were in logarithmic phase they were infected with different HIV-1 isolates. 1 ml of the virus supernatant was inoculated into each of the wells. After 48 hours, supernatant from the

cultures was discarded and replaced with fresh RPMI medium supplemented with 10% FBS. Thereafter the infection was monitored every 48 hours for the production of p24 protein in supernatant. Once the p24 antigens concentration reached above 20 ng/ml, the cultures were expanded to T75 flasks and more cells (1 x 10^6 cells) were added.

2.4.2.4 Determination of p24 antigen levels

The p24 antigen levels in culture supernatant were determined using a commercial ELISA (NEN Life Science Products, USA), according to the manufacturer instructions. Briefly, samples were diluted in 5% Triton-X100, and 100 µl was added onto the supplied plate. Four-fold dilutions (range from 10.0 to 0.01 ng p24) of the positive control were prepared and added onto the appropriate wells and the diluent was used as the negative control (blank). The samples were incubated at 37^{o}C in CO_2 for an hour. After washing the plate six times in wash buffer, 100 µl of the Detector Antibody was added to all wells excluding the blank. The plate was then incubated at 37^{o}C in CO_2 for an hour before washing the plate six times. Then 100 µl of a 1:100 diluted horseradish-peroxidase strepavidin conjugate was added to all the wells, again excluding the blank. The plates were incubated at room temperature for 1 hour before being washed four times. One tablet per 11 ml substrate diluent was prepared and 100 µl of this was added to all the wells. The readings were taken immediately in kinetic mode at 490-630 nm (VersaMax) and the results were analyzed using SoftMaxPro software.

2.4.2.5 Making viral lysates

In T75 flasks, STR5 T-cell lines were chronically infected with Du23, Du151, Du179, Du421, LTNP28 and TM8 viruses while only Du179 and Du151 viruses were grown in D3R5 and C816645 cells. Du23, Du421, LTNP28 and TM8 could not infect D3R5 and C816645 cells. Once more than 20 ng/ml of p24 antigens were detected, culture supernatants together with cells were harvested on a weekly basis and treated with 1% Triton-X100 to inactivate the virus particles and solubilize the viral glycoproteins. The quality of the antigen stocks was evaluated as described below (section 2.4.2.6). The rationale for using this type of antigen preparation was to try to keep the viral envelope intact, at its native state as far as possible. The antigen stock will contain gp120 and gp41 in dissociated and solubilized states with disrupted trimers, hence the "native" state refers to antigen preparation that have not been denatured by reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (β-ME). Harvested antigen stocks were kept frozen at -20^{o}C, and thawed at 37^{o}C for use as and when needed.

2.4.2.6 Evaluating antigen stocks

The quality of the antigen preparations was evaluated in an ELISA by determining their ability to bind A32, 1.7B, 7B2 and C11 mAbs. High-binding ELISA plates (Corning) were coated with 100 μl of 20 μg/ml goat anti-human IgG (Fc-specific) (Sigma) and incubated at 37^{o}C for at least 2 hours before use. Coated plates were stored at -20^{o}C until needed. 100 μl of 20 μg/ml of each of the above mAbs was added and incubated at 37^{o}C for an hour. After washing the plate four times in a wash buffer consisting of PBS in 1% Triton-X100, the plate was blocked with RPMI supplemented with 10% FBS and 10% defibrillated human serum

(DHS) (Irvine Scientific, USA) (blocking agent) and incubated at room temperature for 30 minutes. In all steps plates were washed four times in the wash buffer as described. A dilution of 10% by volume of defribrilated human serum was used as the blocking agent, in various steps of screening, to saturate all the anti-human IgG-Fc sites, and prevent non-specific binding. The blocking reagent was discarded, after which 100 µl of individual harvests of the antigen stocks plus 10% DHS were plated and incubated at 37^{o}C for an hour. The plate was washed following which a mixture of biotinylated mAbs A32, 1.7B, 7B2 and C11 each diluted 1:2000 was prepared in diluent solution containing 4% whey in PBS, 0.5% Tween and 10% DHS, and 100 µl of this was put into each well. After incubation at 37^{o}C for an hour, the plate was washed and 100 µl of horseradish peroxidase-streptavidin (HPO-SA) conjugate diluted 1:2000 in diluent solution was placed into each well. The plate was incubated at room temperature for an hour before being washed. 200 µg/ml of 3, 3', 5, 5'-tetramethylbenzidine (TMB) subtrate was added to 10 ml of 0.1 M Sodium Acetate solution at pH 6 plus 3.3 µl of hydrogen peroxide (H_2O_2) (Sigma-Aldrich). 100 µl of the substrate was added to the plate and allowed to develop over 3 minutes. The reaction was stopped by adding 100 µl of 10 mM sulphuric acid (H_2SO_4) (Sigma-Aldrich). The readings were taken using automated ELISA plate-reader at 450 nm (VersaMAx) and results analyzed by SoftMaxPro software. Antigen stocks that produced OD_{450nm} signals greater than 1.0 against a background of 0.4 or less were considered suitable and these were used in the screening assays.

2.4.2.7 Use of antigen stock

For the purpose of screening for new and novel antibodies from culture supernatant of EBV-transformed cells, the antigen stocks were used as a cocktail of antigens derived from different cell lines infected with different viruses. For each of the transformations done, the cocktail contained the virus antigen preparation from which the transformed B-cells were derived plus at least one other viral preparation to make a mixture of antigens presenting different epitopes. For example, for screening transformation plates done using PBMC from Du23, at least one of the antigen preparations was from Du23 grown in STR5, and viral preparation from Du179/STR5. The rationale for this was to broaden the chance of capturing potential mAbs that might bind to any of the different antigens/epitopes that are presented by these different antigen preparations.

2.5 Production of soluble CD4 using CHO cell lines

The CHO ST4.2 cell line (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr Dan Littman) kindly provided to us by Dr James Robinson, was used for the production of soluble CD4 (sCD4) that consists of the entire extracellular region of CD4. The sCD4-containing supernatant from the CHO ST4.2 cell line was used together with antigen preparations in the screening process to ensure that those epitopes that are induced by sCD4 are exposed for antibody binding and they could be identified. 1 X 10^6 CHO ST4.2 cells were seeded in T75 flask in 40 ml of Dulbeco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% FBS. The cells were grown to confluence at 37^{o}C in 5% CO_2, and the supernatant was harvested after 7 days. Supernatant was centrifuged at 1,000 rpm for 10 minutes to pellet any remaining cells. 50 ml

aliquots of the sCD4-containing supernatant were stored at -20^{o}C for future use. Cells were passaged using 0.25% trypsin as described previously (section 2.3.2).

2.6 Production of EBV

The marmoset cell line B-95/8 (American Type Tissue Collection, ATCC, B-95/8 cell line ECACC 85011419) is known to release high titres of transforming EBV and as such this cell line was used as a source of EBV to establish continuous B-cell lines from human donors. 1 x 10^6 B-95/8 cells were seeded in T75 tissue culture flasks containing RPMI supplemented with 10% FBS and grown to confluence. Supernatant was harvested and filtered through a 0.45 μm pore size filter after centrifugation to pellet the cells. The filtered EBV containing supernatant was aliquoted into 1 ml volumes and stored in -70^{o}C. Cells were passaged by 0.25% trypsinization as described previously (section 2.3.2).

2.7 Transformation of B-cells using EBV

EBV-transformation of B-cells from cryopreserved PBMC obtained from various HIV-1 subtype C infected individuals (Table 1) was performed using a modified procedure as previously described (Gorny et al., 1989; Robinson et al., 1998; Robinson et al., 1990; Xiang et al., 2002). The PBMC cells were thawed and washed three times, and 5-10 x 10^6 PBMC cells in 15 ml tube were incubated with 5 ml EBV containing supernatant for a minimum of 2 hours at 37^{o}C in 5% CO_2. The transforming lymphocytes were then inoculated with RPMI supplemented with 20% FBS and seeded at 10^3 to 10^4 cells per well in 96-well culture plates containing γ-irradiated mature macrophages as feeder cells. The 5 ml EBV-cell suspension was mixed with approximately 35 ml of RPMI supplemented with 20% FBS. Then 100 μl

per well of the transformation cells were plated onto 93 wells of the 96-well plates containing macrophages. The remaining 3 wells were left empty to accommodate the inclusion of controls in the ELISA screening process. The transformation cultures were incubated at 37^{o}C with 5% CO_2 for 2-3 weeks before screening for total IgG and anti-HIV-1 reactivity. After every screening, the cultures were maintained in RPMI supplemented with 10% FBS and 50 μg/ml Gentamycin and 20 μg/ml Ciprofloxycin to suppress bacteria and mycoplasma, respectively.

2.8 Screening and subcloning of transformation cultures

2.8.1 Determination of IgG content (primary cultures)

IgG in culture supernatant (IgG^{+}) from 96-well transformation plates was determined by ELISA. ELISA plates were coated with goat anti-human IgG as described previously (section 2.4.2.6). After 2 hours of incubation at room temperature, plates were washed four times with wash buffer containing PBS at pH 7.2 plus 0.1% Tween 20. Using a 96-well transfer device fitted with 96-well cartridge (TranStar, Corning) 100 μl supernatant samples was transferred from the culture plates into the ELISA plates, and appropriate human IgG (Sigma) standard control was used at the final concentration of 20 μg/ml. After one hour incubation period, the plates were washed three times with the wash buffer. Following the washing, the plates were blocked with 4% skim milk in 1X PBS and 1% Tween-20. Then, 50 μl of goat anti-human (Fc-specific) antibody conjugated with alkaline phosphatase (Sigma) diluted at 1:1000 (20 μg/ml) in dilution buffer containing PBS at pH 7.2, 0.1% Tween-20 and 1% bovine serum albumin (BSA) (Sigma) was used as a secondary antibody. The plates were then incubated for 1 hour at 37^{o}C with 5% CO_2. After washing the plates four times

with washing buffer from AMPAK kit (Dako Diagnostics), 50 µl of AMPAK substrate was added and incubated for 1 hour. Following the incubation, 50 µl of AMPAK amplifier was added and incubated for 3 minutes. The reaction was stopped with 50 µl of 0.5M sulphuric acid (H_2SO_4) before the plates were read at 490 nm (VersaMax plate reader) and the results were analyzed using the SoftMaxPro software. The percentage of IgG^+ wells and visual inspection were taken as an indication of the efficiency of transformation.

2.8.2 Screening for anti-HIV-1^+ specific antibodies (primary cultures)

Supernatants from EBV-inoculated PBMC cultures were screened for IgG antibodies binding to HIV-1 envelope glycoproteins (anti-HIV-1^+), using "reverse capture" ELISA as previously described (Fig 14B) (Robinson et al., 1998; Xiang et al., 2002). ELISA plates for screening were prepared by coating with 100 µl of 20 µg/ml goat anti-human IgG antibody as described previously (section 2.4.2.6). Two weeks after the incubation of the transformation cultures, 100 µl of supernatant samples were transferred from the culture plates as described above (section 2.8.1). The plates were incubated at 37^{o}C for an hour to allow secreted antibodies from the transforming B-cell culture supernatants to be captured. After the incubation period, the plates were washed and blocked as described previously (section 2.4.2.6). The blocking reagent was discarded and 100 µl of detergent-solubilized antigen preparations in 10% DHS was then incubated in the plate for an hour to allow the HIV-1 viral antigens to bind to immobilized antibodies. Depending on the transformation experiment to be screened, the antigen mixture was prepared as described previously (section 2.4.2.7). After the one hour incubation period and washing, 100 µl of a 1:2000 dilution of each of non-competing biotinylated mAbs 1.7B, A32, C11 and 7B2 was used to detect those transformed wells

containing B-cell lines secreting anti-HIV-1 antibodies. We used a mixture of these mAbs to ensure that if the captured human mAbs competed for the binding with one of the labelled mAb, one of the other in the mix would still detect the bound antigen. The development of the ELISA plates to screen for novel epitopes was done as described earlier (section 2.4.2.6). Positive wells were considered to be those with high OD_{450nm} readings (Fig 11, step 1). In general, wells which showed OD_{450nm} signals of greater than 1.0 with a background of less than 0.4 OD_{450nm} were considered positive (anti-HIV-1^{+}) in our screening system.

Each transformation culture was screened at least three times before sub-culturing and selection of HIV-1 specific clones. The screening was developed with HPO-SA and TMB substrate as described previously (section 2.4.2.6) using biotinylated A32, 1.7B, C11 and 7B2 mAbs. The transformation cultures were maintained for about 6-8 weeks while being screened on a weekly basis after the first 2 weeks. This allowed identification and selection of any potential clone that might be slow growing and thus detected later in the screening process. Moreover, potential clones that had already been identified could be confirmed.

2.8.3 Sub-culturing of anti-HIV-1^{+} wells (secondary cultures)

Tissue cultures microwell plates that contained antibody-producing cells that were HIV-1 specific were sub-cultured at low cell densities of between 100-1,000 cells per well and re-screened for antibody production (Fig 11, step 2). From these cultures, anti-HIV-1^{+} wells at the highest dilution that remained positive were sub-cloned. Depending on the rate of cell growth and the density of cells in the 96-well plate, this initial sub-culturing procedure was repeated two or three times. Macrophage feeder plates were used in all sub-culturing

experiments. For each sub-culture, the screening was done three times before the cultures were propagated further. The screening was developed with HPO-SA and TMB substrate as described previously (section 2.4.2.6) using biotinylated A32, 1.7B, C11 and 7B2 mAbs.

2.8.4 Selecting cell lines producing anti-HIV-1 antibodies (tertiary cultures)

Once stable, antibody-producing sub-clones were established two or three wells from each sub-cloned cultures derived from single well were identified and selected for cloning. These were then cultured at the densities of 10, 5 or 1 cell/well (1:100, 1:1000 and 1:5000 dilutions) in 96-well culture plate and incubated at 37^{o}C in 5% CO_2 (Fig 11, step 3).

After screening three times on a weekly basis, anti-HIV-1 positive wells were selected at highest dilutions (i.e. 1 cell/well). This cloning step was repeated two or three times before the cultures were expanded for antibody production. Monoclonality of a cell line was assumed by selecting clones at the highest dilution after 3 cloning steps, when all growing wells showed identical IgG content. Stable cell lines that were considered clonal were selected for expansion and antibody production. The screening was developed with HPO-SA and TMB substrate as described previously (section 2.4.2.6) using biotinylated A32, 1.7B, C11 and 7B2 mAbs.

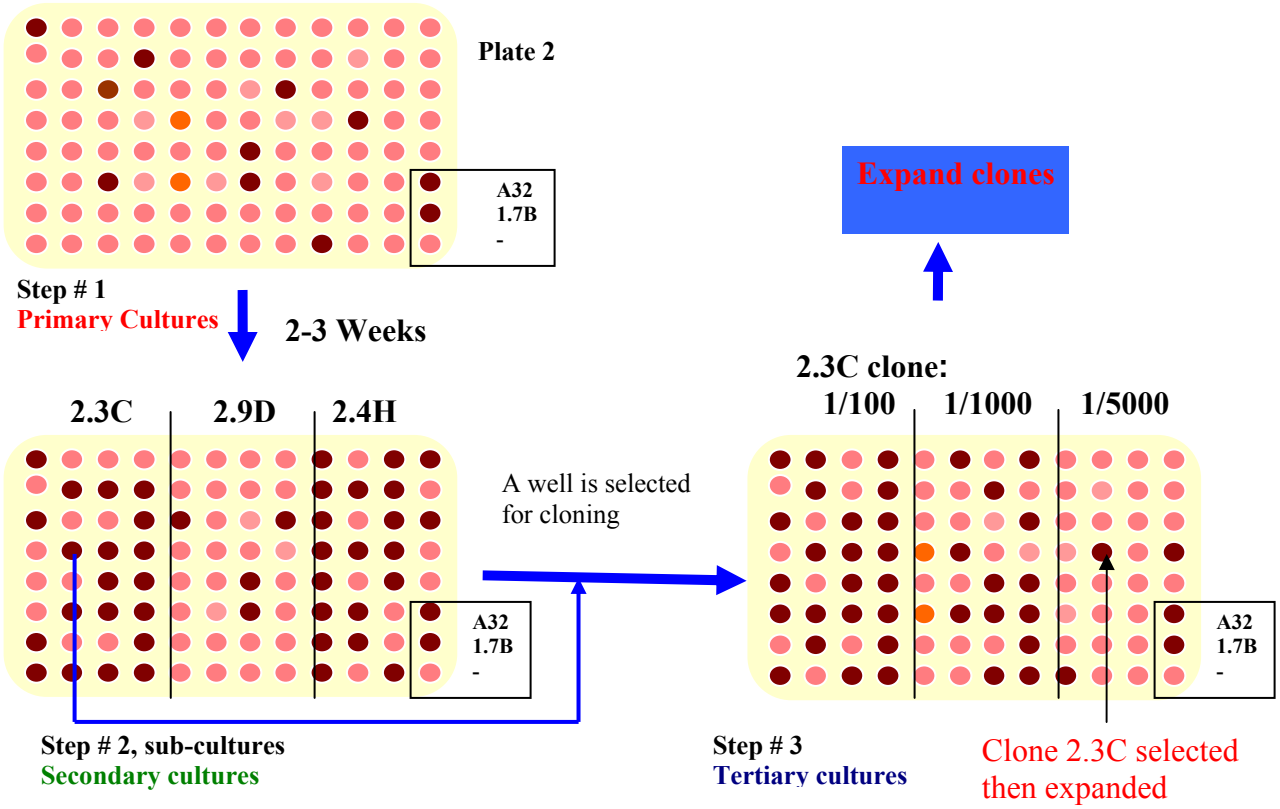

Fig 11. Diagram showing the main procedures used in cloning. For illustrative purposes, an example of generating clone 2.3C from primary (initial) cultures through to sub-cultures (secondary) to selecting or determining clonality (cloning process) in 96-well tissue culture plates is demonstrated. For simplicity, steps 1-3 are indicated as primary, secondary and tertiary cultures. In reality, sub-culturing in step 2 or sub-cloning in step 3 could each be repeated several times. The positions of positive controls, A32 and 1.7B, are indicate and a dash (-) for a negative control (medium only) in the screening process.

2.9 Expansion of HIV-1$^+$ clones and production of antibodies

Anti-HIV-1 clones were expanded from 96-wells into 24-well culture plates. When cells reached confluence several wells were pooled together and the cells were cultured in T75 flask. Cells were passaged into several flasks to reach high density to be expanded into antibody production flasks. High concentration of antibodies was produced in Integra CL 1000 culture flasks as previously described (section 2.2.3).

2.10 Purification and biotinylation of Du23 mAbs

Supernatant from the flasks were centrifuged at 1,000 rpm for 10 minutes to pellet the cells. Clarified supernatant was filtered through 0.22 µm filter and collected for purification using Protein A-sepharose (Sigma) affinity chromatography as described previously (section 2.2.4). The antibody protein concentration (purified IgG) was determined using the bicinchoninic Acid (BCA) Protein Assay Reagent Kit according to manufacturer instructions (Pierce, USA). The procedure for protein determination is as previously described (section 2.2.5). The novel mAbs were biotinylated using Biotin-X-NHS (Calbiochem, USA). The biotinylation was carried out as previously described (section 2.2.6).

2.11 Generation of HIV-1 subtype C envelope glycoproteins

2.11.1 Plasmid DNA

The Du151 gp150 envelope in pcDNA3.1 (Invitrogen) (appendix 7.3) with a CD5 leader sequence was made by Sibusiso Nkosi. The gene was 2,552 bp and contained BspE1 and Xho-I restriction sites. Du151 gp150 envelope proteins, including modified proteins, were used in binding assays to map epitopes for novel mAbs. Changing of the leader sequence to CD5 as shown below was for the purpose beyond the scope of this thesis. This information was crucial in designing primers used in mutagenesis experiments.

CD5 leader sequence:

ATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG GGG ATG CTG GTC GCT TCC GTG CTA GCT

2.11.2 Propagation of plasmid DNA

The Du151 gp150 plasmid DNA was transformed into stbl2^{TM} *E. coli* competent cells (Invitrogen) according to manufacturer instructions. Briefly, the cells were thawed on ice, mixed and then 100 µl aliquots placed into pre-chilled polypropylene tubes. Du151 gp150 DNA (0.1 µg) was added to the cell aliquot and the suspension mixed by gently tapping the tube. The cells were incubated on ice for 30 minutes before being heat-shocked for 30 seconds in a 42^{o}C water bath, without shaking. Cells were then placed on ice for 2 minutes before adding 900 µl of S.O.C medium. The transformation reaction was incubated at 30^{o}C with shaking at 225 rpm, for 90 minutes (Lab-Line Incubator-Shaker, Orbit, USA). Transformed cells were selected on agar plates (Difco^{TM} Luria agar base-Miller, Becton Dickinson) containing 50 µg/ml ampicillin after incubation at 37^{o}C for 12 hours (Precision Economy Incubator, Jouan Inc).

A single bacterial colony representing transformed cells was picked and inoculated in 5 ml Luria-Bertani (LB) broth medium (Miller, Fisher Biotech) containing 50 µg/ml ampicillin and incubated at 37^{o}C for 12 hours with vigorous shaking at 200 rpm. The overnight culture was then used as starter culture and diluted 1:1,000 in 500 ml LB medium containing 50 µg/ml ampicillin. The culture was again grown at 37^{o}C for about 16 hours with vigorous shaking at 200 rpm to reach a cell concentration of 3-4 x 10^{9} cells per ml. The cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4^{o}C. The bacterial pellet was processed to obtained pure plasmid DNA containing the HIV-1 *env* gene of interest. Maxi-prep DNA was performed using Gene Elite Kit (Sigma) as described by manufacturer.

Purified plasmid DNA was analyzed by restriction digest on 1% agarose gel electrophoresis as described later (sections 2.11.4.2 and 2.11.4.4).

2.11.3 Primers used in PCR

We designed the following primers used in deleting the C1 region or V1/V2 regions from Du151 gp150 glycoprotein (Table 6). For deleting the C1 region, the set of primers were designed such that the first 22 nucleotides sequence of the forward primer (indicated in red) span the region of the CD5 leader sequence just upstream of the C1 region. The other half of the primer (23 nucleotides) (in green) covered the portion of the Du151 gp150 sequence immediately beyond the C1 region (i.e. the upstream portion of the V1 region). The reverse primer was a complement and inverted sequence of the forward primer.

Table 6. Reverse and forward primers used for deleting C1 or V1/V2 regions

Primer	**Sequence**
5'-DELC1(forward)	5'-G CTG GTC GCT TCC GTG CTA GCT ACC CTT AAC TGT ACT AAC GCC CCC-3'
3'-DELC1 (reverse)	5'-GGG GGC GTT AGT ACA GTT AAG GGT AGC TAG CAC GGA AGC GAC CAG C-3'
5'-DELV1/V2(forward)	5'-GAA GTT GAC ACC TCT CTG TAA CAG CTC TAC AAT TAC TCA G-3'
3'-DELV1/V2 (reverse)	5'-CTG AGT AAT TGT AGA GCT GTT ACA GAG AGG TGT CAA CTT C-3'

2.11.4 Transient expression of HIV-1 Env glycoprotein derived from Du151 gp150

Transfection of Du151 gp150 HIV-1 envelopes was performed using the 293T cell line following the Effectene^{R} transfection protocol (Qiagen) with some modifications. Briefly, 293T cells were plated in a 6-well tissue culture plate at the concentration of 2.5 x 10^5 cells/ml in 2 ml DMEM growth medium (Gibco) supplemented with 10% FBS. The culture was incubated overnight at 37^{o}C, with 5% CO_2 to reach 70% confluence. Once 50% confluence was reached, cells were transfected using 2 μg DNA, 3.2 μl enhancer reagent and 10 μl effectene (Effectene Transfection Reagent kit, Qiagen). Expression was activated with 1 μg HIV-1 *tat* protein (kindly provided by Dr Robinson). The HIV-1 envelope glycoprotein expression was continued for 48 hours after which the supernatant together with the cells were harvested. Lysis was performed using 1% Triton-X100 in the presence of 1% protease inhibitor (Sigma). These Du151 gp150 protein preparations were used in ELISA binding experiments to evaluate the novel mAbs. Also, the same transfection protocol was used to transfect Du151 C1 and V1/V2 deleted constructs. These proteins were also harvested and treated in the same fashion and used in ELISA binding experiments.

2.11.5 Mutagenesis

The Quickchange mutagenesis kit (Strategene) was used to generate two sets of constructs from Du151 gp150 envelope protein: in one construct we deleted C1 region, while in another construct we deleted both V1/V2 regions together. The principles upon which the mutagenesis system works are illustrated in Fig 12 below.

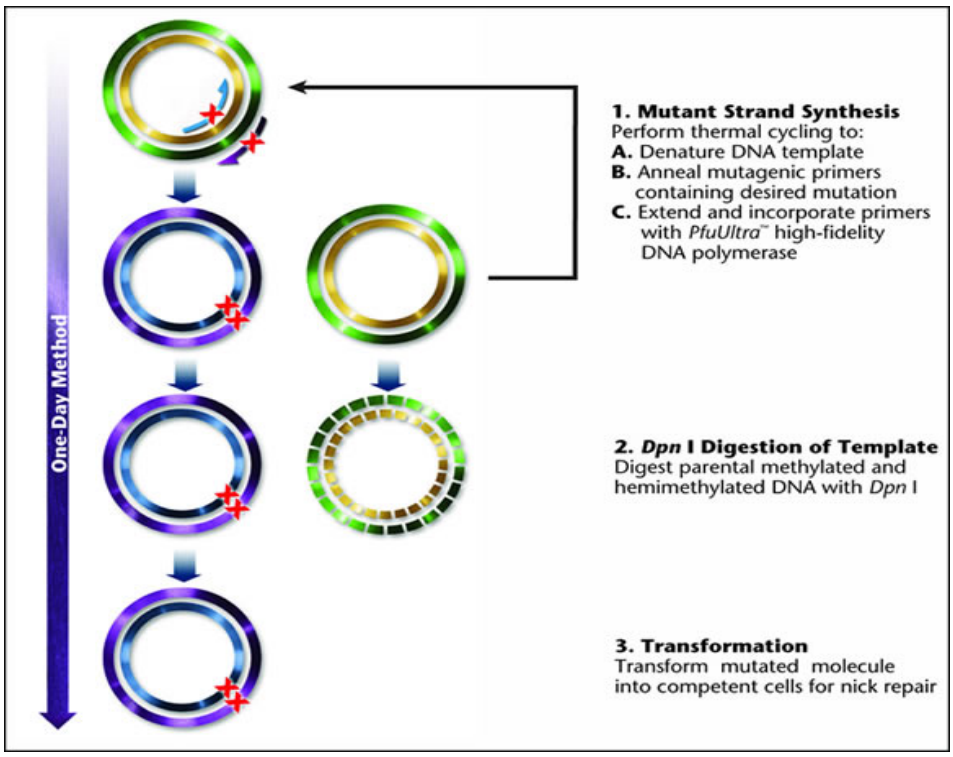

Fig 12. Schematic representation of the principles of the QuickChange site directed mutagenesis method. The diagram is obtained from the instruction manual (Stratagene).

2.11.5.1 Deletion of C1 region

The primers for deleting the C1 region were designed such that half of the upstream portion of both the forward and reverse primers overlapped with the CD5 leader sequence (Fig 13). The C1 region (297 bp) was deleted by using a combination of 5'-DELC1 and 3'-DELC1 primers (Fig 13). The sample reactions were prepared using 15 ng of Du151 gp150 plasmid DNA, 140 ng each of the oligonucleotide primers, 2.5U of *PfuUltra* HF DNA polymerase, 1 µl dNTP (50 mM) mix and 3 µl QuikSolution in a 50 µl reaction volume. The cycling parameters were as follows: 95^{o}C for 1 minute, 95^{o}C for 50 seconds, 60^{o}C for 50 seconds 72^{o}C for 8 minutes, and final extension at 72^{o}C for 7 minutes. Following this, 10U of Dpn I

enzyme was added to the amplification reaction. 2 µl of the Dpn I treated DNA was used for the transformation of 50 µl of XL1-Blue supercompetent cells supplied with the kit (it should be noted that in this particular case, the selection was not based on blue-white colonies). The mixture was heat-pulsed at 42^{o}C for 45 seconds and then incubated on ice for 2 minutes. 500 µl of NZY^{plus} broth, which had been pre-warmed to 42^{o}C was added to each transformation mixture which were then incubated at 37^{o}C for 1 hour, shaking at 230 rpm.

The reaction was divided into aliquots of 250 µl and plated onto selection plates containing 50 µg/ml ampicillin. Plates were incubated at 37^{o}C for not more than 16 hours. Single colonies were picked and grown up in 5 ml LB containing 50 µg/ml ampicillin at 37^{o}C for 12 hours. The presence of the desired mutation, i.e. deletion of the C1 region, was analyzed by appropriate restriction digestions (section 2.11.4.2) followed by 1% agarose gel electrophoresis. Also, the expression of the functional Du151 gp 150 with deleted C1 (Du151 DelC1) was assessed by immunoprecipitation (section 2.12).

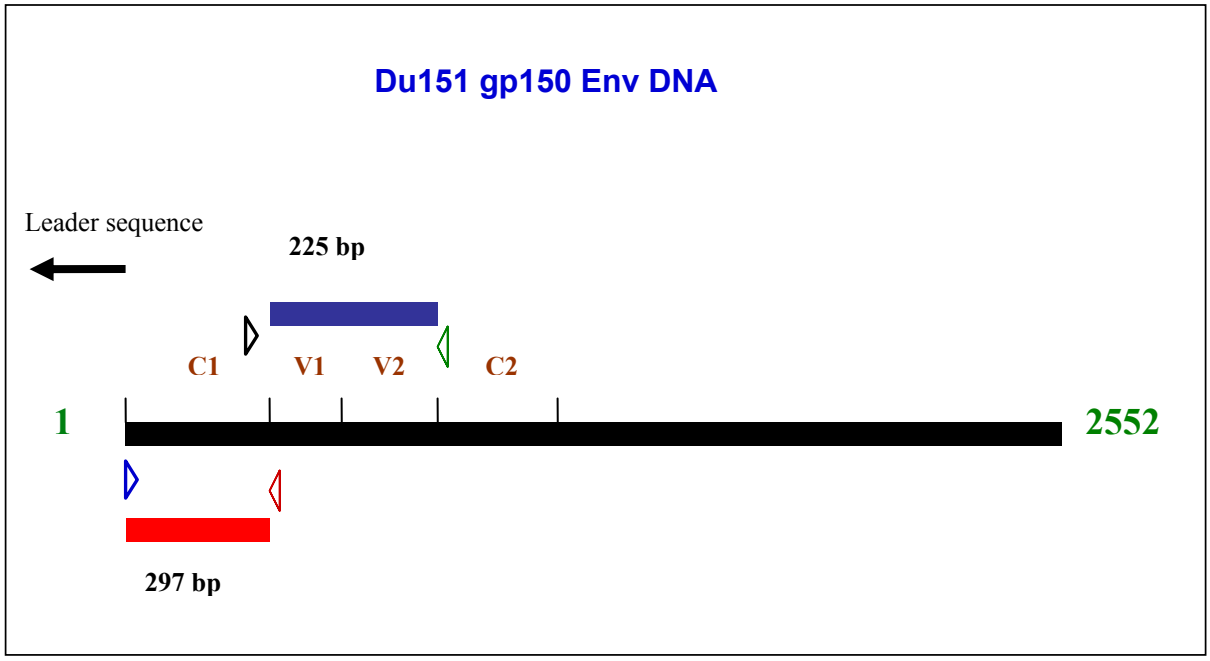

Figure 13. Diagram showing the positions of primers in Du151 gp150 used to generate mutants (diagram not drawn to scale). The positions of 5'-DelC1-forward (blue arrow head) and 3'-DelC1-reverse (red arrow head) primers used for deletion of C1 region covering 297 base pairs are indicated. The positions of 5'-DelV1/V2-forward (black arrow head) and 3'-DelV1/V2-reverse (green arrow head) primer for deleting V1/V2 regions spanning 225 bp are indicated. The position of the leader sequence is indicated by the black arrow.

2.11.5.2 Restriction analysis of deleted C1 region

Two double restriction enzyme digests were performed using parental Du151 gp150 DNA or a construct with C1 deleted. In one 50 µl reaction 5 µg (10 µl) of parental Du151 gp150 DNA was digested with 20,000U (1 µl) each of the restriction enzymes Xho I and BspE I (New England BioLabs, USA) and NEBuffer 2 (5 µl) supplemented with 100 µg/ml BSA (1 µl). 32 µl of double distilled water was added to the reaction mix to bring the volume up to 50 µl. Parental Du151 gp150 DNA was digested in the same manner. The reactions were incubated at 37^{o}C for 2 hours before they were analyzed by gel electrophoresis.

Two single restriction enzyme digests were performed in a volume of 50 µl using parental Du151 gp150 or with a C1 deleted Du151 gp150 construct. In one single digestion reaction 10 µl (5 µg) of parental Du151 gp150 DNA was digested with 1 µl (20,000U) of Xho I (New England BioLabs, USA) restriction enzyme, and 5 µl of NEBuffer 2 (10X) supplemented with 1 µl of 100 µg/ml BSA. 33 µl of double distilled water was added to the reaction mix to bring the volume up to 50 µl. The reaction mix was incubated at 37^{o}C in a water-bath for 2 hours. Parental Du151 gp150 DNA was also digested with Xho I in the same manner.

2.11.5.3 Deletion of V1/V2 region

To delete the V1 together with the V2 regions, the 5'-DelV1/V2 (forward) primer was designed such that the first 23 nucleotides sequence (blue) covered the region upstream of the V1 region (part of the C1 region) (Fig 13). The other half of the primer (black) spanned the region just downstream of the V2 (first part of C2 region) (Fig 13). The 3'-DelV1/V2 (reverse) primer was a reverse and complement of the forward primer. The V1 together with the V2 regions (225 bp) were deleted using a combination of primers 5'-DELV1/V2 and 3'-DELV1/V2 (Table 6). The V1/V2 regions were deleted in a manner described above for deleting the C1 region. However, because of difference in the T_m the cycling conditions were adjusted accordingly. The cycling parameters were as follows: 95^{o}C for 1 minute, 95^{o}C for 50 seconds, 65^{o}C for 50 seconds 72^{o}C for 8 minutes, and final extension at 72^{o}C for 10 minutes. The reaction was then analyzed as described before (section 2.11.4.4).

2.11.5.4 Restriction analysis of deleted V1/V2 region

Two double digestion reactions in a total volume of 50 µl were done using parental Du151 gp150 DNA or a construct with V1/V2 deleted. Both single and double restriction enzyme digests were performed in same way as in the above section (section 2.11.4.2).

2.12 Immunoprecipitation

The Du151 C1 and V1/V2 deleted constructs were transfected as described previously. 5 ml of supernatant from the transfections was harvested and the protein was immunoprecipitated with 10 µg/ml of IgG1b12 antibody, and the reaction was run at room temperature for 2 hours while shaking at 150 rpm. 50 µl in 5 ml of Protein A agarose beads (Pierce) were added to the mixture and incubated further for one hour, while shaking. The mixture was centrifuged at 1,200 rpm for 3 minutes to collect the beads. The supernatant was discarded, and the beads were washed with 1 ml 0.5 M sodium chloride (NaCl). After centrifugation, the NaCl wash was discarded and the bead pellet was then washed with 1 ml PBS. After centrifugation, the bead pellet was mixed with 20 µl of protein sample buffer (0.1M Tris pH 8, 1% bromophenol blue, 2% glycerol, 4% SDS, and 1% β-mercaptoethanol) and then boiled for 5 minutes. 10 µl of each of these preparations was loaded onto SDS-PAGE gel with protein marker (Promega) and electrophoresed at 200 Volts. The gel was stained with Coomassie blue for 30 minutes followed by destaining with buffer consisting of 70 ml acetic acid, 400 ml methanol and made up to a litre with 530 ml of water.

2.13 Characterization of Du23 mAbs

2.13.1 Determination of IgG subclass

The immunoglobulin subclass of the novel mAbs was determined using a four-step ELISA. 100 µl of 20 µg/ml sheep anti-human IgG subclass 1, 2, 3, or 4 (Sigma) was coated to high-binding ELISA plate, and incubated for at least 2 hours before use. PBS-Tween was used for washing in all the steps, and four washes per step were done. The plate was washed and blocked with 4% skim milk in PBS. Then 100 µl sample dilutions were transferred to the test plate and incubated at 37^{o}C for 1 hour. After washing, second antibody murine monoclonal antibodies specific for the different IgG subtypes (Sigma-Aldrich, South Africa) were used. 100 µl of horseradish peroxidase-streptavidin conjugated goat anti-mouse IgG diluted 1:2000 was added (Sigma). The plate was developed as previously described (section 2.4.2.6).

2.13.2 Determination of IgG light chain

Light chain type was determined by ELISA, using 100 µl of 25 µg/ml mouse IgG κ or λ light chains (Sigma-Aldrich, South Africa) to coat the ELISA plates. The same ELISA procedure to develop the plates was followed as described above (section 2.13.1).

2.13.3 Western blotting

2.13.3.1 SDS-PAGE

A 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared by making a separation gel in total volume of 15 ml by mixing 6.5 ml water with 4.5 ml of 30% acryl-bisacrylamide mix (Sigma-Aldrich, South Africa), 3.8 ml Tris-HCl, 150 µl SDS, 150 µl of 10% ammonium persulfate (APS) (Sigma-Aldrich, South Africa) and 6 µl of

TEMED (Sigma-Aldrich, South Africa). The gel was cast into the gel tray and alllowed to saturate (10-15 minutes). Once saturated, the stacking gel was then prepared by mixing 2.7 ml water with 500 µl of 30% acryl-bisacrylamide mix, 500 µl of 0.5M Tris-HCl, 40 µl SDS, 40 µl APS and 4 µl TEMED. This was poured into the tray on top of the separation gel and allowed to set. The Du151 gp150 protein was prepared in 2X SDS PAGE sample buffer and then boiled was run into the at 200 Volts (20 mA) in 1X SDS PAGE Running buffer.

2.13.3.2 Transfer of protein from gel to membrane

The gel was equilibrated with the transfer buffer for one hour, with shaking. Hybond-C nylon membrane (Amersham Life Sciences), 3 mm Whatman absorbent papers and were cut equal to the to the size of the gel. The membrane was also equilibrated with transfer buffer for 15 minutes while the absorbent papers were moistened in the buffer. The nitrocellulose was dried for 15 minutes, after which it was blocked with 5% skim milk dissolved in Tris-Buffered Saline (TBS) for one hour. After washing the nitrocellulose three times in 50 ml TBS, each wash for a period of 5 minutes, the membrane was cut into strips. In all subsequent steps washes are carried out using 50 ml TBS, and they are repeated three times each over a period of 5 minutes. 10 µg/ml of each of the antibodies novel mAbs to be tested was prepared in 10 ml of 0.5% BSA in TBS and each of this antibody was incubated with a strip for four hours. After the incubation, the strips were washed and then incubated for an hour in alkaline-phosphatase conjugated goat anti-human IgG (Fc-specific) diluted 1:1000 in 0.5% BSA in TBS. After washing, the strips were submerged and incubated for an hour in substrate solution. Antibody-antigen complexes were detected as colour bands using NBT-BCIP as substrate. The NBT/BCIP substrate was made by dissolving one tablet in 10 ml of

distilled water. The reaction was allowed to develop in the dark until visible bands are observed in approximately 10-30 minutes. Then the strips were removed, washed in distilled water and dried by blotting in filter paper.

2.13.4 Antibody binding assays

2.13.4.1 Binding Du23 mAbs to viral antigen stock

The mAbs were evaluated for their ability to bind to different antigen species. This included antigens generated using STR5 cells infected with subtype C viruses Du179 or Du151; Du179 grown in C8166R5; purified Yu-2 gp120 protein, viral antigen preparations SF162, ADA, and 89.6 using reverse capture immunoassay as previously described (Robinson et al., 1998). This enabled us to determine which virus antigen preparation is best suited to use for subsequent experiments to characterize the novel mAbs. Furthermore, these experiments could be used an indication of viral subtype specificity. We could determine whether binding of the novel mAbs was restricted to subtype B or C viral strains or broadly cross-reactive. The plates were developed and analyzed as described previously (section 2.4.2.6).

2.13.4.2 Cross-competition assays

Isolated mAbs were tested for binding competition with each other to determine whether they recognized overlapping or nonoverlapping sites according to published methods (Choe et al., 2003; Cole et al., 2001; Farzan et al., 2000; Robinson et al., 1998; Xiang et al., 2002). We used two main ELISA competition formats in many of our experiments: reverse capture or direct coating with an appropriate mAbs or antigen as illustrated in Figure 14 below.

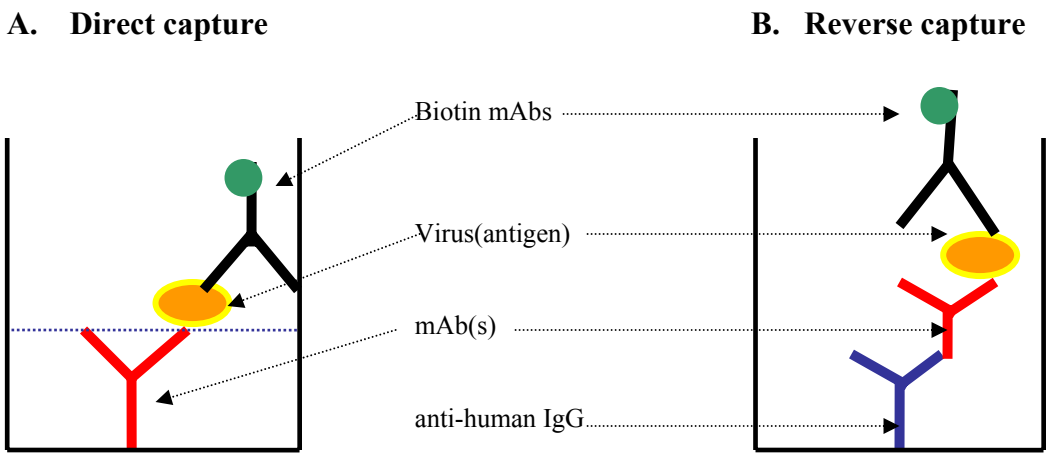

Fig 14. Diagram showing two main ELISA formats used in analysis of Du23 mAbs. (A) (Direct capture) mAb of choice was captured onto ELISA plate to which an antigen is bound. The horizontal dotted line (blue) in (A) indicates the variation in format in which the antigen can be directly captured onto the ELISA plate followed by the respective mAb and then biotin mAb(s) or conjugated (secodary) mAb. (B) (Reverse capture) a capture mAb is bound to anti-human IgG coated plate before binding the antigen. The secondary mAb(s) (biotin) and development of the signal is common to both setups.

2.13.4.2.1 Direct capture ELISA

High-binding ELISA plates were directly coated with 100 µl per well of appropriate mAbs at the final concentration of 5 µg/ml diluted in PBS. The plates were incubated at room temperature for an hour after which they were washed four times in PBS containing 1% Triton. All subsequent washing steps were repeated four times using this wash buffer. The plates were then blocked with total human IgG described previously. Following washing step, 75 µl of Du23 mAbs including controls antibodies at a final concentration of 20 µg/ml diluted in RPMI-1640 supplemented with 10% FBS were captured into relevant wells and incubated for 30 minutes. Without washing the plate, 75 µl per well of appropriate biotinylated mAbs diluted 1:2000 were captured across the plate in their respective wells.

After washing the plates were thereafter developed with HPO-SA and TMB substrate as described before (section 2.4.2.6).

2.13.4.2.2 Reverse capture ELISA

High-binding ELISA plates were coated with goat anti-human IgG (Fc-specific) antibody (Sigma) antibody as described in section 2.8. To these plates, 100 µl of Du23 mAbs at a final concentration of 5 µg/ml were captured into appropriate wells. Appropriate controls were included and the plates were incubated for an hour, before washing and blocking with 200 µl per well of RPMI supplemented with 10% FBS plus purified total IgG at the concentration of 400 µg/ml and incubated for 30 minutes. The blocking reagent was discarded and 100 µl of relevant antigen preparations were added to the plates, and incubated at 37^{o}C for an hour. The plates were washed and 100 µl of biotinylated Du23 mAbs diluted at 1:2000 were captured onto the plate, each mAb into separate well. Thereafter the plate was developed with HPO-SA and TMB substrate as described before (section 2.4.2.6).

In similar competition assays we examined the effect of soluble CD4 (sCD4) at the concentration of 10 µg/ml on binding of biotinylated mAbs to captured antigen preparations (purified sCD4 was kindly provided by Dr Robinson). The plates were developed with HPO-SA and TMB as described previously (section 2.4.2.6).

2.13.5 Testing the dependence of Du23 mAbs on protein conformation

The Du23 mAbs were also tested for their dependence on the conformation of the viral antigen species by ELISA. The high-binding ELISA plates were directly coated with 100 µl

of 89.6 antigen preparations, Yu-2 gp120 and Yu-2 Del V1V2 at 2 μg/ml diluted in PBS (Fig 14A). The plates were incubated at room temperature for an hour, and then washed four times in PBS-Triton-X100. All subsequent washing steps were done with PBS-Triton-X100 and repeated four times. Blocking was done using RPMI supplemented with 10% FBS. In one plate, 100 μl of PBS was placed in each well while on the other plate 100 μl of 50 mM Dithiotreitol (DTT) was placed into each well and the plates were incubated for 30 minutes at room temperature to denature the antigen protein. After washing, 100 μl of the novel mAbs, EH21, 39F, 1.7B and KX16 mAbs at the concentration of 5 μg/ml was placed into respective wells for both DTT treated and non-treated plates. The plates were incubated at 37^{o}C for one hour. After the incubation 100 μl of goat anti-human IgG-Horseradish peroxidase coupled conjugate diluted 1:2000 in PBS-Tween-Whey was added. The plates were then incubated for an hour at room temperature. The plates were developed with TMB substrate as described before (section 2.4.2.6).

2.13.6 Neutralization assays

2.13.6.1 Viruses

The viruses Du23, Du151, Du179, Du421, LTNP28, and TM8 are subtype C viruses that were passaged in STR5 cell line while 615-004-04 virus, also subtype C was grown in D3R5 cell line over several generations. These viruses were adapted in the respective T cell lines which may have rendered them more sensitive to neutralization.

2.13.6.2 Luciferase assay

2.13.6.2.1 Evaluation virus titres

The JC53 cell line was grown to confluence in T75 culture flasks using DMEM supplemented with 10% FBS. After washing three times with HBSS, the cells were trypsinized and harvested into 50 ml falcon tubes. HBSS was used to fill up the tubes, and then centrifuged at 1,000 rpm. The pellet was suspended in complete medium made up of DMEM with 10% FBS and 20 μg/ml DEAE dextran. The DEAE dextran is used in the medium to enhance infectivity. 200 μl of 5 x 10^3 cell/ml cells were plated into each well. Appropriate virus dilutions were made in complete medium and plated accordingly. The virus used here were Du151 and Du179 that were grown in STR5 cell line which were passaged more than 10 times. Viruses Du23, TM8, LTNP28, Du421 and 615-004-04 were also used. The 615-004-04 virus was grown in D3R5 cells while the other viruses were grown in STR5 cells, and all of these viruses were passaged more than 10 times. The infected cultures were incubated for 48 hours. After the incubation period, the medium was aspirated from each of the culture preparation, and 100 μl of GloLysis Buffer (Promega, USA) was added. After a 10 minute incubation, 50 μl of the lysed cell suspension was transferred into white-coloured Nunc-immuno™ maxisorp™ plate (Nunc™) to which 50 μl Bright-Glo assay substrate (Promega, USA) was added, and the reaction was analyzed immediately.

2.13.6.2.2 Neutralization using cell line grown viruses

Dilutions of viruses were prepared in complete medium as described above as: Du151 at 1:100, Du179 at 1:400, Du23 and 615-004-04 at 1:10 dilutions were used. SF162 was

included as control and it was used at 1:400 dilution. The neutralization assay was performed by incubating 200 µl of appropriate dilutions of the viruses each with 200 µl of the novel mAbs and 21E (control) each at final concentration of 20 µl/ml. The mix was incubated at 37^{o}C 5% CO_2 for an hour. The JC53 cells were prepared in 96-well tissue culture plate to which the virus/mAb mixture was added and incubated for 48 hours. All experiments were done in duplicate and neutralization for Du179 and Du151 was repeated more than twice.

2.13.6.2.3 Neutralization using pseudovirions

The ability of the isolated mAbs to neutralize Du151.2 pseudovirions was evaluated using the JC53 cells in a luciferase assay (this assay was performed courtesy of Ms Natasha Taylor). The mAbs were used at concentrations of 20, 40, 80 and 160 µg/ml (100, 200, 400 and 800 dilutions). 50 µl of the diluted pseudovirus (1:65) was mixed with 50 µl of diluted mAbs, plus 2 controls (sample 106 and IBU-21) and incubated at 37^{o}C for an hour. Cells were prepared at 10^5 cell/ml in DMEM medium supplemented with 10% FBS and 75 µg/ml DEAE dextran. Then 100 µl of the cell suspension was added to the pseudovirus/mAbs in the 96-well tissue culture plate. The plate was incubated at 37^{o}C in 5% CO_2 for 48 hours. Luciferase activity was measured by removing 150 µl of medium from each well, and replaced with 100 µl of Bright-Glo Reagent and then the plate was incubated at room temperature for 2 minutes. After mixing, 150 ml was transferred into luminometer and the readings were taken immediately.