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The role of residues 153 and 283 in the non-macrophage-tropic phenotype of HIV-1 R5 envelopes from transmitted viruses

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ABSTRACT

The ability of HIV-1 to interact with receptors on the host cell plays a major role in determining its tropism. Several point variations in the envelope gene of clade B R5 viruses have been shown to confer tropism towards primary macrophages. The T283N mutation in the CD4bs of HIV has been shown to drastically affect macrophage tropism. More recently an E153G mutation located in the V1 loop which modulates the V3 loop to prime low CD4 use has also been shown to confer high levels of macrophage infectivity. This shift may also increase the envelopes sensitivity to neutralizing antibodies. This project explores whether these residues involved in the non-macrophage-tropic phenotype of transmitted R5 viruses or effect sensitivity to entry inhibitors, soluble CD4, b12 and Maraviroc. The results presented confirm that substitutions at 153 and 283 affect macrophage-tropism of some viruses and/or shift sensitivity to entry inhibitors. Importantly, my data implicates the presence of further, unknown and dominant determinants of non-macrophage-tropism.

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BACKGROUND

HIV History

The Human Immunodeficiency Virus (HIV) is a retroviral lentivirus which leads to Acquired Immunodeficiency Syndrome (AIDS) in humans. HIV is a retrovirus characterized by a long and variable incubation period. The first clinical observations of AIDS like symptoms, known at the time as Pneumocystis carinii pneumonia (PCP), were published in 1981 (Drew, 1981). This was then linked to a rare form of skin cancer known as Kaposi's sarcoma (KS) and eventually led to the discovery of a new retrovirus initially named HTLV-III but later renamed HIV. Since its discovery the virus has infected and killed many millions leading to it being considered a pandemic by the World Health Organization. Currently, there are at least 33.3 million people infected with HIV (UNAIDS, 2010).

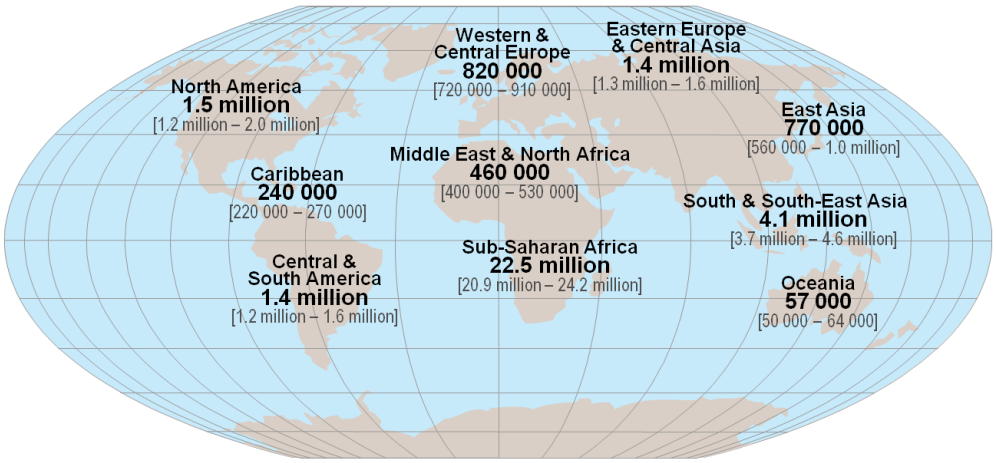


Figure 1: Global HIV Pandemic. The world health organization 2009 report shows that the worldwide pandemic has reached 33.3 million since its discovery in the 1980's (UNAIDS, 2010).

Pathology of HIV Infection

The HIV virus is thought to have originated in non-human primates and transferred to humans in Sub-Saharan Africa more than a century ago. Two main variants of the virus have been found, HIV-1 which is the cause of most of the cases worldwide and HIV-2 which is less virulent and is found mostly in West Africa (Coffin, 1997). Both are closely related to Simian Immunodeficiency Virus (SIV) with HIV-1 being more closely linked with SIV_{cpz} which infect chimpanzees and HIV-2 being more close to SIV_{smm} which infects sooty mangebey monkeys (Knipe and Howley, 2001). HIV is divided into 3 groups: M – the main group, O – an outlier group, and N – the group being neither M nor O. The M group is then separated in subtypes, or clades, from A-J with B being most prevalent in developed nations (Knipe and Howley, 2001) (**Figure 2**).

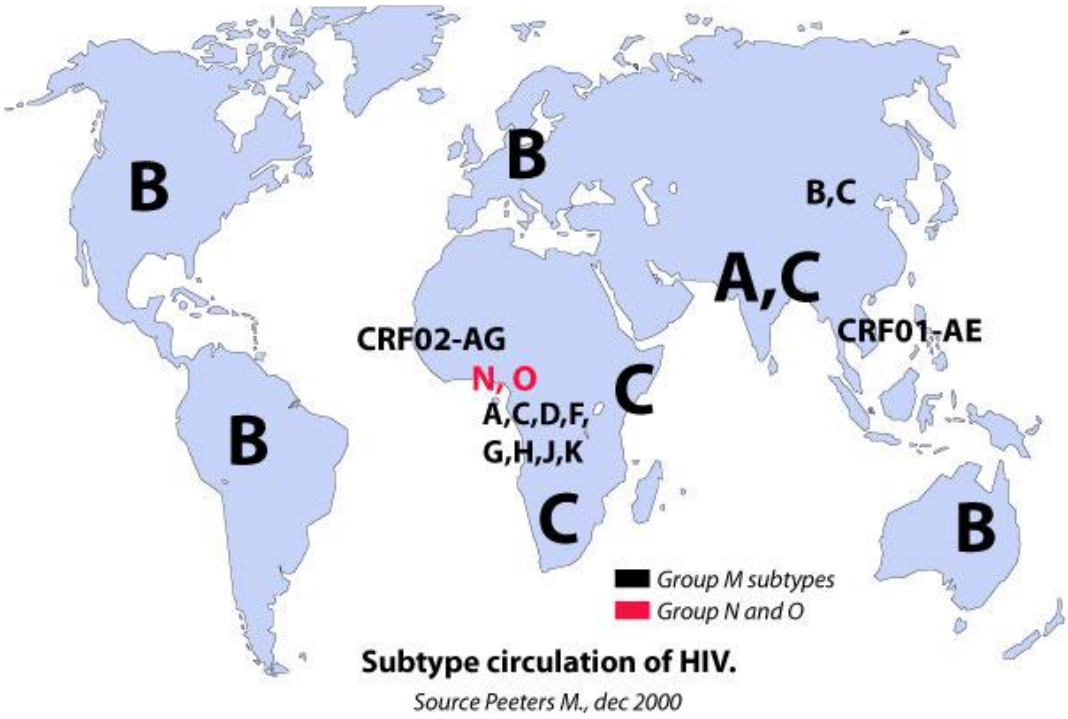


Figure 2: HIV-1 Subtype distribution by geography. Map shows HIV subtypes by locations, the developed world is predominantly B type while Africa shows all three groups as well as many group M subtypes, this (Vidal, 2000).

HIV is the only lentivirus known to infect humans (Knipe and Howley, 2001). The typical course of an HIV-1 infection is highly variable with death occurring approximately a decade after infection (**Figure 3**). The initial acute phase of the disease which happens immediately following infection results in rapid viral replication. This increase in viral copy is accompanied by a drop in $CD4^+$ T cells. As the disease progresses it enters a chronic asymptomatic phase which is characterized by clinical latency in which viral loads drop and $CD4^+$ T cells levels stabilize. Once the T cell levels fall below 200 cells per microliter the body's cell-mediated immunity is no longer functional. At this point the disease progresses to a symptomatic final phase: AIDS. The onset of AIDS is often accompanied by the infection from opportunistic microbes which eventually lead to death.

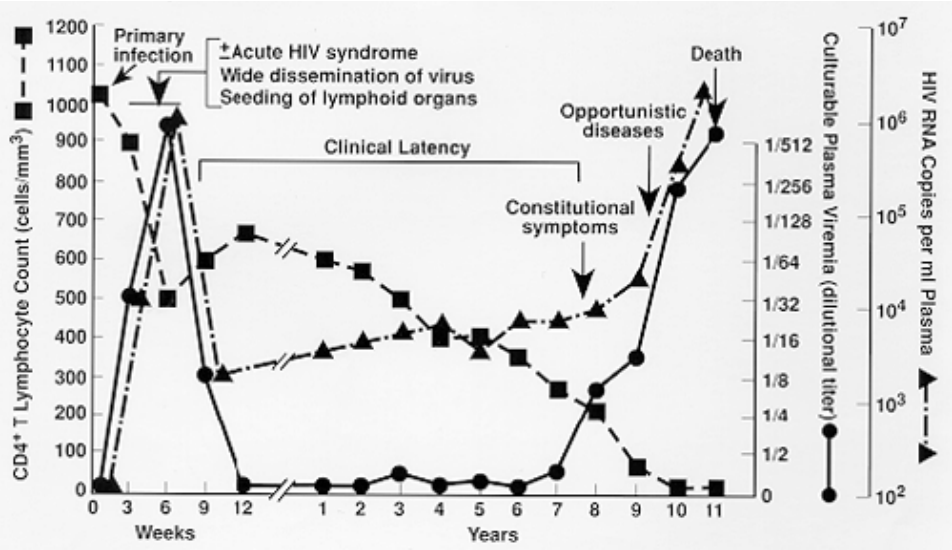


Figure 3: The course of HIV infection. The graph shows the levels of $CD4^+$ T lymphocytes, HIV RNA copies per ml of plasma, and plasma viremia over the course of the infection. There are three distant phases seen, acute, asymptomatic, and symptomatic/AIDS (Fauci, 1996).

The symptoms of the disease pathology are brought on by the underlying characteristics of the virus itself. HIV uses reverse transcriptase to turn its RNA genome into DNA so that the host's cells can replicate the virus. This process is extremely error prone, making 1 mistake per

10,000 base pairs copied; this results in one error per round of replication. The difficulty in treating HIV is that it is not one virus but rather many slightly different viruses known as quasispecies. This also allows it to evade the immune system which isn't able to keep up with all the variations.

Viral Composition

Like all retroviruses HIV contains several structural genes including: *gag*, *pol*, and *env* (Wiley, 2001). However HIV is more complex and contains at least 6 more genes including: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (Wiley, 2001). These genes are summarized in **Table 1** by name as well as the protein encoded and its function.

Table 1: HIV Composition

Gene Name	Proteins Encoded
Group-specific Antigen (*gag*)	Structural Protein; Precursor for the Matrix (MA), Capsid (CA), Nucleocapsid (NC), and p6 proteins.
Polymerase (*pol*)	Structural Protein; Protease (PR), Reverse Transcriptase (RT), and Integrase (IN).
Envelope (*env*)	Structural Protein; Envelope glycoproteins
Trans-activator of viral transcription (*tat*)	Regulatory Protein; Enhances transcription.
Regulator of Viral Protein Expression (*rev*)	Regulatory Protein; Regulates RNA splicing.
Negative Factor (*nef*)	Accessory Protein; Enhances viral replication. Down modulation and degradation of CD4 and MHC class I.
Virus Infectivity Factor (*vif*)	Accessory Protein; Crucial for infectious virons specific to cell type. Causes degradation of host defense protein, APOBEC 3G.
Viral Protein R (*vpr*)	Accessory Protein; Stimulates viral gene expression. Important for infection of non-dividing cells.
Viral protein U (*vpu*)	Accessory Protein; Enhances virus release by removing tetherin from the cell surface. Also degrades CD4.

(Tables contents from: Wiley, 2001; Knipe and Howley, 2001).

Structural Proteins – gag, pol and env

The HIV-1 genome contains the same structural proteins as other retroviruses and are created via a polyprotein precursor (Knipe and Howley, 2001). The first of these is the group specific antigen (*gag*) protein which is cleaved to form four other proteins: matrix (MA), capsid (CA or p24), nucleocapsid (NC) and p6 proteins (Watts, 2009). The MA protein is important for targeting gag and gag-pol precursor polyproteins into the plasma membrane prior to viral assembly (Frankel 1998). The CA protein forms the core of the virus and also contains some of the most highly conserved domains of the virus (Frankel 1998). The NC protein is the third part of the *gag* polyprotein and its primary role is to bind to the packaging signal and deliver two copies of full-length RNA into the assembling virion (Frankel 1998). **Figure 4** shows the genome of HIV-1 as well as the locations of each of the related proteins.

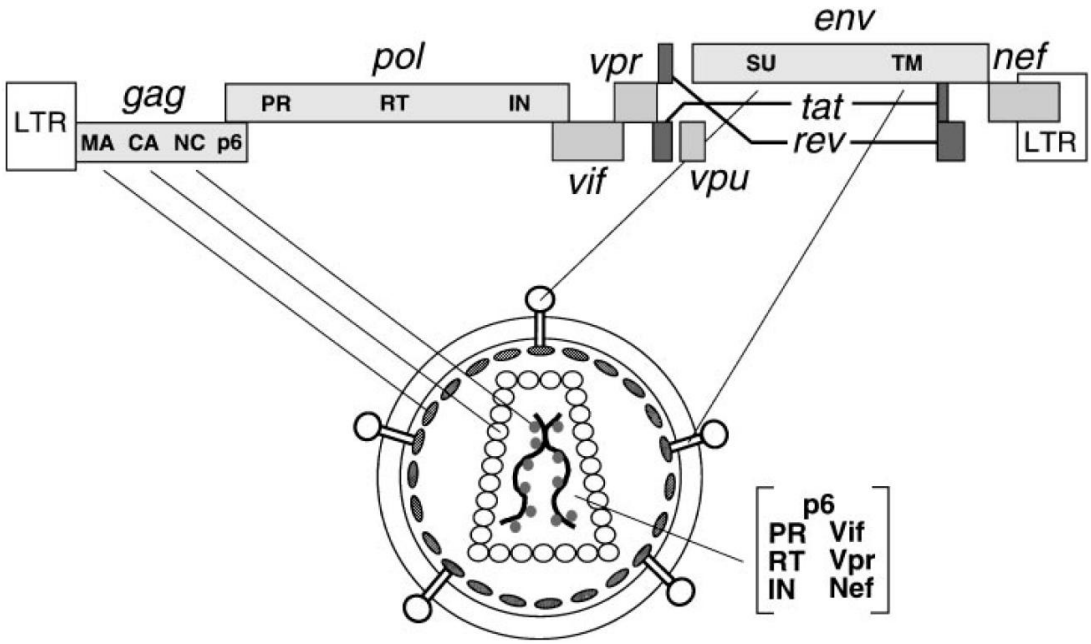


Figure 4: Map of the HIV genome. Organization of the HIV-1 genome and virion (Frankel, 1998). SU = gp120, TM = pg41.

The second of the precursor proteins is polymerase (*pol*) which is cleaved to form three proteins: viral protease (PR), reverse transcriptase (RT), and intergrase (IN) (Watts, 2009). PR functions as a dimer enzymatically cleaving polyproteins to produce MA, CA, NC, p6, PR, RT and IN proteins (Frankel 1998). PR is a prime site for chemotherapy research because of its importance in the replication phase of the virus (Frankel, 1998). Prior to integration into the hosts chromosome, the viral RNA must first be reverse transcribed into dsDNA, this is the role of RT (Frankel, 1998). RT is also the primary cause of genetic variation and as such is also a target of drug design (Frankel 1998). The IN protein catalyzes a series of reactions which integrates the viral genome into the host chromosome (Frankel 1998) and has recently been targeted for anti-HIV drugs.

The envelope (*env*) gene is the last of the structural polyproteins and produces gp120 and gp41 (Watts, 2009). These proteins are grouped into trimers on the virion which consists of a gp120 surface protein and a gp41 transmembrane subunit (Dunfee, 2006). These are both formed from the precursor, gp160 being cleaved in the golgi apparatus (Clapham and McKnight, 2002). The gp120 protein is the focus of much research because it facilitates entry into the target cell. It also contains five variable loops which can modulate the tropism of the virus (**Figure 5**).

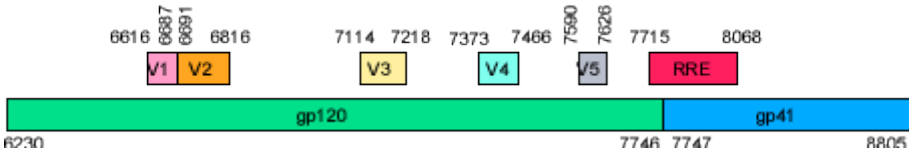


Figure 5: The Envelope Gene. The polyprotein env is cleaved to form gp120 and gp41. The figure shows the location of the variable loops. The env gene also encodes the rev response element (RRE), an RNA structure involved in nuclear export of HIV transcript. Numbers represent the nucleotide sequence (LANL).

Regulatory Proteins

The HIV genome also includes two regulatory proteins: the transactivator of viral transcription (*tat*) and the regulator of viral protein expression (*rev*). The *tat* protein up regulates the production of viral mRNAs up to 100 fold. It does not bind to DNA but instead to an RNA hairpin known as the trans-activating response element or TAR (Frankel 1998). Without *tat, RNA* polymerase II will only transcribe for a few hundred nucleotides (Frankel 1998). The second regulatory protein, *rev*, contains a nuclear export signal which allows it to shuttle between the nucleus and cytoplasm (Frankel, 1998). Rev binds the RRE present on incompletely or unspliced viral mRNA allowing it to be exported from the nucleus without extensive splicing (Frankel 1998).

Accessory Proteins

HIV-1 also has four accessory proteins that are unique to lentiviruses and do not appear to be necessary for viral replication *in vitro*; they are: *nef*, *vif*, *vpr*, and *vpu*. Both nef and vpu down regulate cellular levels of CD4 (Frankel, 1998). This is beneficial to the virus because the gp160 precursor protein is sometimes held in the endoplasmic reticulum through interaction with newly synthesized CD4 molecules (Frankel, 1998). The *vif* protein disrupts the human antiviral protein APOBEC and also has some role in the assembly or maturation of the virus (Rose, 2004; Frankel 1998). The vpr protein mediates the rapid transportation of nucleoprotein complexes following the fusion and entry into the cell (Frankel 1998). Vpr's nuclear localization ability is especially important in non-dividing cells such as macrophages (Frankel, 1998).

Viral Entry

The entry of the HIV virion into the cell is mediated by the high affinity interaction between gp120 and CD4 which induces a conformational change in the structure of gp120 and exposes the co-receptor binding site (Dunfee, 2006). This then leads to a conformational change in gp41 which enables fusions and entry into the cell (Dunfee, 2006). The variable loops discussed above may help the virus to escape antibody-mediated neutralization (Clapham and McKnight, 2002).

The CD4 receptor which is used by HIV-1 to gain entry to the cell is most commonly found in the following cells: T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. In the body, CD4 recognizes class II major histocompatibility complex (MHC) molecules (Ryu, 1990). This recognition plays a role in mediating an efficient immune response however it can also be subverted to become the receptor for HIV-1 infection (Ryu, 1990). HIV-1 is usually unable to infect cells without CD4, this signifies that the presence of CD4 is a major determinant of HIV-1 tropism.

Co-receptor and Tropism

As discussed the primary receptor for HIV is CD4, however in order for the virion to enter the cell a coreceptor is needed. This is fulfilled by seventransmembrane (7TM) chemokine receptors, either CCR5 (R5) or CXCR4 (X4). In some cases a virus is able to use both of these receptors. It has been suggested that it is possible for HIV-1 to use other 7TM chemokine receptors *in vitro* however their significant use *in vivo* is not currently supported (Clapham and McKnight, 2002). R5 viruses infect memory T-cells and primary macrophages while X4 viruses mainly target naïve T-cells. This makes the co-receptor a secondary determinant of tropism.

The co-receptor is especially important in deciding what type of T-cell the virus can infect. The main receptor for transmitted variants of HIV-1 is R5 with X4 viruses often becoming detectable with the onset of AIDS which leads to the rapid decline of $CD4^+$ T-cells and eventually death (Caverell, 2009). However the ability of R5 viruses to infect primary macrophages is highly divergent and varies over 1000 fold (Musich 2010). Several mutations in R5 viruses have been identified which increase the macrophage tropism; these include E153G and T283N mutations.

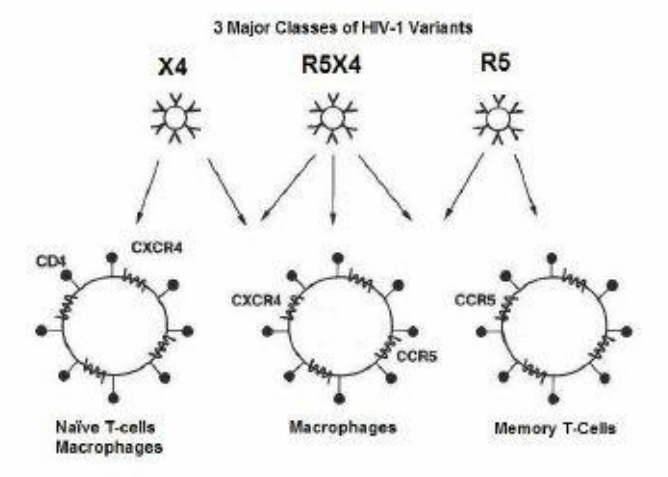
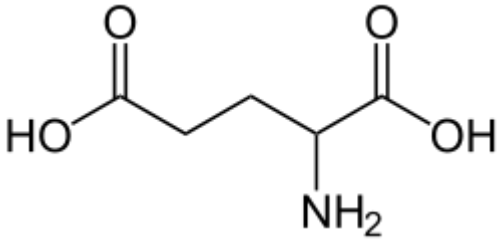
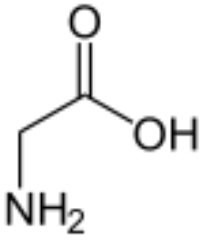


Figure 6: HIV-1 Co-receptor usage. HIV-1 is able to use either X4, R5, or a combination of co-receptors to infect an array of CD4 producing cells (Berger, 1999)

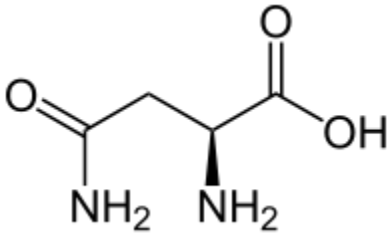
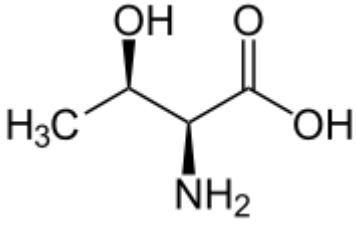
E153G Mutation

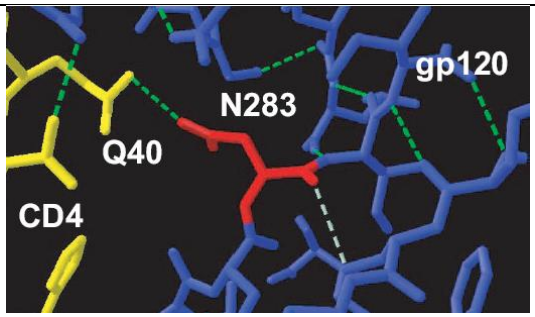
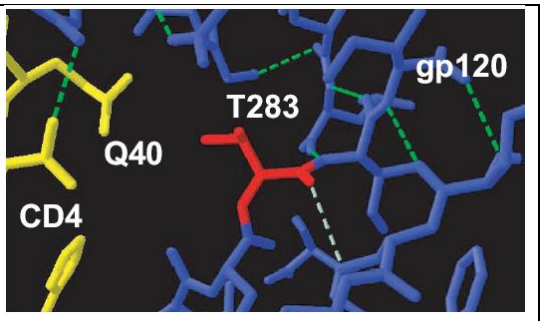
It has recently been reported by Musich et al. that a single point mutation at residue 153 in the V1 loop can modulates macrophage-tropism (Musich 2010). It was reported that there were dramatic shifts in sensitivity to soluble CD4 (sCD4) and a V3 loop mab 447-52D as well as modest shifts in b12 sensitivity (Musich, 2010). It is possible that the shift in sCD4 suggests a conformational change which allows binding to suboptimal levels of CD4 (Musich, 2010).

Table 2: E153G Amino Acid Comparison		
Amino Acid	Glutamic acid (E)	Glycine (G)
Structure		

T283N Mutation

Amino acid location 283 is one part of the conformational CD4 binding site on gp120. It has been shown that when this location is an asparagine (N) gp120's affinity for CD4 is increased (Dunfee, 2006). This mutation has been reported to occur specifically in viruses associated with brain envelopes involved in dementia (Dunfee, 2006). It has also been shown that the introduction of this mutation into an envelope with T or I resulted in increased macrophage tropism (Duenas-Decamp, 2009). It is possible that this increased affinity is the result of asparagines ability to form an additional hydrogen bond with Q40 on CD4 as seen in **Table 3** (Dunfee, 2006).

Table 3: T283NAmino Acid Comparison		
Amino Acid	Asparagine (N)	Threonine (T)
Structure		

Functional difference		

The T283N change increases the potential for a hydrogen bond from Q40 to CD4 (Dunfee et al, 2006).

PROJECT PURPOSE

The purpose of this MQP is to investigate two known mutations to the HIV-1 envelope gene that can affect its macrophage-tropism. The first was recently identified by the Clapham Lab and is an E153G mutation which is located in the V1 loop of gp120. The second mutation is in the CD4 binding site of gp120 which was shown to be present in later stages of the disease, in particular those with HIV-associated dementia. This mutation has been shown to increase affinity for cells with low levels of CD4, thus making them more macrophage-tropic (N283 Citation). This project will explore if these point mutations alone are responsible for this increase in tropism. The virus panel used includes samples from early transmitted virus. Our aim was to establish whether these determinants were involved in the non-macrophage-tropic phenotype of transmitted R5 viruses, or affected their sensitivity to entry inhibitors.

MATERIALS AND METHODS

Selection of Virus Envelopes

Virus envelopes were selected from a panel of HIV-1 subtype B envelopes provided by the NIH AIDS Research and Reference Reagent Program. The envelopes 6T and 20T were selected for their resistance to sCD4 and b12 while 11T and 15T were selected for their sensitivity. In addition two of the envelopes contained the point mutations of interest and were included as controls (5T and 9T). Other envelopes were used in some experiments as known controls, these are: LN8, LN40, B59 and VSVG.

Table 4: Envelopes

	Env Clone Name		IC50 b12 ug/ml	IC50 sCD4 ug/ml	Coreceptor	Residues	
	Short	Long				153	283
Transmitted Panel Envelopes	6T	63358-04.P3.4013	>50	538	R5	E	T
	20T	PCB931-06.7C3.4930	22.7	1000	R5	E	T
	11T	PRB926-04.A9.4237	0.5	93	R5	E	T
	15T	700010040.C9.4520	0.7	97	R5	E	T
	5T	1012-11.TC21.3257	>50	331	R5	E	N
	9T	62357_14.D3.4589	47.5	83	R5	G	T
Patient Controls Envs.	LN8						
	LN40						
	B59						
	VSVG						

DNA Preparation and Construction of Clones with Single Point Mutations

The envelope gene is contained within a pcDNA3.1D plasmid which is under a CMV promoter and while in bacteria confers an ampicillin resistance. Competent bacteria glycerol stocks are kept at -82°C and an inoculation loop was used to transfer a small amount to a 14-ml tube with 5 ml of LB-Ampicillin and incubated while shaking overnight. It is then centrifuged at

4,000 rpm for 15 minutes and the supernatant removed. At this point a miniprep kit from Qiagen was used to extract the DNA which was eluted in 60-80 µl water.

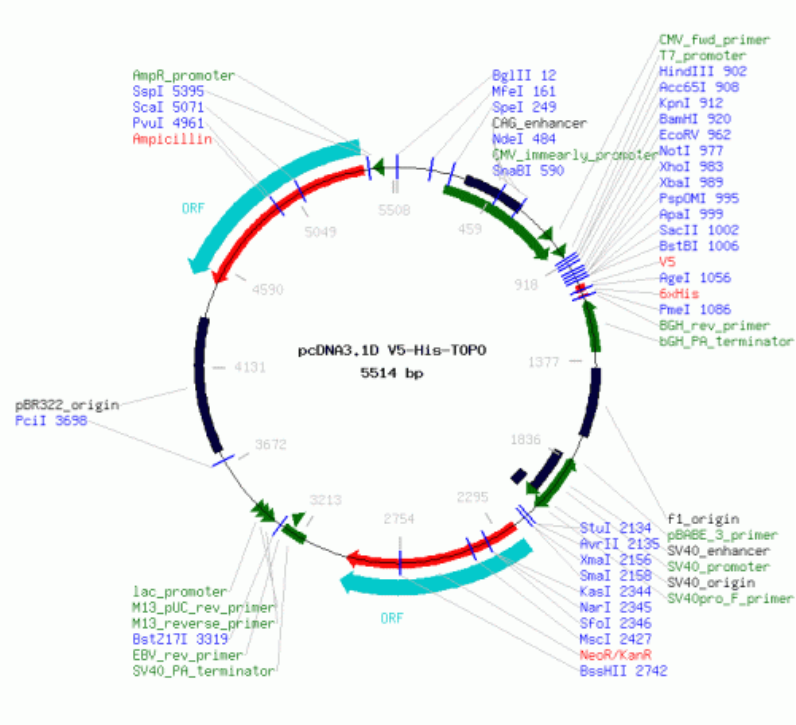


Figure 7: Plasmid. The features of the pcDNA3.1D plasmid are shown. There are two origins of replication which allows it to be used in both bacteria and mammalian cell lines.

Mutagenesis of the Transmitted Panel Envelope Vectors

Mutagenesis was performed using an in vitro site directed PCR technique (QuikChange Kit, Strategene). Prior to this however, eight sets of mutagenic primers were designed and ordered through the Center for AIDS Research. Considerations in the design of the primers included the following: primers between 25 and 45 bases in length with the mutation near the center of the primer with at least 10 base pairs of correct sequence on both sides and containing a minimum GC content of 40%. This last consideration was not always possible as this region is very TA rich and in one case a primer of 47 base pairs was used in order to increase the GC concentration. The primers used are summarized in **Tables 5 & 6**.

Table 5: E153G Mutagenic Primers	
Env	**Primers**
6T	GGAGAGAAAGGAGGAATAAAGAACTGC GCAGTTCTTTATTCCTCCTTTCTCTCC
11T	GGAGAGAGGAGGAATAAAAAACTGC GCAGTTTTTTATTCCTCCTCTCTCC
15T	CGGGGAAATGATGGAGAAGGGAGGAGTAAAAAACTGTTCTTTCAAG CTTGAAAGAACAGTTTTTTACTCCTCCCTTCTCCATCATTTCCCCG
20T	CGGTGGAGAGAGCAGGAATGAAAAACTGC GCAGTTTTTCATTCCTGCTCTCTCCACCG

Table 6: T283N Mutagenic Primers	
Env	**Primers**
6T	CGAACAATGCCAAAAACATAATAGTACAGCTG CAGCTGTACTATTATGTTTTTGGCATTGTTCG
11T	CGAACAATGCTAGAAACATAATAGTACAGC GCTGTACTATTATGTTTCTAGCATTGTTCG
15T	GTGACAATGCTAAAACAATAATAGTACAACTG CAGTTGTACTATTATGTTTTTAGCATTGTCAC
20T	CGGACAATGCTAAAAACATAATAGTACAGC GCTGTACTATTATGTTTTTAGCATTGTCCG

Once the primers were received they were diluted to 100 μM for use in PCR. The sample reaction was prepared in a PCR hood using 5 μl of reaction buffer, 125 ng of oligonucleotide primer 1 and 125 ng of oligonucleotide 2, 1 μl of dNTP mix and 1 μl of PfuTurbo DNA polymerase. The polymerase was added last to prevent the stock from being degraded. The tubes were then returned to the main lab where 50 ng of dsDNA template was added. The reaction was then placed in a thermocycler; the first step is an initialization step for 30 seconds at 95°C, which activates the polymerase. The samples are then cycled through three steps, denaturation of the dsDNA happens at 95°C for 30 seconds then the reaction temperature is dropped to 55°C which allows annealing of the primers to the DNA and then the temperature is raised to 68°C and the polymerase extends the primer and creates a new strand. The samples are cycled 20 times and then the temperature is dropped to 4°C indefinitely (at least 2 minutes) to stop the reaction.

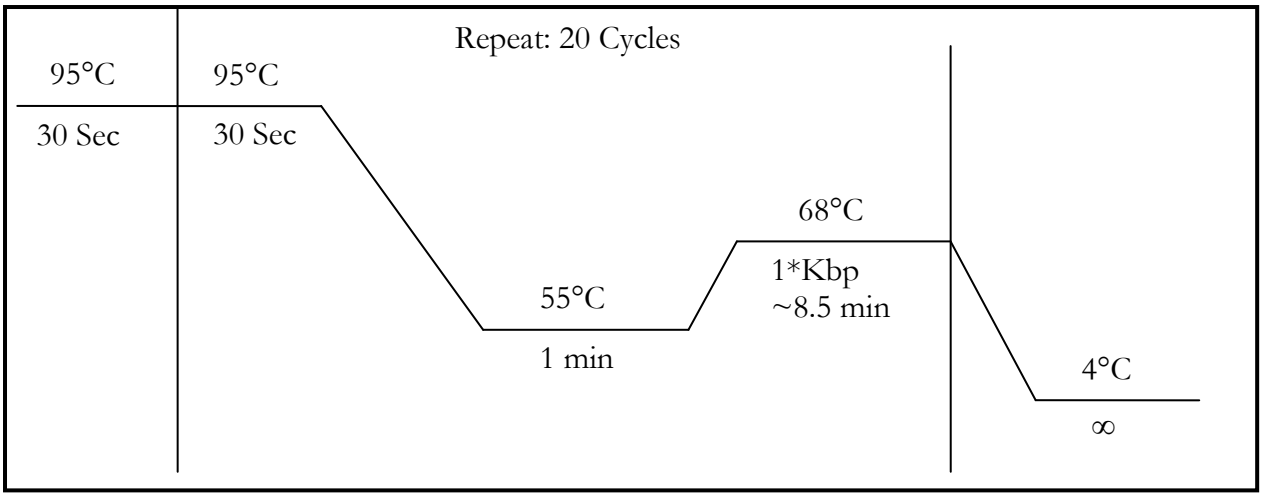


Figure 8: PCR Strategy. Step one is initialization, step two causes denaturation of the DNA, step 3 causes annealing of the primers and step 3 alls the polymerase to extend the primer, the last step stores the new DNA at an appropriate temperature. Steps 2-4 are repeated for 20 cycles.

The samples are then digested using 1.5 μl of DpnI for 1 hr at 37°C to remove methylated nonmutated parental DNA. Microcentrifuge tubes are then prechilled in ice and 50 μl of XL-1 blue supercompetent cells are aliquoted into them. To the cells is added 1.5 μl of the DpnI digested PCR reaction which is then incubated on ice for 30 minutes. This is followed by a heat shock at 42°C for 45 seconds and put back on ice for at least 2 minutes. Prewarmed SOC medium (~500 μl) is added and incubated in the 37°C shaker for at least 1 hour. This is then plated at 100-300 μl onto a carbinacillin plate and incubated overnight at 37°C. The next day colonies are picked and grown in 5 ml LB-amp overnight for miniprep the next morning. The rest of the colonies are picked using a sterilized wooden tooth pick and placed on a gridded plate which also contains colonies from the colonies picked for miniprep. The samples were then sent to GeneWiz for sequencing to confirm the mutations were present.

Production of env+ Pseudovirions

In order to create pseudovirions, both backbone DNA as well as env expressing plasma must be cotransfected into 293T cells. The backbone is derived from the NL4.3 standard and contains all of the HIV-1 genes but has a premature stop codon in the env gene. Transfections were preformed on 293T cells that were plated in 6-well plates at $1x10^5$ cells/mL with 2 mL per well. The medium used is Dulbecco's Modified Eagle Medium (DMEM) with 4% Fetal Bovine Serum (FBS) and gentamicin. The plates were incubated at 37°C and 5% CO2 overnight. The concentration of env clone DNA as well as the backbone DNA is 1.25 μg/μl. The transfection was done using two tubes each, in the first tube was both sets of DNA, 10.34 μl 2M CaCl2, and enough nuclease free water to bring the final volume to 83.40 μl. The second tube contains 83.40 μl 2X HEPES buffered saline. The first tube was added drop wise while the second tube was being vortexed. After incubating at room temperature for 30 minutes 166.8 μl from the tubes were added to each well of the 293T plates. After 24 hours the medium was changed and the supernatant was harvested at 48 hours and clarified by centrifuging for 10 minutes at 1,200 x g. This centrifuging caused all cell debris to go to the bottom of the tube, the supernatant was then aliquoted into 1 mL tubes and submerged in liquid nitrogen which would kill any cells that are left and then stored at -152° C.

Titration on TZM-bls

TZM-bls are a HeLa cell line, an immortal cell line derived from cervical cancer cells. They have a 'built in' long terminal repeat controlled β-galactosidase reporter gene which identifies infected cells (Wei, 2002). Quantification relies on pseudovirions only undergoing a single round of replication. Focus forming units/ml can be estimated by counting individual or small groups of blue spots (Duenas-Decamp, 2008). Cells were plated at 5 x 10^4 cells/mL on 48

well plates the day prior to titration. Four dilutions per virus were used, the first being a neat well with full strength virus, the second and following containing 270 μl of medium and 30 μl from the previous well. A separate tip is used between each well when moving from a higher concentration well to a lower one to reduce contamination. Once the dilutions were set up media was removed from the cells one well at a time and 100 μl of the virus dilution was added with two wells per dilution. The addition of virus dilution was done from least concentrated to most. The plates were placed in the incubator for 3 hours and 0.5 mL of growth medium was then added.

After 48 hours the cells were fixed and stained using the following protocol. Media was removed from each well by aspiration and washed in 1x PBS. This was aspirated and replaced with 0.5 mL PBS/0.5% gluteraldehyde and incubated at 4°C for 10-15 minutes which caused the cells to become fixed. This was then removed by aspiration and cells were rinsed twice with PBS/0.1% azide. In order to stain the cells 200 μl of yellow PBS containing 0.5 mg/ml (1:80 dilution) of X-Gal was applied (yellow PBS contains 3mM potassium ferrcyanide, 3mM potassium ferrocyanide and 1mM magnesium chloride). Plates were then incubated at room temperature and counted after approximately 2 hours. The substrate solution can then be replaced with PBS/0.1% azide and kept at 4°C until counted.

Titration on Macrophages

Elutriated monocytes were provided by the University of Massachusetts Medical School Center for AIDS Research. They were plated in 14 cm bacterial culture plates with DMEM with 10% human plasma for 5-7 days in order to differentiate them into macrophages. The plates were then treated with versene and a cell scraper used to remove the adhered cells and the final concentration was brought to 2.5 x 10^5 cells/mL. This concentration was then plated in a 48

well plates and left to adhere in the incubator overnight. The next day 100 µl of DEAE dextran (diluted 1:100 to 10ug/ml) with macrophage media was added to each well and incubated for 30 minutes. Virus was diluted and added the same as with TZM-bls and then "spinoculated" at 1200 rpm for 45 minutes at room temperature. After 3 hours or the next morning media was added and then incubated for about a week. Media is changed as necessary.

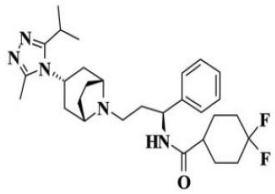
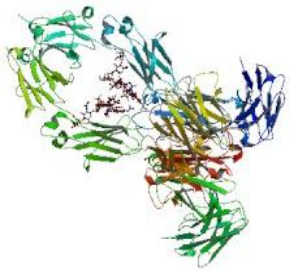
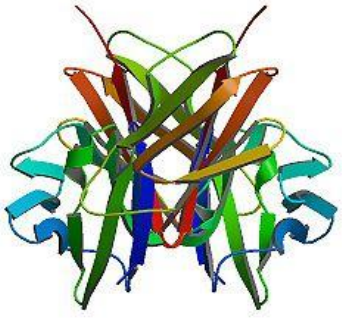
After the week the cells are fixed using a methanol-acetone protocol and stained using an indirect immunostaining technique using two antibodies. To do this media was removed from the cells by aspiration and rinsed with 1x PBS and then removed by aspiration again. The cells were then fixed by applying 0.5 mL of methanol-acetone at -20°C for 5-10 minutes and then rinsed with PBS and again with PBS/0.1% azide/1% FBS. A 1:1 mix of monoclonal antibodies 3896K and EF7 was used to stain for p24, it was diluted 1:30 and 100 µl of the dilution was added to each well and incubated at room temperature for 1 hour. The cells were then washed twice with PBS/0.1% azide/1%FBS. The secondary antibody is a goat anti-mouse beta-galactosidase conjugate, 100 µl of the antibody diluted 1:200 was added to each well and incubated for an hour at room temperature. The cells were then washed once with PBS/0.1%azide/1% FBS and once with plain PBS. The same x-gal staining procedure used for TZM-bls was then used to stain the macrophages. The plates were then incubated at room temperature for at least 3 hours but not more than overnight and counted by light microscopy.

Neutralization and Inhibition Assays

In these assays different compounds are used to inhibit infection. The three compounds used were: Maraviroc - a CCR5 inhibitor, b12 - a monoclonal antibody that binds to the virus envelope, and soluble CD4 (sCD4) - which mimics CD4 that is normally used for cell entry. Two hundred µl of TZM-bl cells at 1.6x10^5 cells/ml were plated in 96 well luminescence plates

(white wall, clear bottom) one day prior to infection. The medium used was DMEM with 4% FBS and gentamicin. The next day, a serial dilution of the treatment was created and was incubated with 200 FFU of virus (except for Maraviroc which was incubated with the cells) for 1 hour at 37°C. Medium was then aspirated off the cells and 50 µl of the virus/inhibitor mix was added to the cells (30 µl of virus in the case of Maraviroc) and left to incubate at 37°C for approximately 18 hours. After the incubation the medium was changed (for the Maraviroc the inhibitor was added back in the same dilutions instead of plain medium). The next day the medium was aspirated and replaced with 100 µl clear DMEM (without phenol red) and 100 µl of beta-glo. Beta-glo invokes a luminescent response in the cells expressing the beta-galactosidase reporter gene. The plates were then incubated in the dark at room temperature for 30 minutes and then read on the luminometer.

Table 7: Assay Treatments – Structure and Function

Treatment	Maraviroc	b12	sCD4
Type	CCR5 inhibitor	Monoclonal Antibody	Glycoprotein
Structure			 (HIV/MHC binding site shown)

(Maraviroc – Dorr, 2005; b12 – Saphire, 2001; sCD4 – Ryu, 1994)

RESULTS

The overall goal of this project was to further examine the determinants of the HIV-1 envelope that increase their macrophage-tropism and to establish their role in the non-macrophage-tropic phenotype of transmitted R5 envelopes. The first phase of the project was to create a total of eight mutants, four for each of the mutations being studied. The second phase was to test if the genotypic change to each of the mutants caused a phenotypic change in macrophage infection or affected sensitivity to entry inhibitors.

Construction and Verification of Mutant Clones

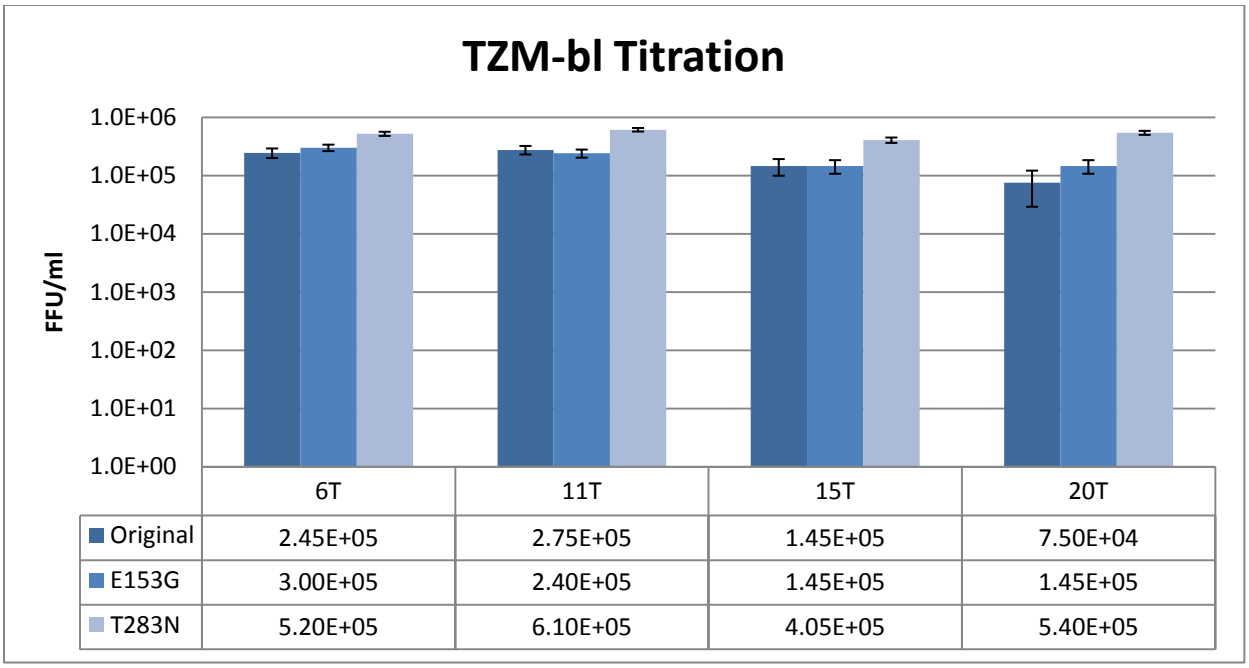
There were two different env mutations made for each of the viruses for a total of eight clones. Following the mutagenesis procedure DNA was sequenced and only sequences with the correct mutation were used. **Figure 9** shows the nucleotide and amino acids for one of the mutants (6T T283N). These sequences were analyzed using MacVector as well as Lasergene MegAlign (shown in **Figure 9**) using the ClustalV alignment method.

```
      +---------+---------+---------+---------+---------+---------+---------+
      1140      1150      1160      1170      1180      1190      1200
      +---------+---------+---------+---------+---------+---------+---------+
6T    ATTAGATCTGCAAATTTCTCGAACAATGCCAAAACCATAATAGTACAGCTGAATGAGTCTGTAAAAATTG
62-5  ATTAGATCTGCAAATTTCTCGAACAATGCCAAAAACATAATAGTACAGCTGAATGAGTCTGTAAAAATTG

      +---------+---------+---------+---------+---------+---------+---------+
     250       260       270       280       290       300       310       320
      +---------+---------+---------+---------+---------+---------+---------+
6T    TGQCKNVSTVHCTHGIKPVVSTQLLLNGSLAEEEVVIRSANFSNNAKTIIVQLNESVKIECMRPNNNTRKG
62-5  TGQCKNVSTVHCTHGIKPVVSTQLLLNGSLAEEEVVIRSANFSNNAKNIIVQLNESVKIECMRPNNNTRKG
```

Figure 9: Sequence Confirmation. The single point mutation in DNA (C to A) causes an amino acid shift from T to N in the 6T envelope gene. Each mutant was confirmed like this, only this mutant is shown.

Once the sequence was confirmed a glycerol stock of the transformed bacteria was made along with for each envelope+ plasmid. Plasmid DNAs encoding each env along with env-minus pNL4.3 (to express the other HIV proteins) were then transfected into 293T cells. Pseudovirions were created and then titrated on TZM-bls to confirm infectivity. TZM-bls are a permissive cell line as they have high levels of both CD4 and R5. All of the mutant strains produced virus that was able to infect the permissive TZM-bl cell line within the same range as the non-mutated envelope+ viruses at or above 10^5 Focus Forming Units (FFU) per ml (**Figure 10**). FFU is a simple count of how many cells appear to be infected. In this case this is then multiplied to account for the dilution factor. These verified virus stocks were then used to perform the macrophage and neutralization/inhibition studies.



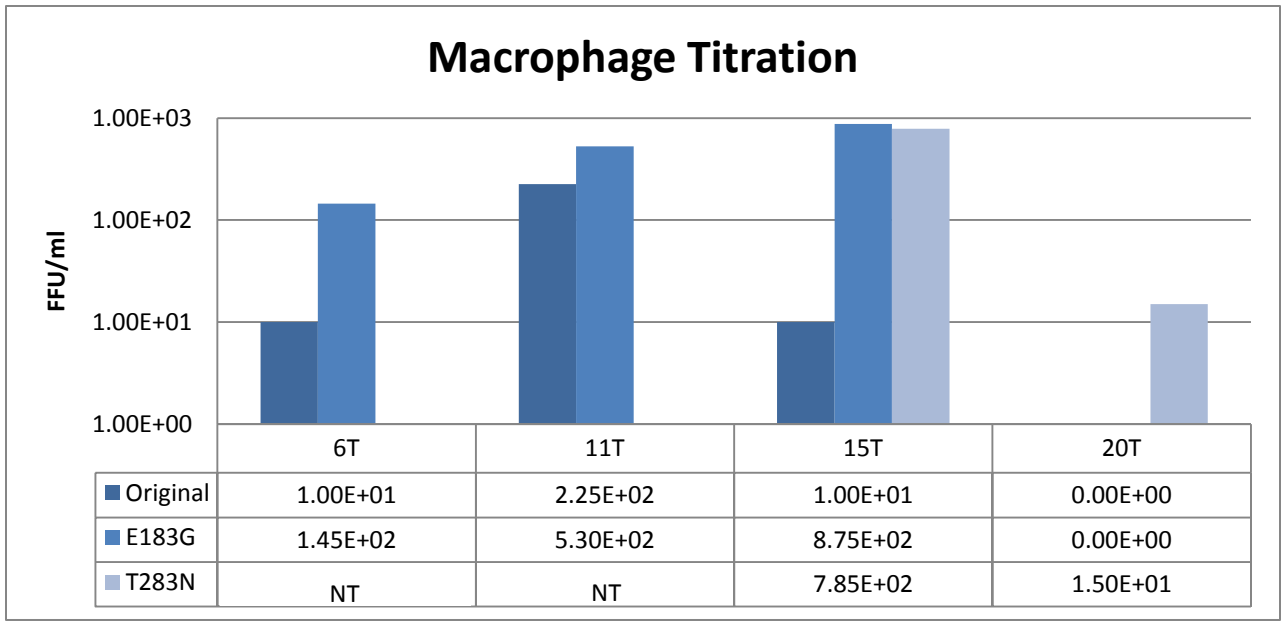
	6T	11T	15T	20T
Original	2.45E+05	2.75E+05	1.45E+05	7.50E+04
E153G	3.00E+05	2.40E+05	1.45E+05	1.45E+05
T283N	5.20E+05	6.10E+05	4.05E+05	5.40E+05

Figure ~~9~~ 10: TZM-bl titration on a logarithmic scale. This data represents the potency of the virus stocks produced in focus forming units per ml.

Macrophage Studies

Once the virus stocks were confirmed by TZM-bl titration they were then titrated on macrophages. The mutants 6T and 15T showed at least an order of magnitude increase in

infectivity. For both 6T and 15T this meant going from 10 FFU/ml to 530 and 875 FFU/ml respectively for the E153G mutation. 11T showed a more modest increase for the same mutation from 225 to 530 FFU/ml, still doubling compared to the original envelope. For 20T the original envelope showed no macrophage tropism, the T283N mutation allowed a small level of infection. However macrophages being primary cells that come from donors these results are not yet confirmed. One of the macrophage experiments resulted in only the VSVG lane staining, even though there were also other macrophage tropic virus controls present. There is also currently no reliable data on the T283N mutation for the 6T and 11T viruses because they had not yet been produced.

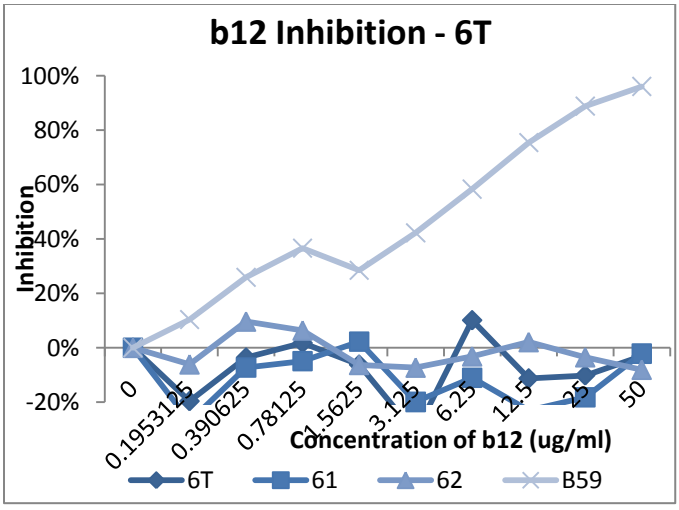


	6T	11T	15T	20T
Original	1.00E+01	2.25E+02	1.00E+01	0.00E+00
E183G	1.45E+02	5.30E+02	8.75E+02	0.00E+00
T283N	NT	NT	7.85E+02	1.50E+01

Figure 10: Macrophage titration on a logarithmic scale. The T283N mutations for 6T and 11T do not have enough reliable data to report here. Both the original 20T envelope as well as the E183G mutant were unable to infect macrophages.

Envelope 6T Inhibition Sensitivity

The 6T envelope was chosen because of its resistance to both b12 and sCD4 (IC50 values of >50 and 538 ug/ml). As expected the b12 neutralization assay shows what little effect b12 has on the original envelope as well as the mutants. The sCD4 assay however shows a marked increase in sensitivity for both the mutants particularly the E153G with 94.5% inhibition at the highest concentration (50ug/ml) compared with a 26.9% inhibition for the non-mutated envelope. All the envelopes are sensitive to Maraviroc at higher concentrations however the mutants become less sensitive at lower concentration starting at 6.25 ng/ml with a 35% difference between the original and mutant envelopes. At 3.13 ng/ml the difference is 78% for the E153G mutant and 58% for the T283N mutant. This result was not expected for Maraviroc which is an R5 inhibitor, it was expected that the mutants would have the same sensitivity as the original envelope. However since the values at the higher concentrations are the same it is possible that this finding is insignificant.



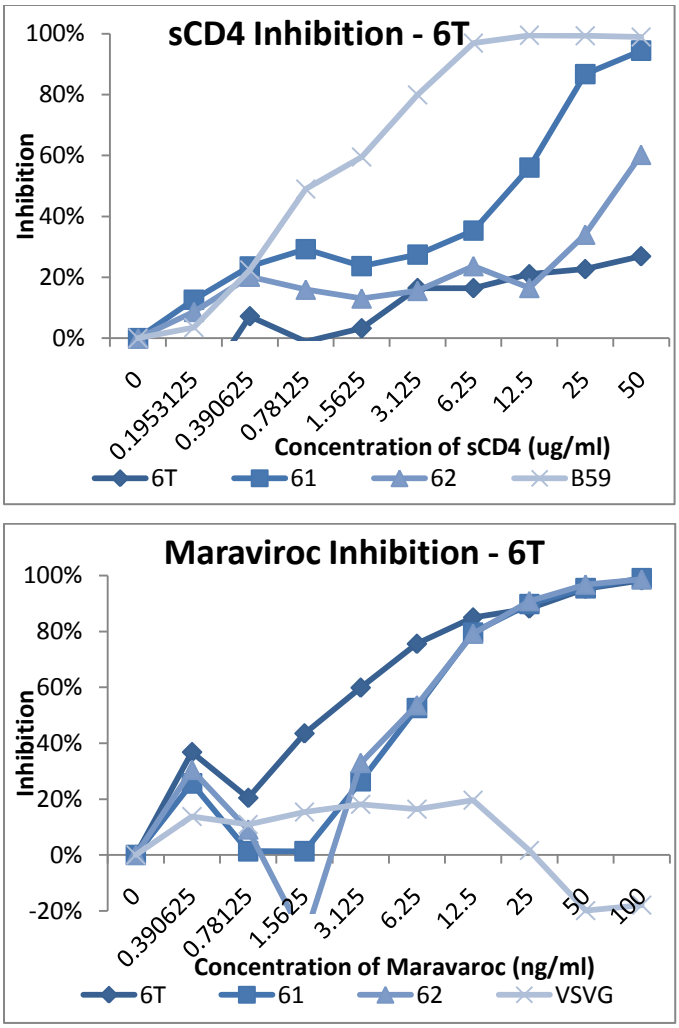
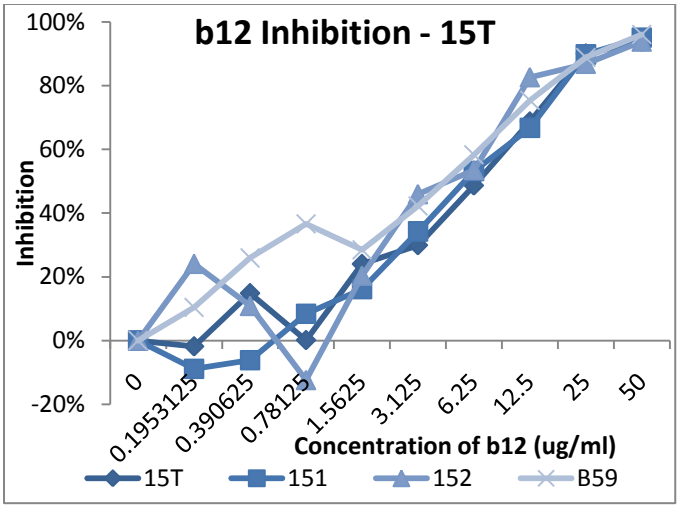


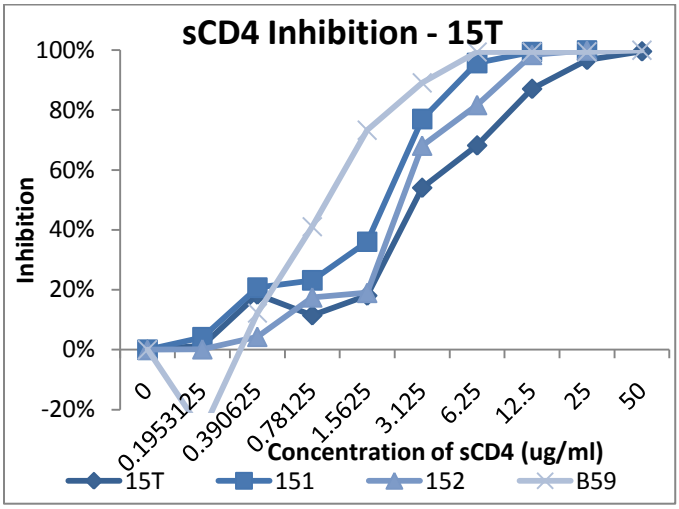
Figure 11: Neutralization assays using envelope 6T and its mutants. The E153G mutation is represented by 61 and T283N mutation is represented by 62. B59 is a positive control for b12 and sCD4, VSVG is a negative control for Maraviroc.

Envelope 15T Inhibition Sensitivity

The 15T envelope was chosen because of its sensitivity to both b12 and sCD4 (IC50 values of 0.7 and 97). The b12 assay shows that all of the mutants show roughly the same sensitivity as the original envelope. The sCD4 assay shows a modest increase in sensitivity for both of the mutations. Starting at 12.5 ng/ml this increase in sensitivity is about 12% for both mutants increasing to 34% for the E153G mutant and 18% for the T283N mutant at 6.25

ng/ml. For 0.78 ng/ml the difference is 67% and 41% respectively. This increase in sCD4 is expected however it is not as drastic as in 6T because there is no restoration of sensitivity the original 6T envelope is sCD4 resistant. All the envelopes are sensitive to Maraviroc with no real shifts as was seen in 6T.





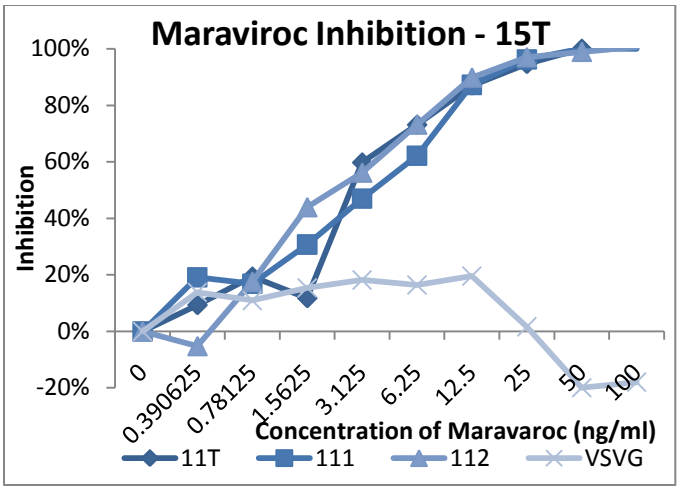


Figure 12: Neutralization assays using envelope 15T and its mutants. The E153G mutation is represented by 151 and T283N mutation is represented by 152. B59 is a positive control for b12 and sCD4, VSVG is a negative control for Maraviroc.

Envelope 20T Inhibition Sensitivity

The 20T envelope was chosen because of its resistance to both b12 and sCD4 (IC50 values of 22.7 and 1000). As expected the b12 assay shows the little effect b12 has on the original envelope as well as the mutants. The sCD4 assay also showed very little sensitivity which was also expected given such a high IC50 value. The Maraviroc assay shows that the mutants are slightly more sensitive at lower concentrations than the original envelope. This begins at 3.13 ng/ml for the E153G mutant with a 10% increase in sensitivity. At 1.56 ng/ml there is a 30% increase in sensitivity for that mutant and a 9% difference for the T283N mutant. Like 6T this only happens at lower concentrations however since the shift is opposite that of 6T it is possible that both of these findings have little significance.

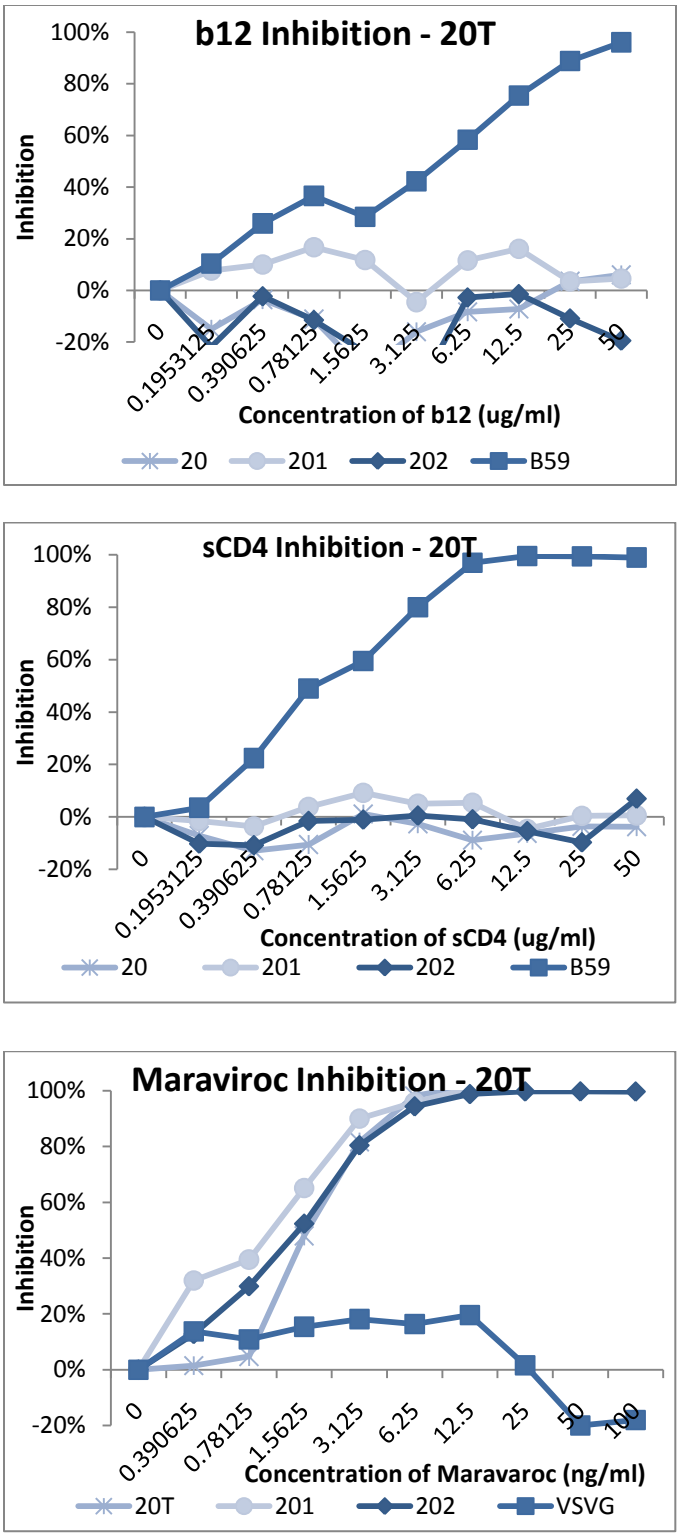


Figure 13: Neutralization assays using envelope 20T and its mutants. The E153G mutation is represented by 201 and T283N mutation is represented by 202. B59 is a positive control for b12 and sCD4, VSVG is a negative control for Maraviroc.

DISCUSSION

The key receptor for HIV-1 infection is CD4 with a chemokine receptor also needed to gain entry to the cell. In most cases of transmission the chemokine CCR5 is used however by the later stage of the disease 50% of AIDS patients have switched to the chemokine CXCR4 as the receptor. This shift allows the infection of T-cells that do not have CCR5 and which normally leads to death. Patients without any X4 virus however also develop AIDS through evolving a broader cell tropism. Viruses were identified in patients with HIV associated dementia that had evolved a broader macrophage tropism. This project explores the role of envelope determinants for the non-macrophage-tropic phenotype of transmitted R5 viruses.

Here, I have studied two substitutions. The first is a T283N amino acid substitution in the CD4bs of HIV-1 reported by Dunfee et al. to confer broader macrophage tropism. The second is a distant V1 loop change, E153G, that has affects in the V3 loop as well as the CD4 binding site and also modulates macrophage infection via low levels of CD4. The T283N change is proposed to act via a mechanism in which the affinity of gp120 towards CD4 is increased by an additional hydrogen bond to Q40 on CD4. The data presented here seems to confirm that for some viruses the T283N mutation does confer additional macrophage tropism. For one of two envelopes tested (15T) in which the non-mutated envelope was unable to infect macrophages, both E153G and T283N conferred substantial increases in macrophage infectivity. In contrast, T283N conferred only a modest increase in macrophage infectivity for 20T, while E153G had no effect. These observations indicate the presence of other unknown and dominant determinants in 20T that maintain the non-macrophage-tropic phenotype.

Previously, non-macrophage-tropic envelopes were shown to be highly resistant to sCD4 inhibition (Peters et al, 2008), while macrophage-tropic envs were generally sensitive. Of the

three non-macrophage-tropic envelopes tested here (6T, 15T and 20T), two were resistant to sCD4 and the third highly sensitive. Both E153G and T283N increased sCD4 sensitivity for 6T. However, neither 15T (sCD4 sensitive) nor 20T (sCD4 resistant) showed altered sCD4 sensitivity following E153G and T283N substitutions. The results are interesting and suggest that transmitted envelopes may have distinct properties from late disease stage envelopes previously studied by this lab (Peters et al. 2008). Neither E153G nor T283N affected sensitivity to b12, an indicator of the exposure of the CD4 binding site, while shifts in Maraviroc sensitivity were minor.

Further macrophage experiments are needed to confirm the results presented and to evaluate the effect of donor variation. There is no reliable data available for the T283N mutation for 6T or 11T because they were more difficult to make and were not yet produced when the reliable macrophage studies were done.

The data presented as well as that by Dunfee et al. and Musich et al. show that a single amino acid substitution are able to modulate macrophage tropism for some clade B R5 envelopes. The data showing that neither T283N nor E153G have major affects on the non-macrophage-tropism of 20T especially interesting. The non-macrophage-tropism of this envelope may therefore be determined by other envelope sites that are dominant over changes at 153 and 283. Further investigation is therefore warranted to identify such determinants. It is also not known if substitutions at 153 and 283 are complementary so the affects of double mutations should also be examined. For example, the data presented here is consistent with the possibility that the T283N mutation is a determinant of macrophage tropism while the E153G is able to modulate or over-ride that tropism. This is supported by the proposed mechanisms

given by Dunfee et al. and Musich et al. in which the T283N enhanced CD4 binding site may be protected by the V3 loop via changes at residue 153.

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