Abstract

Although several studies have determined key differences in envelope motifs between TF and chronic HIV-1, it is still not known what the overall glycosylation profile is that is selected for in a transmission event, as well as what contributes to this selection. Using our approach of modifying specific viruses, determining their transmission fitness in an ex vivo tissue explant assay, and determining their glycan content, we have laid the basis for determining the overall glycan structure which is selected for in TF HIV-1. Preliminarily, this study has shown that C-type lectins represent a stringent barrier to transmission and provides future studies with several modified viruses to be tested to determine a glycan profile that is most similar to TF HIV-1. Knowledge of the overall glycan motifs which are selected for in a transmission event provides the means necessary to target TF in a prophylactic therapy.

Keywords

Transmitted/Founder, HIV-1, glycosylation, C-type lectin, mucosa, Transmission, gp120, envelope, transmission, carbohydrates

Summary for Lay Audience

HIV-1 is a genetically diverse virus which produces thousands of different variants during infection in a host. When male-to-female transmission occurs, out of the thousands of variants present, a single virus is able to establish infection in 80% of cases. This virus is called the transmitted/founder virus (TF). As someone is infected with HIV-1 for an extended period of time, the viral population present in the host is distinct compared to the virus which first transmitted, the TF. For example, they differ from one another through a protein on their surface known as gp120. These proteins act as spikes which bind to cells to infect them. They contain sugars on their surface which function to shield the virus from the immune system. TF viruses have been shown to contain less sugars on the surface of their gp120 proteins compared to chronic viruses, but it has not been shown why this is the case. The female genital tract contains many defense mechanisms which function to prevent HIV-1 from infecting its host. One of these defense mechanisms are sugar-binding proteins known as C-type lectins. These proteins have been shown to bind to HIV-1 and signal other cells to destroy it. We propose that HIV-1 TF viruses with less sugar on their surface, are able to escape C-type lectins and are thus better able to infect the host. In this thesis, we looked to determine what the best sugar content for transmission was by modifying the viral sequence, growing viruses in the presence of sugar content inhibitors, and cleaving the sugars off of the viruses with enzymes. The original virus's transmission fitness was tested using a tissue model developed to mimic a transmission event. Further, an assay was optimized to measure binding strength of our viral gp120 proteins to C-type lectins. This will help determine if certain modified viruses are better able to avoid trapping by this defence mechanism. Overall, this lays the groundwork by generating the modified viruses necessary to determine the sugar content of the TF virus. Future studies can build to develop therapies targeting the TF virus.

Co-Authorship Statement

Studies presented in this thesis were completed by Adam Meadows with the assistance of past and present members in the laboratories of Dr. Eric Arts and Dr. Carole Creuzenet and collaborators as listed:

Dr. Immaculate Nankya performed the cloning of acute env sequences into the pREC_nflNL4-3env backbone.

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Chapter 1

1 Introduction

1.1 The Global HIV Burden

There were 36.7 million individuals infected with HIV reported at the end of 2015, with 2.1 million new cases being reported by the end of that year[1,2]. New HIV infections among children have declined by 50% since 2010, however there have been no declines in new HIV infections among adults[1,2]. Eastern and Southern Africa account for 46% of the global total of new HIV infections and new HIV infections declined by 14% between 2010 and 2015 in these regions[1]. In addition, new HIV infections declined by 8% between 2010 and 2015 in Western and Central Africa[1,2]. Between 2010 and 2015, the number of AIDS-related deaths in North America and Western and Central Europe decreased by 24%[1.2]. At the end of 2015, US$ 19 billion was invested in the AIDS response in low- and middle-income countries[1]. These figures demonstrate how AIDS continues to represent a significant burden across the globe, which outlines the significant need for continued research on the infectious disease.

2 Crossing the mucosal tissue: HIV Transmission

Transmission of HIV primarily occurs following unprotected sex[1]. Transmission rates are low, as demonstrated by a 0.65-1.7% chance of transmission for anal intercourse and 0.03-0.5% chance for heterosexual, vaginal-insertive intercourse[1]. HIV is a virus which requires infection of cells which express the CD4 receptor and either the CXCR4 or CCR5 co- receptor such as T cells or macrophages[3,4]. Protein spikes on the HIV exterior, known as gp120, bind to these receptors in order to facilitate fusion and entry into the

target cell[5]. Prior to this step during transmission, HIV-1 crosses a mucosal barrier which consists of the epithelial layer and secreted factors such as mucus and specific peptides[5](Fig.1.). In most instances this begins with cell-free virus or an infected cell crossing the epithelial cell layers[5]. It has been shown that viruses achieve this either by passing between junctions of epithelial cells or by being taken up by epithelia-resident Langerhans or immature dendritic cells through the epithelial cell layer[5]. Once a virus has bypassed the mucosal barrier, it must find a cell with its cognate receptors, T cells and more rarely macrophages[,3]. Once a T cell has been infected it will contain stably integrated HIV DNA and thus through cells propagation, this DNA will persist for an infected individuals' lifetime[6,7].

Due to HIV-1's high mutation rate which has been established at ~4[-3] per base per cell, it has been shown that a viral quasispecies arises during transmission, meaning, thousands of distinct viral variants are present in a donors transmission fluid. Depending on the route of transmission, only a single to a few specific variants in this quasispecies contain qualities such as reduced glycosylation which are believed to be selected in order to cross the mucosal barrier and establish infection in the new host[8]. In fact, in 80% of male-to-female heterosexual (MTF HET) transmission events, a single/variant virus establishes systemic infection[9]. These variants are known as the Transmitted/Founder (TF) viruses. Unsurprisingly, these viruses have been of much interest since their discovery and insights into these viruses have noted many distinct characteristics, one being the envelope protein.

HIV-1 contains a host-derived envelope with protruding trimer membrane spikes which function to promote fusion and entry into a target cell[3,10]. During the protein processing, a larger trimer gp160 is cleaved into two subunits known as gp41 and gp120[10]. The gp41

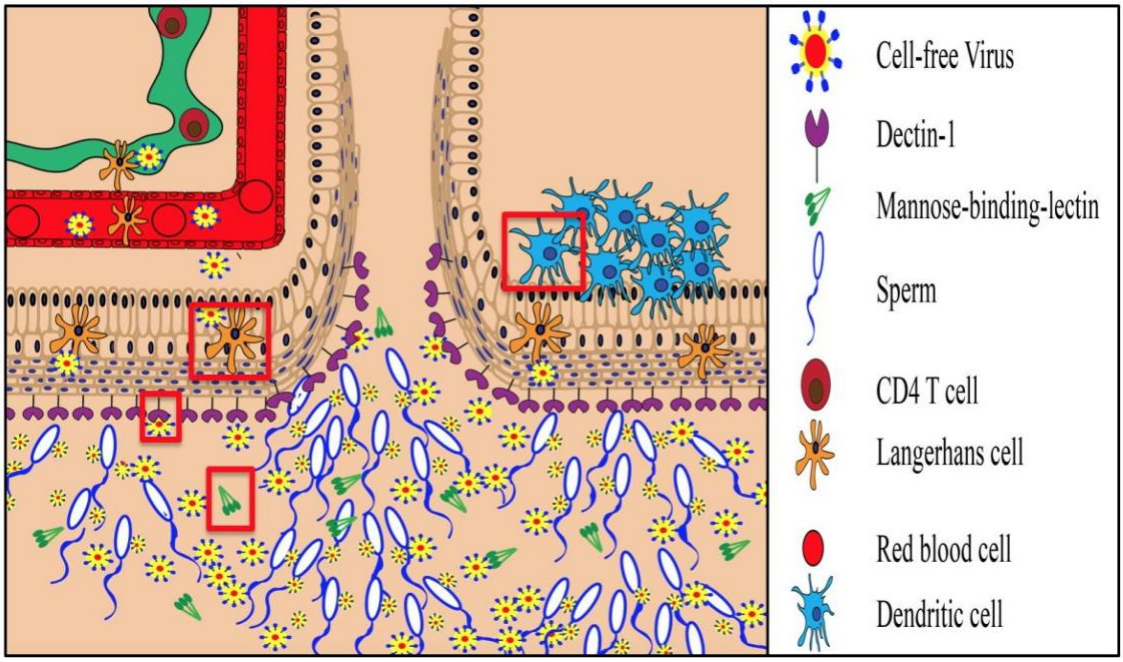


Figure 1. Schematic of male-to-female heterosexual transmission of HIV-1 and outlined lectins/cells which may contribute to transmission prevention in the female genital tract.

In heterosexual, male-to-female HIV transmission, thousands of distinct variants of HIV exist in the donor inoculum. The majority of these variants are unable to traverse the mucosa either due to inability to cross epithelia, inability to penetrate mucus in the vagina, or due to neutralization via binding of glycans on their envelopes to soluble mannose-binding-lectin or epithelia-bound Dectin-1. Those that do cross the first few layers of epithelia, may bind Langerhans or dendritic cells via lectin binding of langerin or DC-SIGN, respectively. These viruses are carried to nearby blood vessels or lymphatic vessels where they drain into lymph nodes to encounter $CD4_+$ T cells and establish infection in the recipient.

portion includes the transmembrane as well as intracellular portion of the heterodimer while gp120 forms the majority of the extracellular region of the protein[10]. Studies have shown that the gp120 subunit can vary significantly in glycosylation as well as length between variants due to high mutation rates during viral replication which may have implications on transmission[11].

As mentioned earlier, HIV requires the binding of the CD4 receptor along with either CXCR4 or CCR5 coreceptors[3]. Many cells express these receptors including dendritic cells, macrophages/monocytes, but most abundantly on T cells[12]. Diversity of the HIV virus allows it to enter by one or both of the co-receptors[12]. Viruses which use CXCR4 co-receptors are termed X4 tropic while CCR5 viruses are termed R5 tropic[12]. Studies have found that about 90% of transmitted/founder viruses are R5 tropic, suggesting an advantage in using this particular co-receptor upon transmission[13].

2.1 HIV-1 envelope and glycosylation

The envelope of HIV contains trimeric proteins known as gp120 which facilitate fusion and entry into a target cell via binding to the CD4 receptor and CXCR4 or CCR5 co-receptors[3,4]. Over 50% of the mass of these proteins consist of N-glycosylation modifications which led to many studies examining the mechanisms by which the virus utilizes these modifications to propagate its infectious cycle[10,14].

The gp120 protein contains anywhere from 24-27 N-glycosylation sites which have been shown to be required for proper folding of the trimeric structure[15-16]although more recent studies have found certain sites to be dispensable for monomeric protein formation[17]. Further, since these heavily glycosylated proteins are on the surface of the HIV envelope and are therefore the main portion of the virus accessible to exterior factors such as antibodies, it is not surprising that the glycosylation plays a major role in HIV-1 antibody

sensitivity[18-20], which has implications in both transmission and chronic infection. Further, several known neutralizing antibodies have actually been shown to contain these glycans in their binding epitopes[21-27]. More importantly, past studies have shown that different glycosylation profiles in what is termed the 'glycan shield' result in different neutralization sensitivities which demonstrate the importance of these modifications during transmission and infection of the virus[28,29].

Env glycosylation is determine by peptide sequence and steric hindrance via host cell machinery[30,31]. The polypeptide gp160 is first folded and oligomerized into trimers in the endoplasmic reticulum where exposed motif's containing NX(S/T) are targeted for glycosylation with a high mannose type glycan ($GlcNAc_2Man9$)[32,33] . These trimers are then trafficked to the Golgi where they are subsequently cleaved into gp120 and gp41 heterotrimers[32,33]. Once in the golgi, mannosidases are the first of several glycosidases which process the high mannose glycans into complex glycans[32,33]. Steric hindrance due to close proximity or high density of N-linked sites reduces access by mannosidases which could prevent the ability of these regions to vary in terms of glycan modifications[31]. In addition to site specific differences, the ratio of high mannose to complex glycans may be altered depending on the cell type infected and cell cycle stage[34]. An example outlining the importance of specific N-linked sites is shown in the V3 loop of HIV-1 gp120. This region typically only contains 1-2 N-glycosylation sites which act as an electrostatic modulator that influences the global structure and diversity of the interaction surface with CCR5 of the gp120 outer domain[35-37]

Further demonstrating the importance of steric hindrance in env glycosylation is outlined in differences between monomer and trimer associated glycan signatures. A conserved high

mannose patch is found in monomeric gp120 due to its structure sterically preventing the accessibility of mannosidases around the N332 glycan[38-40]. This patch also exists in trimeric gp120 but changes in position and size over the course of infection[41,42]. A trimer-associated mannose patch also exists which includes the N156, N160, N197, N262, N276, N301, and N637 glycans[39]. As such, monomer glycosylation differs from native virion trimer. Specifically, it was shown that highly conserved glycosylation sites were predominantly high-mannose structures in both monomer and trimer, but glycans at positions N88, N160, N276 and N411 were mainly complex type in monomeric form while high mannose type in the trimer[43]. Altogether, several factors should be taken into account when considering glycosylation of a native virion trimer and further research needs to be performed in order to determine native HIV-1 glycosylation.

2.2 Role of glycosylation on Env folding, structure, and infectivity

It was previously thought that recombinant unglycosylated Env was unable to carry out necessary functions such as binding to its cognate receptor CD4[15], however, it has now been shown following the mutation of each N-linked site in gp120 resulted in little effect on folding of the monomer[41] and trimer[44]. Further, simultaneous mutations of 14 of 15 N- glycosylation gp120 core sites were also found to be dispensable for proper Env folding[45]. Given this, its perhaps unsurprising that mutating individual glycosylation sites does not significantly affect viral surface env expression[46]. Additionally, complex glycans were found to be dispensable for folding[47].

Others features of N-glycosylation that have implications in viral infectivity are their position relative to amino acid cysteine on gp120. Reduced infectivity was seen when N-

linked site is at positions -5, -4, or -3 of cysteine 385[48]. Further, these mutations appear to have different effects on infectivity depending on subtype. Site mutagenesis studies in clades B and C demonstrated that glycosylation sites N262, N332 and N386 are necessary for infectivity in clade B whereas clade C infectivity depends on a greater number of sites[46,49]. Overall, further research needs to be done to determine specific N-linked sites which are indispensable for transmission.

2.3 Routes of transmission

Depending on the route of transmission systemic infection is caused by a single to a few TF clones. Some studies have implicated that a significant factor in differences between transmission stringencies is due to the differences in the epithelial barrier type at the site of transmission[50]. For instance, the anal epithelial barrier consists of simple columnar epithelia, leaving the underlying tissue and potential target cells only protected by a single layer. Compare this to the stratified squamous epithelial in most of the vagina and cervical tract—a barrier that is built to better withstand breaches of barrier function due to abrasion. Given these barrier differences, it has been shown that multiple distinct TF clones are more likely to successfully infect in men who have sex with men (MSM) transmission events compared to male-to-female (MTF) events perhaps due to this robust mucosal barrier difference. However, this does not tell the entire story. For instance, a potential caveat to this theory of barrier function being the major bottleneck to transmission arises in the case of blood-to-blood transmission events, as in the case of IVDU. Three notable studies observed early blood of newly infected intravenous drug users (IVDU) and found in 40-80% of these transmission events, a single TF clone was present in the blood[51-53] which suggests a combination of factors likely contribute to this phenomenon.

Nonetheless, barrier function clearly represents a major bottleneck, which has led to the study of the viruses which transmit more frequently compared to others, as understanding these barriers could lead to promising prophylactic vaccine designs.

2.4 Transmission Bottlenecks

The human immune system has implemented many defense mechanisms over time in order to prevent infection from pathogens which put selective pressure on incoming pathogens such as HIV-1 to adapt to a specific phenotype to bypass these defense mechanisms or bottlenecks. In the donor blood, it has been established that the viral population is heterogeneous in nature[54,55]. Before transmission, viruses in the blood, either cell-free or cell-associated must enter the donor genital tract before entering the donor transmission fluid[56-58]. Virus which enters the donor genital tract may either establish foci of replication and produce a clonal population at a particular site, or several distinct viruses may replicate independently, producing a heterogeneous population at the site[59]. These viruses are now under new selective pressure due to the fact that the genital tract contains susceptible cells as well as inflammation which results in bursts of distinct viral populations[60].

Another bottleneck arises in the donor transmission fluid where neutralizing antibodies and host lectins among other factors have been shown to bind the highly mannosylated gp120 envelope protein, preventing the virus from entering its fusion-competent state and thus infect its target cell[61-69]. Further, the donor transmission fluid has been

shown to promote an inflammatory environment in the recipient, potentially contributing to infection[70]. A study using an ex vivo explant model of human cervical tissue displayed how seminal plasma increased inflammation in these tissue explants and compromised barrier function[71]. Further, it has been shown that specific proteins in the donor semen can form amyloid fibrils and increase infectivity[70,72]. Studies have also demonstrated that donor semen promotes infection of epithelial cells along with promoting R5 tropic transmission[73,74]. It should also be noted that the vast majority of TF variants are R5 tropic which is likely a factor that is selected for during transmission.

In MTF HET transmission, a single variant out of a diverse pool in the donor transmission fluid establishes infection in 80% of cases[8]. Epithelia in the vagina and the ectocervix are multilayered squamous epithelia which are capable of withstanding damage due to physical abrasion. Between the ecto- and endocervix is a transitional zone where this multilayered squamous epithelia changes into single layered columnar epithelia. Although it has since been up to debate, this region is thought to be the most vulnerable site of transmission since viruses have less of a barrier to potentially pass through[75]. Protecting the epithelial layers is the cervicovaginal mucus which is home to protective microbes known as the microbiome as well as other factors such as lectins and neutralizing antibodies. The factors bind pathogens and elicit protective immune responses, as well as help maintain homeostatic inflammatory levels of the tissue, thus maintaining a healthy barrier[62-69,76-77]. At this point in the transmission event, the resulting virus which is selected for is believed to be the TF virus which could mean that this particular step represents the most important bottleneck during transmission for study. A study looking at sequence diversity in the recipient genital tract compared to the

recipient blood during acute/early infection found that there was 3-fold less diversity of the C2-V3 region of gp120 following this transmission compared to virus in the genital tract[50]. This group presented three potential models for this bottleneck. The first is that several viruses actually penetrate the female genital tract mucosal barrier, establish localized infection and the virus which has the highest fitness and produces enough virus to be carried by host DCs to the lymph node for trans-infection is the transmitted founder[50]. In other words, several viruses enter and replicate in the recipient genital tract, but only one is disseminated to cause systemic infection. Another model they presented displayed the most stringent part as passing the initial mucosal barrier. Here, they reason that due to several reasons such as glycosylation, that only a single virus actually crosses the epithelia and thus only a single virus can be taken up by migratory DCs to host T cells. The last model they proposed is like model two, in that a single virus enters the recipient genital tract and due to the presence of immune pressure from APCs and other innate immune aspects, cause the virus to replicate and evolve more quickly than it would in the blood, producing several distinct viruses. Going from the recipient genital tract to the blood may represent an additional barrier in this case, perhaps at the level of DCs or tissue resident Langerhans cells or due to homing receptors such as $\alpha 4\beta 7$, although these theories are up to debate[78-80]. The authors postulate that transmission likely involves a combination of all three models, however little is known as to what is actually occurring at these stages.

2.5 C-Type lectins as a barrier to HIV transmission

C-type lectins are proteins which are common in their carbohydrate-rich-domain (CRD) which have evolved to carry out a wide variety of functions dependent on calcium for

binding. These proteins exist in several dimeric forms and have evolved to bind to sugars as well as other proteins in a wide variety of functions[81]. Existing as transmembrane proteins or in secreted forms, some of the functions of these proteins include immune signaling as well as adhesion molecules as in the case of selectins[81].

In the mucosal tissue such as the female genital tract, some of the main C-type lectins present are Dectin-1, DC-SIGN, and Mannose-binding-lectin (MBL). Dectin-1 exists in transmembrane form on the surface of phagocytes and functions as a signaling molecule as a pattern recognition receptor[82,83]. Primarily, these proteins exist in anti-fungal immunity, binding to beta-glucans to trigger immune responses as well as the NF-kappaB pathway[83,84]. They have also been implicated in other anti-bacterial and anti-bacterial immune responses, along with signaling for T cells[85-87].

DC-SIGN is a surface-bound C-type lectin which contains its carbohydrate binding domain on its C-terminus[88]. They are expressed exclusively on dendritic cells and are involved in binding the receptor ICAM-3 to initiate the process of MHC-peptide presentation[88]. Furthermore, they have been heavily implicated in enhancing infection of T cells by HIV[89-91]. DC-SIGN has been shown to bind to gp120 on the surface of HIV-1[92]. Further, it was first noted in vitro that binding of DC's to HIV-1 increased its infectious lifespan for up to four days, which was later shown to be due to the ability of DCs to internalize the virus through this DC-SIGN interaction[93,94]. The process is known as 'trans' infection since the gp120-DC-SIGN interaction itself does not increase the affinity of the CD4-CCR5 interaction, but instead, the virus can hijack the DCs migratory abilities to be brought to target cells as well as the interaction brings the virus in close proximity to T cells when MHC presentation is occurring by DCs[95].

On the contrary, lectins such as Mannose-binding-lectin have been implicated in preventing HIV infection among other pathogens. Mannose-binding lectin is a secreted C-type lectin that functions as a pattern recognition receptor and binds to several different bacteria, viruses, and protozoa[96,97]. In addition to activating the complement system of innate immunity, MBL also functions to opsonize pathogens it binds to, promoting their immune clearance[98]. One of the first studies that brought a connection of C-type lectins to HIV was published in the Lancet by Garred and colleagues[99]. Patients with variant alleles coding for reduced levels of mannose-binding-lectin; a lectin present in the mucosa of healthy individuals, actually increased these individuals' susceptibility to HIV transmission. Several other studies have found similar results which suggest lectin binding may influence transmission events[100-103]. A study in macaques also found that transmission rates of SIV or SHIV were reduced when administered cyanovirin-N (CV-N), suggesting these lectins likely contribute to the bottleneck in the genital tract[104]. Although less clear, it has been shown in vitro that MBL can neutralize 10% of primary isolates through its interaction with gp120[98]. The DC-SIGN-gp120 interaction has also been shown to be competitively inhibited by the interaction of HIV-1 with MBL[98]. Overall, MBL appears to function in several ways to prevent transmission of HIV and may be important in the transmission bottleneck of HIV-1 as it may block essential interactions required for cellular entry.

3 Transmitted/Founder HIV

Several interesting properties have been found to be common among these clones. The most prevalent subtype C HIV TF viruses have been shown to contain shorter V1/V2

loop region in their gp120 envelope protein, which has implications in reducing neutralization sensitivity as well as binding to other host defense mechanisms such as lectins[105]. Further, less prevalent Subtypes A and D TF's have been found to have reduced potential N-linked sites (PNGS) in acute sampled clones compared to their chronic counterparts[106]. The same phenomena has not been seen as definitively in Subtype B TF's and still continues to be studied[107,108]. The role of glycosylation in terms of transmission has been understudied as most research to date has looked at glycosylation in the context of immunogenicity and in evading host immune responses during chronic infection[21]. In the context of transmission, little is known on glycan binding in the recipient genital tract or tissue other than binding to lectin receptor DC-SIGN on DCs[88,89]. In this case, reduced glycosylation on the TF could be thought of as counter-intuitive since this interaction has been implicated as being highly beneficial in promoting infection, is dependent on sugar-lectin binding[88,89]. Further, studies have shown that specific 'lost' N-linked sites such as position 12 have been shown to increase env expression, perhaps detailing how loss of glycans could play a larger role than initially thought[109].

3.1 Characteristics of TF viruses

Understanding the factors that enable a TF virus to bypass the mucosal barriers and evade the host innate immune system is key to developing preventative vaccines against HIV-1 transmission. As was mentioned previously, TF Env contain shorter surface-exposed variable loops, fewer N-linked glycosylation sites and more sialylation compared to chronic viruses[110-112]. This is in contrast to chronic HIV whereby mutations occur in Env

to escape broadly neutralizing antibodies targeting trimeric Env which increases the length of variable loops, adds N-glycosylation sites, and subsequently alters the high mannose patch of gp120 subunits[38,39,42].

Like other pathogens, HIV-1 is under selective pressure from adaptive immunity as the emergence of antibodies targeting Env causes genetic changes to alter Env composition. However, virions derived from the original TF genotype remain in the diverse circulating HIV-1 clones in the blood of chronically infected patients despite the pressure of the adaptive immune system. This suggests the virus is forced into such diversity as changes in its genotype to overcome one type of immune pressure, cause it to become susceptible to another. Thus, in order to achieve a robust preventative measure against HIV-1, it may be important to consider preventative theories that target both innate and adaptive components.

3.2 Glycosylation of Env in Chronic and TF viruses

To date, glycosylation differences between chronic and TF HIV-1 has primarily focused on the presence or absence of potential N-glycosylation sites. Under the current methods of study, researchers have had little success in determining a glycosylation profile that distinguishes between the two. Five pairs of glycosylation sites have been shown to have conserved glycosylation status across all Subtype M HIV-1 viruses and circulating recombinant forms (according to reference HXBc2 gp120 amino acid numbering: 88 and 241, 88 and 262, 197 and 262, 241 and 262, and 262 and 276[113]. This is significant as these sites could represent important regions selected for during a transmission event.

However, due to the inability to perform these studies on native TF gp120, it is not known whether this can be translated *in vivo*, and whether these sites are important for transmission.

3.3 Studying envelope glycosylation

Beyond the presence or absence of glycosylation sites, site specific glycosylation data are extremely lacking in the field. This largely lies in the fact that on average there are 25 N-glycosylation sites in gp120, 4 in gp41, and glycosylation is highly heterogenous[38,114] Furthermore, the gold standard for glycopeptide analysis is mass spectrometry, however, glycopeptides ionize and fragment less efficiently than unglycosylated peptides. To overcome this, hydrophilic-based enrichment, complimentary enzymatic deglycosylation and glycan profiling with ESI and MALDI-TOF mass spectrometry have been used[34,10,114-116]. Even so, mass amounts (~75 ug) of Env are required in order to comprehensively study the glycan profile of Env using the current methods which makes it incredibly time consuming to perform these studies[34,110,114,115]. For this reason, monomeric forms of Env have been used but as was mentioned earlier, native trimeric Env glycosylation differs drastically from recombinant monomeric Env glycosylation in terms of high mannose versus complex and hybrid glycan content. Thus, these recombinant forms do not represent a good model for studying glycosylation of native virion derived Env that exist as gp120-gp41 homotrimers.

A further improvement in terms of achieving a more representative gp120 to perform glycopeptide studies on was the development of ‘native-like’ trimers known as BG505 SOSIP.664 trimers (cleaved SOSIP.664 gp140 trimers based on the subtype B transmitted/founder strain, BG505)[117]. Briefly, these trimers were made in vitro from the subtype A TF virus using transient transfection in HEK294T cells[117]. Two groups

characterized these trimers and found nearly 90% similarity in terms of glycosylation[39,43]. These two studies used deglycosylation with Endo H (cleaves hybrid and high mannose glycans) and PNGase F (cleaves N-glycans except those with α1-3-linked core fucose residues) to determine site- specific glycosylation as opposed to glycan structures. Cao *et al.* (43) detected more glycosylation sites and glycoforms than Behrens *et al*. (44) which led to discovering how more sites contain complex glycans than previously described.

This study aimed to elucidate the glycosylation profile which results in the phenotype that is selected for in 80% of male to female heterosexual transmission events. Currently, there are no feasible methods to analyze the glycosylation of the TF HIV-1 virus following a transmission event. Current methods of analyzing gp120 glycosylation involve the production of mass amounts of stable recombinant proteins and analysis through mass spectrometry. These studies bypass the issue with protein production required for mass spectrometry analysis, however, it is still controversial how similar these recombinant proteins glycosylation pattern is to native HIV-1 TF gp120. This study took an alternate approach to studying the TF glycosylation profile by making several sequence and enzyme dependent changes to whole acute and chronic subtype B viruses, determining their transmission fitness using an *ex vivo* model, and in the future, analyzing the virus which transmits most readily in a model created by Drs Eric Arts and Robin Shattock using mass spectrometry. The rationale behind this project is that it may not be possible to analyze *in vivo* TF HIV-1 glycosylation due to lack of total protein available to analyze, but if a glycosylation pattern can be determined that allows efficient transmission in a human tissue model which serves to mimic the *in vivo* transmission event, it is likely this glycosylation pattern is similar to those selected for during an *in vivo* transmission event.

Chapter 2

4 Materials and methods

4.1 Acute and chronic Env chimeric viruses

For all virus studies, acute subtype B envelopes were obtained from the Center for HIV-1/AIDS Vaccine Immunology (CHAVI) acute infection studies[9] along with a chronic envelope from a patient sample in a Belgian cohort acquired at Case Western Reserve University[118,119]. These envelopes were used to create HIV-1 *env* chimeric viruses with a common backbone. Using the yeast-based recombination cloning method previously described, envelope genes from acute and chronic clones were inserted into pREC_nfl_NL4-3_Δenv/URA3. Following insertion of the env into the pREC backbone, infectious virus was produced by transfection of the construct with a supplementary vector pCMV_cplt in 293T cells using the Effectene lipid system (Qiagen, USA) according to the manufacturers protocol. The viruses were then propagated through U87.CD4.CCR5 cells, and the progeny virion infectious titre was quantified using the 50% tissue culture infective dose ($TCID_{50}$) assay as described previously.

4.2 Cell culture

U87.CD4.CCR5 cells were obtained from the NIH AIDS reagent database. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Sigma) supplemented with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Sigma), 300ug/mL G418

(geneticin)(Thermofisher Scientific), and 1ug/mL puromycin (Thermofisher Scientific) for a maximum of 10 passages with trypsin (Sigma).

A3R5.7 cells were obtained from the NIH AIDS reagent database. Cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Sigma) supplemented with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Sigma), and 1mg/mL G418 (geneticin) (Thermofisher Scientific) for a maximum of 10 passages prior to infection.

PM1 cells were obtained from the NIH AIDS reagent database. Cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Sigma) supplemented with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Sigma) for a maximum of 10 passages prior to infection.

TZM-bl cells were obtained from the NIH AIDS reagent database. Cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Sigma) for a maximum of 10 passages prior to infection.

4.3 Viral propagation

U87.CD4.CCR5 cells were cultured in T-175 culture flasks (Corning) until 50-60% confluency. Viruses were propagated by infecting each flask with 1x10^5 -1.5x10^5 infectious units (IU) as determined by reverse transcriptase activity along with 30 mL of culture media. Infection was determined by visual syncytia formation. Viral supernatant was harvested every 3 days until day 14 of infection and stored at -80°C for future use.

4.4 Reverse Transcriptase (RT) Assay

Reverse transcriptase activity of viruses was used to quantify infectivity for future tissue and cellular infections. Briefly, 10uL of viral supernatant was added to 20uL of RT assay buffer consisting of 50mM Tris-HCl, 5mM DTT, 5mM MgCl2, 150mM KCl, 0.05% NP-40 and oligo-dT poly-rA and incubated at room temperature for 20-30 mins. Radioactive dTTP at a final concentration of 4uCi/mL was added to the same RT buffer described earlier. 5uL of this mixture was then added to each viral sample per well and subsequently incubated at 37°C for 2 hrs. After this time, 10uL of the mixture was spotted onto DEAE filter mats and allowed to dry for 10 mins at room temperature. The filter mats were then washed 5X for five mins on a shaking incubator in 1x saline-sodium citrate buffer, followed by two, five min washes with 85% ethanol. The filter mat was then dried for 15 mins on a 65°C heat block. Once dry, the filter mat was exposed overnight on a Phosphor screen and imaged using the Bioquant 800 Phosphorimager. The samples were then analyzed for positive reverse transcriptase activity using ImageJ software.

4.5 Median Tissue Culture Infectious Dose ($TCID_{50}$)

Infectivity assays for all viruses were performed using human U87.CD4.CCR5 cells. Cells were plated in a 96 well plate at a density of 10,000 cells per well and allowed to adhere overnight. To measure viral infectivity, virus supernatant was diluted 1:4 in a 96 well plate 10 times, and 100uL of virus dilution was added to the plated U87 cells in triplicate. 6-8 hours post-infection, viral supernatant was removed and 150uL of fresh

DMEM was added. RT assays were performed on day 7 post-infection supernatants and each well was considered positive or negative based on a negative column containing only media. The Reed and Muench method was used to calculate the Real TCID50/ml infectious units for future infection use.

4.6 Tissue explant assay

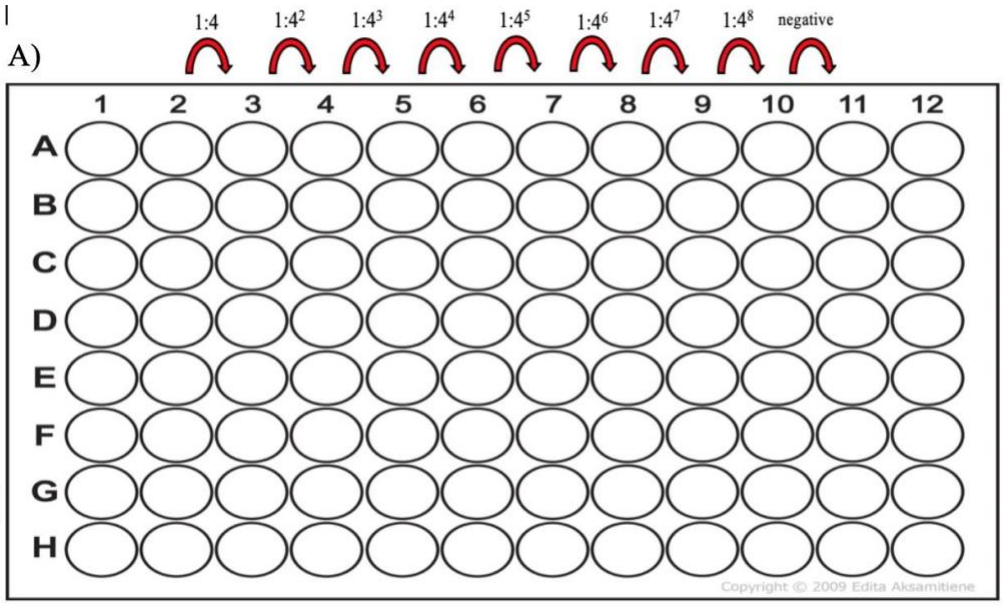
Frozen endocervical explants were acquired through the National Disease Research Interchange (NDRI) tissue bank and stored in liquid nitrogen until future use. Tissue explants were cut into approximately 3mmx3mmx3mm pieces while completely submerged in complete growth medium (RPMI). 3-5 pieces were placed in each well of a 48 well plate with 300uL of growth medium. 750 IU (as determined by reverse transcriptase activity) of the acute B4 and chronic Q0 viruses were mixed in culture media and added to each well for 6-8 hrs at 37°C, 5% CO2. Following this time frame, tissue pieces were washed 3 times with warm PBS to remove any virus which has not entered and bound to tissue. Tissue pieces were then added to a new tissue culture plate with fresh media. Migratory cells were then collected by aspirating with warm PBS in each well. 500ul of each migratory cell sample were added to Eppendorf tubes and washed 3 times by centrifugation at 1500g for 5 mins. Following this process, cells were added to a new plate with fresh media. Both the tissue and the migratory cells were cultured for 10 days, replacing 100ul of media every 2-3 days.

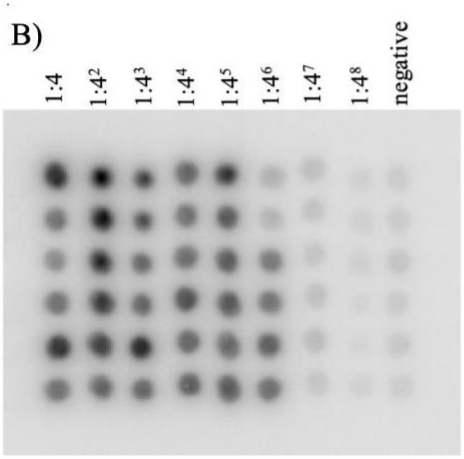
4.7 DNA extraction

Following the ex vivo tissue explant assay, tissue samples were digested in 600 mAU/ml of proteinase K at 56°C for 12-16hrs. Migratory cells were digested in the same final concentration of proteinase K for 20 minutes. DNA was extracted according to the Qiagen DNeasy blood and Tissue kit Protocol.

4.8 Platinum Taq PCR Protocol

All PCR reactions were performed using Platinum Taq DNA polymerase kit (Thermofisher). A single reaction mix contains: 5uL of 10x Platinum Taq PCR Buffer, 1.5uL of 50mM MgCl2, 1uL of 10mM dNTPs, 1.0uL of each forward and reverse primer at a concentration of 10uM, 0.4uL of Taq Polymerase, 20uL of template and milliQ H2O to bring the final reaction mixture to 50uL. The following thermocycling conditions were used : initial denaturation for 2 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C, extension at 72°C for 1min/kb of the final product length, followed by a final 10 minute extension step at 72°C and hold at 4°C.





C)

Virus		IU/mL	IU/uL
Acute viruses			
B4		201033.776	201.033776
B7		1766.4	1.7664
B8		158931	158.931
B9		2181972	2181.972
Chronic viruses			
K44		388016	388.016
Q0		357494.225	357.494225
I10		69000	69

Figure 2. Dilutions and plate set up for a reverse transcriptase assay ($TCID_{50}$).
(A) Serial dilution set up for dilution of Q0 and B4 HIV-1. (B) Sample RT assay spot results demonstrating positive and negative wells via p32 radioactivity. (C) Table detailing infectious units per mL calculated via the Reed and Muench method.

Primer name	Primer Sequence	Description
ED14	TCTTGCCTGGAGCTGTTTGATGCCCCAGAC	Forward primer for external 1st round PCR amplification of the C2-V3-C3 region of env
EnvB	AGAAAGAGCAGAAGACAGTGGCAATGA	Reverse primer for external 1st round PCR amplification of the C2-V3-C3 region of env
E80	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCA ATTCCCATACATTATTGTG	Forward primer for external 2nd round PCR amplification of the C2-V3-C3 region of env
E125	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCA ATTTCTGGGTCCCCTCCTGAGG	Reverse primer for external 2nd round PCR amplification of the C2-V3-C3 region of env
UNIV-GA4	TTGCCAAAGAGTGACCTGAGGGAA	Forward primer for external PCR amplification of the gag region prior to qPCR
gag-cons1	GAGAGAGATGGGTGCGAGAGCG	Reverse primer for external PCR amplification of the gag region prior to qPCR
gSCAF	CATGTTTTCAGCATTATCAGAAGGA	qPCR forward primer for gag proviral DNA
gSCAR	TGCTTGATGTCCCCCCACT	qPCR reverse primer for gag proviral DNA
gSCA probe	6-Fam-TACTGGGACAGCTACAACCATCCCTT-BHQ	qPCR probe for gag proviral DNA
B4xQ0Fwd	CAAAGCCTAAAGCCATGTGTAAAATTAACCCCA CTGTGTGTTACCTTAAATTGCACTAATGTGAAT	B4 forward primer for B4 V1/V2 region of env with homologous overhangs for the Q0 envelope
B4xQ0Rev	TCAGTCATTACACAGGCCTGTCCAAAGGTATCCT TTGAGCCAATTCCCATAGGAGTTACAATTTATCA ACGTATAGCTTGTATT	B4 reverse primer for B4 V1/V2 region of env with homologous overhangs for the Q0 envelope
URA3Q0Fwd	TGGGATCAAAGCCTAAAGCCATGTGTAAAATTAA CCCCACTGTGTGTTACCCCGCGGAGATTGTACTGA GAG	URA3 forward primer for entire URA3 selection factor with homologous overhangs for the Q0 envelope
URA3Q0Rev	TGGGATCAAAGCCTAAAGCCATGTGTAAAATTAAC CCCACTGTGTGTTACCCCGCGGAGATTGTACTGAGA G	URA3 reverse primer for entire URA3 selection factor with homologous overhangs for the Q0 envelope

Illumina Tag

Table 1. Primers used in this study

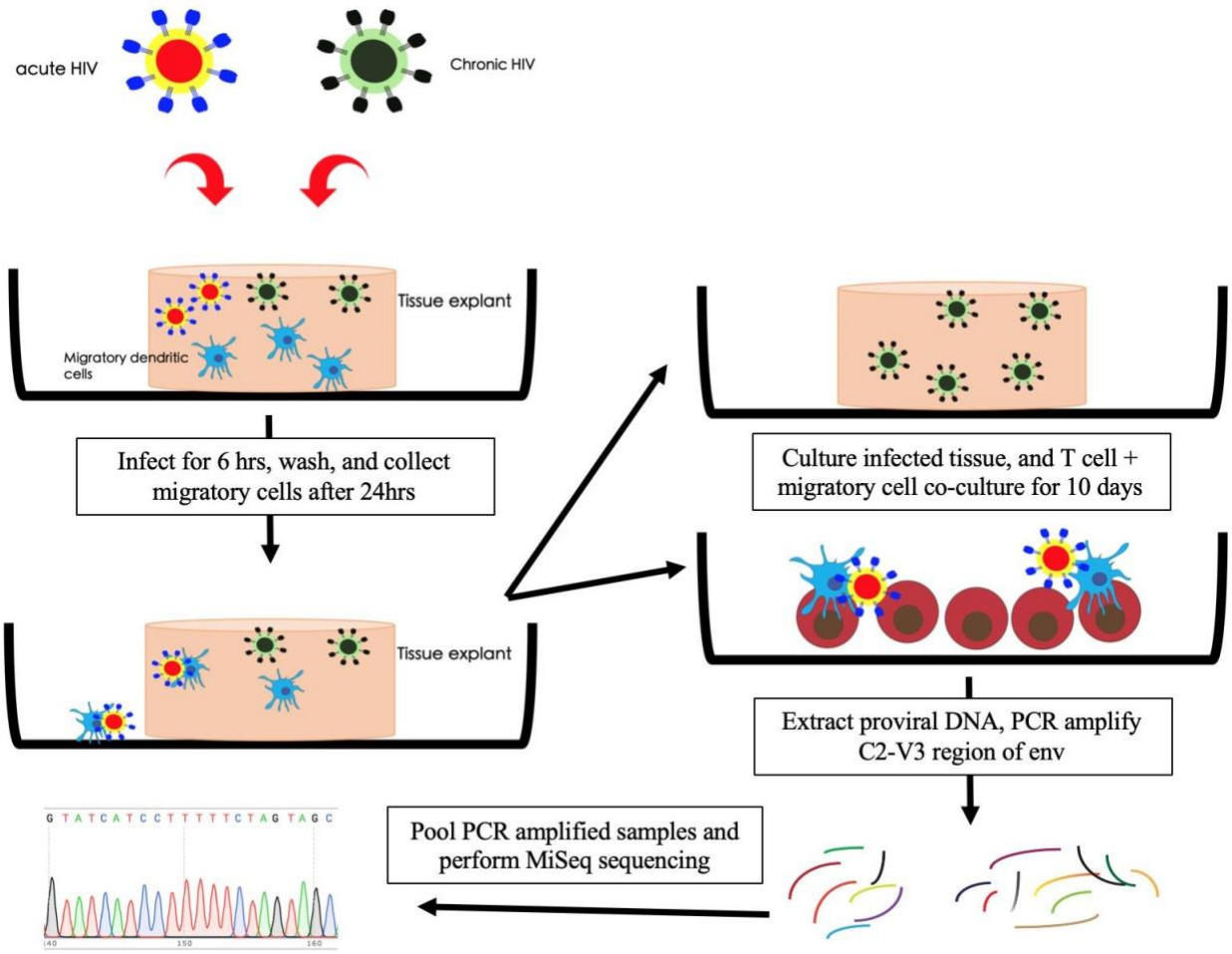


Figure 3. Schematic of the ex vivo Tissue explant assay as a measure of transmission fitness.

Example workflow of the ex vivo tissue explant assay whereby acute and chronic viruses were competed against one another and transmission fitness was measured by next generation sequencing of proviral DNA.

4.9 NGS analysis of multi-virus infections

Two rounds of PCR amplification were performed on extracted proviral DNA. The C2-V3-C3 region was amplified using 1st round external primers EnvB and ED14, and 2nd round nested primers E80 and E125 under the conditions described above. The PCR products were confirmed on a 1% agarose gel to confirm the ~500bp band size. Any samples with smearing or double bands were either gel extracted, and ethanol precipitated, or the PCR amplification was redone under varied conditions to yield pure bands. The amplified PCR products were purified using the Agencourt AMPure XP bead system with a bead:DNA ratio of 0.7:1 according to the Roche manual. The sample library was quantified using the Quant-iT PicoGreen ds DNA assay kit (Invitrogen) and diluted to 4uM. The purified and normalized samples were then tagged with unique Illumina tag sequences according to the Nextera XT DNA Library Preparation Kit (Illumina) protocol. Briefly, the process involves an 8-cycle PCR in which a unique pair of Illumina adaptor sequences are attached to the tag sequences on the ends of each PCR amplified C2-V3-C3 sample. This process ensures each sample can be properly mapped/differentiated from one another during Illumina MiSeq Metagenomic Sequencing (Illumina). Sequencing by Illumina's MiSeq System was then performed at London Regional Genomics Centre at the Robarts Research Institute at Western University. The samples were sequenced using the MiSeq 600 cycle Reagent Kit v3. Sequence data was subsequently analyzed by Dr. Jeff Bailey's group at Brown University using their SeekDeep pipeline.

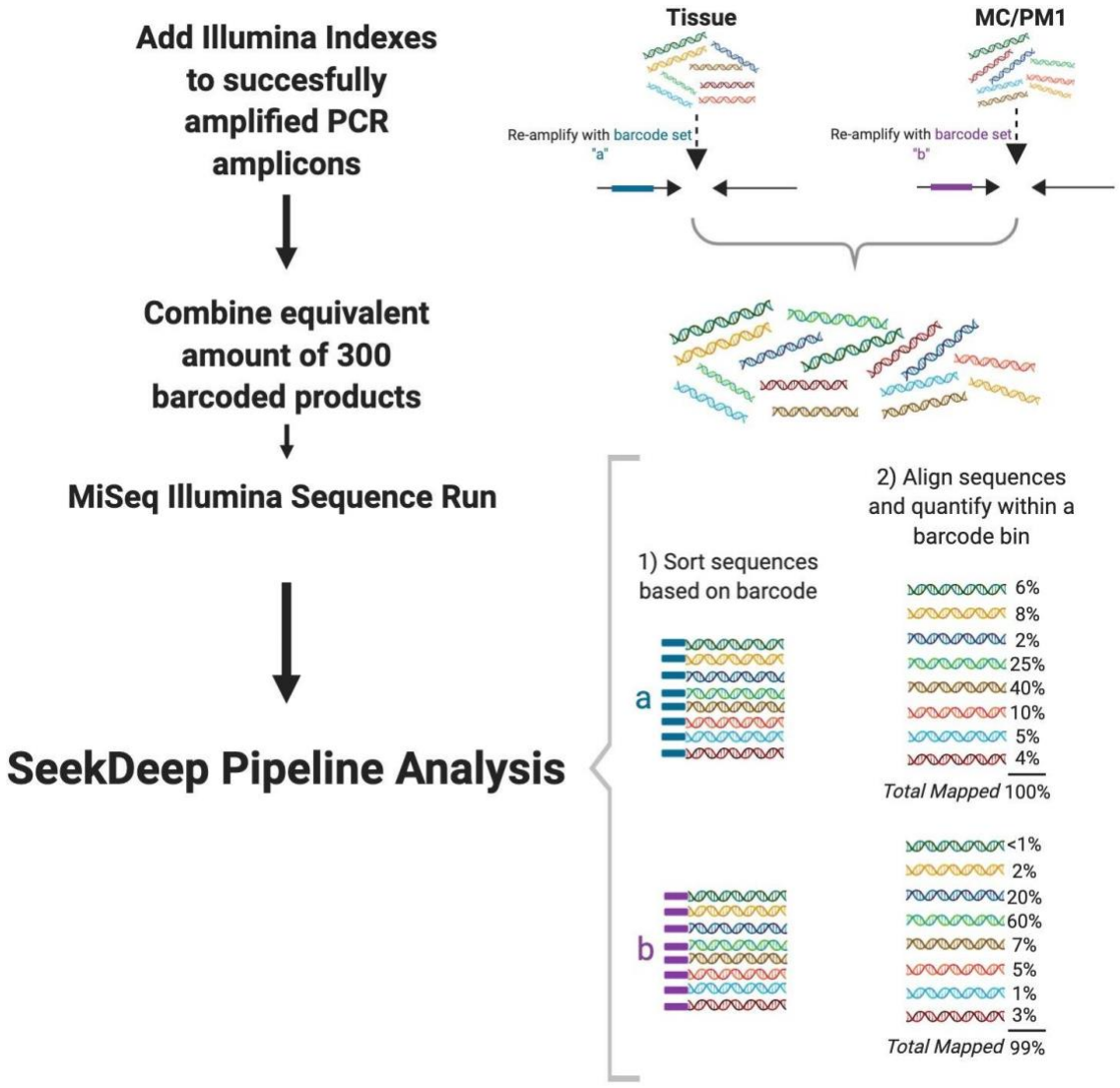


Figure 4. Experimental workflow for sample preparation and barcoding for Illumina Miseq.

PCR amplified C2-V3 fragments are indexed using specific barcodes to be able to distinguish samples from one another. Barcoded samples are then pooled and sequenced using Illumina Miseq platform and sequences are analyzed using SeekDeep Pipeline analysis.

4.10 Quantitative polymerase chain reaction (qPCR) of gag

Following DNA extraction, a pre-PCR of the gag region under the following conditions was performed. 95°C for 2 minutes, 35 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, and 72°C for 10 minutes. Primers: Forward: 5'-TTGCCAAAGAGTGACCTGAGGGAA-3', Reverse: 5'-GAGAGAGATGGGTGCGAGAGCG-3'. Amplified PCR product of each sample was then quantified using qPCR under the following reaction conditions: 60°C for 2 minutes, 95°C for 20 seconds, and 40 cycles of 95°C for 1 second, and 60°C for 43 seconds. Samples were quantified using a standard containing Xba1 digested Sbfi-1 pREC NL43 plasmid derived from the Arts lab which was serially diluted from 1 million copies to 5 copies of DNA.

4.11 Yeast recombination of chimeric envelope viruses

In order to determine the effect of specific regions of envelope in each of our viruses, we used homologous yeast recombination to introduce double strand breaks and replace specific regions of interest in our envelope chimeric acute and chronic viruses. *Sacchromyces cerevisiae* is able to repair double stranded DNA breaks and insert regions into the break with homology to that particular region in a plasmid genome. Specific viral envelopes with the pREC NFL backbone (full length genome) and along with the swapped the V1/V2 loop of an acute virus into a chronic virus were used. To properly select for successful inserts, two rounds of yeast recombination needed to be completed with two different selection markers. The backbone contained previously added β-

isopropylmalate dehydrogenase (LEU2) for selection on leucine-deficient media and the first round of yeast recombination involved the insertion of URA3 which is a catalytic enzyme that encodes for uracil. The insertion of URA3 into the region of interest grown on uracil deficient media ensured the original V1V2 loop of our plasmid was successfully removed, all plasmids lacking URA3 would cause colony death without the presence of uracil growth media.

Prior to yeast recombination, plasmids were transformed into Thermo Electrocompetent Stbl4 bacterial cells according to manufacture protocol overnight and grown in 500mL cultures. The plasmid DNA was verified on an electrophoresis gel for size and quantified using a nanodrop. The plasmid was linearized using a partial digest method with Spe1 according to the manufacturers protocol. Inserts were PCR amplified off of the viral plasmid of interest using primers listed in table 1. The primers were designed to amplify the V1/V2 region of interest specific to a virus, as well as having a 35-50bp overhang of homology to the V1/V2 region of interest in the viral genome to be inserted into. Products were confirmed for size and purity on a gel and were gel extracted according to the manufacturers protocol to eliminate any non-specific amplified products.

A single colony of yeast was grown O/N in 50 mL of Yeast extract-Peptone-Dextrose (YPD) media at 30°C. The following day, the yeast culture was extracted by spinning down the culture at 4000rpm for 5 mins. The supernatant was then removed, and the pellet was resuspended in 1mL of sterile milliQ H2O. The resuspended yeast culture was then pelleted at 4000rpm for 5mins and subsequently resuspended in 1mL of fresh, cold TE/LiAc solution consisting of 1x TE (10mM Tris-Cl pH 7.5 and 1mM EDTA pH 8.0) and 1x Lithium Acetate (pH 7.5) and stored on ice. 100µl of the yeast resuspended

solution was then added to 4ug of linearized plasmid, 1ug of the PCR purified insert, 50ug of denatured Salmon Sperm DNA (Thermo Cat. No. 15632011) and 400µl of polyethylene glycol (8 volumes 50% PEG400, 1 volume 10x TE and 10x LiAc). The mixture was incubated in a 1.5ml Eppendorf tube in a shaking incubator at 30°C, 5% CO2 for 1-1.5 hrs. The yeast solution was then heat shocked at 42°C in a water bath for 15 minutes and subsequently pelleted at 14000rpm for 10 seconds. The supernatant was gently aspirated, and the pellet resuspended in 150µl of milliQ H2O and plated on the appropriate growth media plate depending on the selection markers needed. Colonies were selected following 3-5 days until large colonies were formed. The colony was picked and resuspended in 200µl of breaking buffer which consists of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0. The mixture was then added to 0.3g of glass beads and 200µl of phenol/chloroform. The final mixture was vortexed for 2 mins and pelleted at 14000rpm for 5-10 mins. 150µl of the aqueous phase of the solution was removed and added to a new 1.5ml Eppendorf, along with 700µl of 100% ethanol, 170µl of H2O, and 30µl of sodium acetate (3M) and vortexed. The solution was then pelleted at 14000rpm for 10 mins. Following aspiration of the supernatant, 70% ethanol was added to the pellet and the mixture was then pelleted for 5 mins at 14000rpm. The supernatant was subsequently aspirated, and the pellet allowed to dry for 5-10mins. The DNA pellet is resuspended in 20µl of water and then transformed into Thermo Electrocompetent Stbl4 bacterial cells according to the manufacturers protocol. Bacteria is plated on ampicillin plates and grown overnight. The final colonies were screened using sanger sequencing to confirm the sequence insertion. The final round of selection which involves the swapping of URA3 for the region of interest

involves plates without leucine and with 5-fluoro-otic acid which when in the presence of uracil, produces a toxic anabolite that kills colonies containing URA3, thus selecting for the plasmid backbone (with leucine) and insert (without URA3).

4.12 Viral purification

50 ml of virus was propagated on U87.CD4.CCR5 cells as described above. Following harvesting of the viruses from the culture flasks, cellular debris was removed by centrifugation at 1500xg for 5 mins. Virus was then transferred in 15 ml increments to 150KDa Amicon Ultra-15 Centrifugal Filter Unit (Millipore), and centrifuged at 3700 RPM for 25 minutes at 4°C. The solution which remained in the top of the filter unit was removed and placed in a 1.5ml Eppendorf. This process was repeated until all 50ml were purified. Finally, the concentrated viral solution was pelleted at 40,000xg for 1hr at 4°C. The supernatant was removed, and the pellet was resuspended in 50µl of sterile PBS.

4.13 GP120 envelope large scale purification

Three litres of viral supernatant was propagated using the viral propagation method described above. Cellular debris was removed by centrifugation at 1500xg for 5 mins in 50ml aliquots. Further, aliquots were carefully balanced and ultracentrifuged at 32000xg using the Avanti J-3O1 (Beckman Coulter) for 1.5 hrs at 4°C. Pellets were resuspended in 1ml of 50mM ammonium bicarbonate and stored at -20°C until future use. The virus was then lysed using a 2.5% v/v solution of Empigen BB in order to release the gp120 from the membrane alone with 2.5unit/ml of Benzonase and 2mM NaCl in order to remove DNA and RNA in the mixture. The solution was vortexed for 10 seconds and

incubated at room temperature for 2 hrs. The solution was then adjusted to a final NaCl concentration of 0.65M and subsequently centrifuged at max speed for 15 mins. The supernatant was then filtered using a 0.8micron filter unit and prepared for Galanthus nivalis agglutinin (GNA)(Sigma) column purification. 1ml of GNA from resin solution was added to the column and mixed gently. The column was equilibrated with 10 column volumes of 20 mM Tris-HCl pH 7.5, 650 mM NaCl, 0.25% Empigen BB. The solution was removed using a syringe and subsequently the lysed gp120 was loaded. Once the sample had completely flowed through, the column was washed with 10 column volumes of 20 mM Tris-HCl pH 7.5, 650 mM NaCl to remove excess Empigen BB, followed by 10 column volumes of 20 mM Tris-HCl pH 7.5, 1 M NaCl to remove nonspecifically bound proteins, 20 mM Tris-HCl pH 7.5, 150 mM NaCl to remove excess salts, and finally competitively eluted using 10 column volumes of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 M methyl-α-D-mannopyranoside. The eluate was then dialyzed overnight in a molecular weight cutoff 12-14kDa in 50 mM ammonium bicarbonate buffer pH 8.0 to remove sugars, tris, and salts. Following dialysis, the samples were frozen at -80°C overnight and lyophilized until future use. The proteins were quantified and assessed for purity using SDS-PAGE/B13 antibody western blotting.

4.14 GNA lectin ELISA

The lectin GNA from Galanthus nivalis was diluted to a final concentration of 5ug/ml in coating buffer consisting of NaHCO3/Na2CO3. 100µl of the final solution was added to a Greiner high binding 96 well plate (Sigma) overnight and sealed at 4°C. The plate was then washed 3X in wash buffer consisting of PBS and .05% Tween 20. Following the

wash and removal of any residual liquid by tapping the plates, 100µl of dilution buffer containing 10% FBS (Sigma) in wash buffer was added to each well. The plate was then sealed and placed on a shaker for 2 hrs. The standard recombinant gp140 SF162 (NIH Aids Reagent database) was diluted to 100ng/ml in dilution buffer and serially diluted 2-fold to 0.78ng/ml. Samples were lysed in a 1% Triton-X solution and diluted 1:10, 1:30, 1:90, and 1:270. Following the blocking step, the plate was washed as described above and 100µl of standard and samples were added to the plate for 1-1.5hrs on a shaker at room temperature. The plate was subsequently washed as described above and 100µl of the monoclonal anti-gp41 Ab 5F3 (NIH Aids Reagent Database) diluted to a final concentration of 1ug/ml was added to each well for 1 hr on a shaker at room temperature. The plate was then washed as described above and 100µl of the secondary polyclonal Ab GAH-IgG conjugated with peroxidase diluted 1:5000 was added to each well for 1hr on a shaker at room temperature. The plate was then washed as described above and 100µl of room temperature TMB colour solution (Thermofisher) was added to each well for 10-15 mins in the dark. Following this step, 100µl of 2.5M H_2SO_4 was added to stop the colorimetric reaction. The colorimetric reaction density was read using cytation5 imager (Biotek). Binding affinity of different whole viruses was calculated based on the standards used.

4.15 Enzymatic digestion of gp120 with glycosidases

50ml of each virus was purified as described above. 25µl of the purified whole virus solution was then digested with several enzymes according to the manufacturers protocol for 24 hrs at 37°C. Following this time frame, the sugars that had be shed off of the

protein was subsequently removed using a molecular weight cut off filtration unit. Briefly, 500µl of PBS was added to the digested virus solution and the mixture was then added to an amicon Ultra-0.5ml centrifugal filter with a 100kDa MW cutoff. The solution was centrifuged at 14000rpm for 10mins and the digested virus mixture was removed and placed in a new 1.5ml Eppendorf tube. The virus was then measured for glycan binding via ELISA.

4.16 Propagation of viruses in the presence of glucosidase inhibitors

T-175 flasks were seeded with U87.CD4.CCR5 cells which were grown to 60% confluency. Twelve hours prior to infection with chronic or acute viruses, castanospermine was added to each flask at a final concentration of 50µM. Following this timeframe, virus was propagated for 6 days under the propagation conditions described above.

4.17 Surface Plasmon Resonance

All of the binding kinetics assays to C-type lectins involved the use of the tabletop Surface Plasmon Resonance instrument by Nicoya Lifesciences. Prior to any sample injection, the machine was primed with running buffer for 5 minutes with a blank sensor chip provided with the instrument. Following this timeframe, the standard startup procedure was performed which involved first taking a reference spectrum in light and dark states and loading a new NTA coated sensor chip, which was subsequently flown over with running buffer (TRIS, Nicoya lifesciences) until the baseline settled on the

instrument. Following the startup procedure, the NTA sensor chip was activated and coupled to the ligand—a C-type lectin such as mannose binding lectin. Briefly, the instrument's flow rate was slowed to 20µl /min and repeat injections of 200mM imidazole was injected over the sensor chip until the resulting peak on the instrument was consistent. Following imidazole priming, 40mM NiCl2 was injected twice each for a 5-minute interaction time. His-tagged mannose binding lectin was diluted to 10uM in running buffer and 250µl was injected twice over the activated NTA chip to allow the nickel his-tagged binding interaction to occur for 5 minutes. Following this procedure, samples could then be injected to measure binding affinity to mannose binding lectin. For repeat injections, 250µl of regeneration buffer (glycine-HCl, pH 1.5, Nicoya lifesciences) was injected at the maximum flow rate on the instrument and new samples were only injected once the baseline had reached what it was prior to injection of the previous sample dilution. Peaks were analyzed using the TraceDrawer Nicoya Lifesciences software.

4.18 Infectivity Assay

Viruses were serially diluted 4-fold 10 times in complete DMEM. 10,000 TZMbl cells supplemented with 10ug/mL of DEAE dextran were added to each well and 100µl of the viruses was added to each well. The virus-cell mixture was incubated at 37$_{o}$C, 5% CO_2 for 48hrs. The Britelite plus kit (Perkinelmer) was brought to room temperature for 1-2hrs. Following this timeframe, the lyophilised Britelite plus substrate was reconstituted in 10mL of reconstitution buffer. 100µl of media was then removed from each well in the TZMbl infection plate and 100µl of the reconstituted Britelite plus substrate was

subsequently added. The plate was then incubated at room temperature in the dark. 150µl of the incubated mixture was then transferred to a new black flat bottomed 96 well plate and read for luminescence by a cytation5 imager plate reader (Biotek).

4.19 Neutralization Assay

HIV-1 Q0 and B4 primary viruses were propagated in U87.CD4.CCR5 cells and subsequently titrated in TZM-bl cells to achieve a TCID of 15,000 Relative Luminescence Units (RLU) equivalents- 100-fold of negative control.
Broadly neutralizing antibodies PGT121, PG16, PG9, and 2G12 were first diluted to 10 µg/ml followed by fivefold serial dilutions in the 96-well plate and added in a mixture with the titrated viruses. The virus-antibody mixture was then added to 100,000 TZM-bl cells with 10µg/mL DEAE-dextran for 72 hrs. The % neutralization was determined by reduction in relative luminescence units (RLU) compared to the virus control (virus+cell) as described in the Infectivity Assay methods.

4.20 Western blot

For analysis of chronic and acute HIV-1 Env total glycan content, viruses were propagated and purified as described above, and their gp120 proteins were isolated via the methods described above. Gp120 proteins were boiled at 93°C in 5X SDS-PAGE sample buffer (0.312 M Tris pH 6.8, 25% 2-Mercaptoethanol, 50% glycerol, 10% SDS) for 15 minutes and proteins were separated on a 10% SDS-PAGE gel. They were then transferred to nitrocellulose membranes and blocked in 5% non-fat skimmed milk (BioShop Canada, Burlington, Canada) in TBST containing 0.1% Triton X-100 for 1

hour at room temperature. They were then incubated overnight at 4°C with clarified hybridoma supernatant containing mouse anti-gp120 (B13) mAb antibody (Research and Reference Reagent Program, NIAID, NIH, contributed by George Lewis). Membranes were washed and incubated for 1.5 hours with species-specific HRP-conjugated antibodies (1:3000 for B13, Thermo Scientific). All blots were developed and quantified using ECL substrates (Millipore Inc., Billerica, MA) and a Gel Doc EZ Imager (Bio-Rad).

Chapter 3

5 Results

5.1 Global glycan differences between chronic and acute HIV-1 subtype B viruses

Global site-specific analysis of conserved N-linked glycosylation sites of 20 acute subtype B HIV-1 viruses obtained from the CHAVI study were compared to conserved group M, and subtype A, B, C, D sequences obtained from the HIV Los Alamos sequence database. Analysis showed that acute envelope sequences had between 16 and 24 conserved N-linked glycosylation sites, while in comparison the consensus sequences displayed at least 23 (23-25) conserved N-linked glycosylation sites (Fig.5.A). Based on these analyses along with preliminary transmission fitness studies using these acute envelope HIV-1 in penile tissue (Fig.5.B courtesy of Eric Arts) revealing that acute viruses display higher fitness in crossing mucosal barriers compared to chronic viruses, a high transmission fitness acute virus known as B4 along with a low transmission fitness chronic virus known as Q0 were chosen for the bulk of the studies performed. These preliminary transmission fitness studies were performed in penile tissue according to the tissue explant assay protocol described earlier. For the analysis of global and site-specific N-linked glycosylation of the viruses listed above, Western blot analysis against gp120 was used. First B4 and Q0 were propagated in U87.CD4.CCR5 and harvested every 3-4 days until day 14 (Fig.5.C). Following the collection of a large volume of viral propagated supernatant, cellular debris was removed by centrifugation and total virus was pelleted by ultracentrifugation (Fig.5.C). The pelleted virus was then lysed and gp120 molecules were purified using a GNA lectin column (Fig.5.C). Following quantification of the purified gp120 by SDS-PAGE (Fig.5.C), a western blot using mouse anti-gp120 antibody B13 (Fig.5.D) was performed.

Global glycosylation differences appeared to be evident in a representative Western blot against gp120 whereby the presence of a lower molecular weight band at ~70kDA was present in the B4 purified gp120 fraction and was largely absent in the same purified fraction of Q0 chronic gp120 (Fig.5.D). This suggests the presence of a more diverse gp120 glycosylation profile on the high transmission fitness acute B4 gp120 with lower total sugar content compared to the low transmission fitness chronic Q0 gp120. It is important to note that the lower sugar content/more diverse sugar content gp120 molecules corelates with previous transmission studies in penile tissue whereby lower global glycosylation appears to coincide with higher transmission fitness and vice versa (Fig.5 A,B and D).

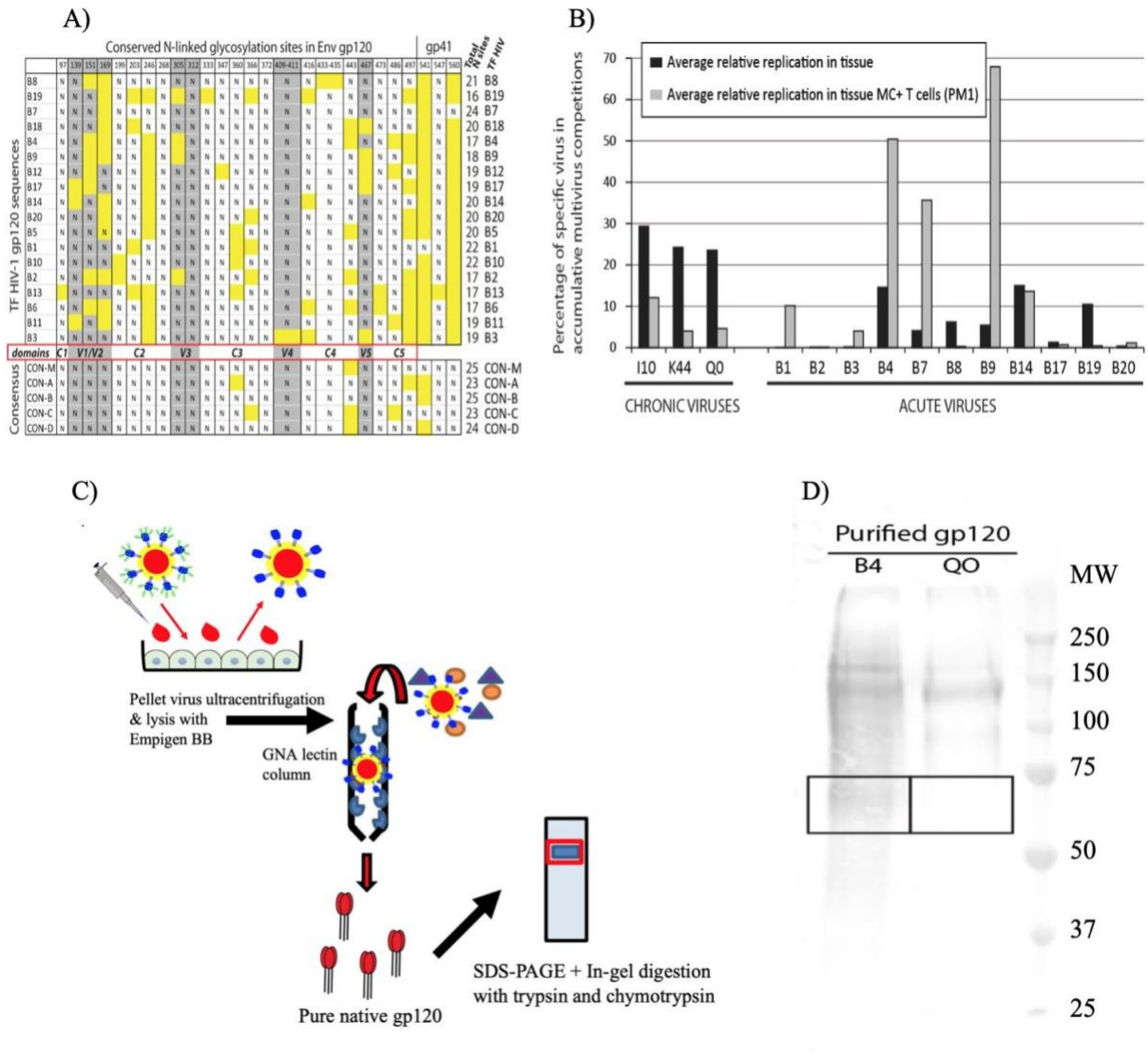


Figure 5. Determination of global differences in glycosylation between acute and chronic HIV-1 strains.

(A) Global envelope glycosylation site analysis of subtype B acute viruses (CHAVI) compared to conserved group envelopes (Courtesy of Dr. Eric Arts). Yellow highlights N-linked sites that were absent in the sequence. (B) Summary of transmission fitness of acute and chronic subtype B env chimeric viruses in terms of replication in tissue vs migratory cells (Courtesy of Dr. Arts). (C) Schematic of large-scale production of whole chimeric acute and chronic HIV viral particles and subsequent purification of native gp120 envelope proteins for glycan analyses. (D) SDS-PAGE using B13 antibody against chronic Q0 and acute B4 native gp120 proteins.

5.2 Neutralization sensitivity of acute B4 virus and chronic Q0 virus appear to be influenced by differences in the V1/V2 loop.

Neutralization sensitivity to bNAbs was assessed to compare specific glycan motifs on the acute B4 and chronic Q0 virus. Specifically, neutralization sensitivity to the bNAbs PG9 and PG16 to assess the V1/V2 loop, PGT121 to assess the V3 loop, and 2G12 to assess the high mannose patch on gp120 was evaluated (Fig.6.A.). Briefly, propagated viruses were titrated onto TZM-bl cells to determine the dilution that results in 15,000 RLU equivalents. The antibodies were diluted to a starting concentration of 10ug/mL and further serially 5-fold diluted. The virus and antibody dilutions were mixed and incubated with 100,000 TZM-bl cells for 72 hours. Reduction in RLU compared to the virus only control was used as a measure of neutralization sensitivity. Both viruses were highly sensitive to neutralization by the antibody PGT121 targeting the V3 variable loop of gp120 with a neutralization percentage of 93.5% for Q0 and 97.5% for B4 (Fig.6.B). While the acute B4 HIV-1 virus was highly sensitive to neutralization by the high mannose patch targeting antibody 2G12 (92.98% neutralization), the chronic Q0 HIV-1 was largely resistant to neutralization by the same antibody with only a 26.9% neutralization (Fig.6.B). An opposite trend was seen with the V1/V2 loop targeting antibodies PG9 and PG16. While the chronic Q0 virus showed 75.9% neutralization for both antibodies, the acute B4 virus was more resistant to these same antibodies with a 41.03% and 16.84% neutralization, respectively (Fig.6.B). This suggests differences between the virus's glycan profile at the high mannose patch as well as the V1/V2 loop of gp120.

5.3 Yeast recombination to swap the V1/V2 loop from the acute high transmission fitness B4 virus to the chronic low transmission fitness Q0 virus.

To evaluate the role of the V1/V2 env glycosylation on transmission fitness we thought to replace the V1/V2 region in the low transmitting virus (Q0) with the V1/V2 of the high transmitter (B4). Primers were designed to amplify the V1/V2 loop of the acute B4 virus envelope along with homologous 50bp overhangs homologous to Q0 to allow the yeast double stranded break repair mechanism to swap this fragment into the chronic Q0 envelop region (Fig.7.A). Prior to this, the gene URA3 was PCR amplified with the same 50bp overhangs to insert into the Q0 envelope V1/V2 loop. The PCR products were confirmed to be of the correct size of 480bp bp by agarose gel electrophoresis (Fig.8.A).To activate the yeast recombination double-stranded DNA break repair mechanism, the Q0 envelope was partially digested for 5 minutes at 37°C to linearize the plasmid (Fig.8.B) . As described above, a 4:1 ratio of linearized plasmid to insert was added to YPD-Leu-URA selection plates and screened for colonies following 3-5 days (Fig.7.B). Following this timeframe, yeast colonies were picked, DNA isolated as described above (Fig.7.B). The URA3 containing Q0 plasmid was then linearized using the Sbf1 according to the manufacturers protocol (Fig.8.C). A 4:1 ratio of Q0 plasmid now with URA3 inserted in the V1/V2 region to the PCR amplified V1/V2 loop of B4 with homologous overhangs was subjected to a yeast transformation and added to a YPD-Leu+5FOA selection plate (Fig.7B). The final colonies contain the B4 V1/V2 loop insert in place of the URA3 selection marker, as the 5FOA plate kills any colonies containing URA3 as described above (Fig.7). The final insert/plasmid was confirmed by sanger sequencing.

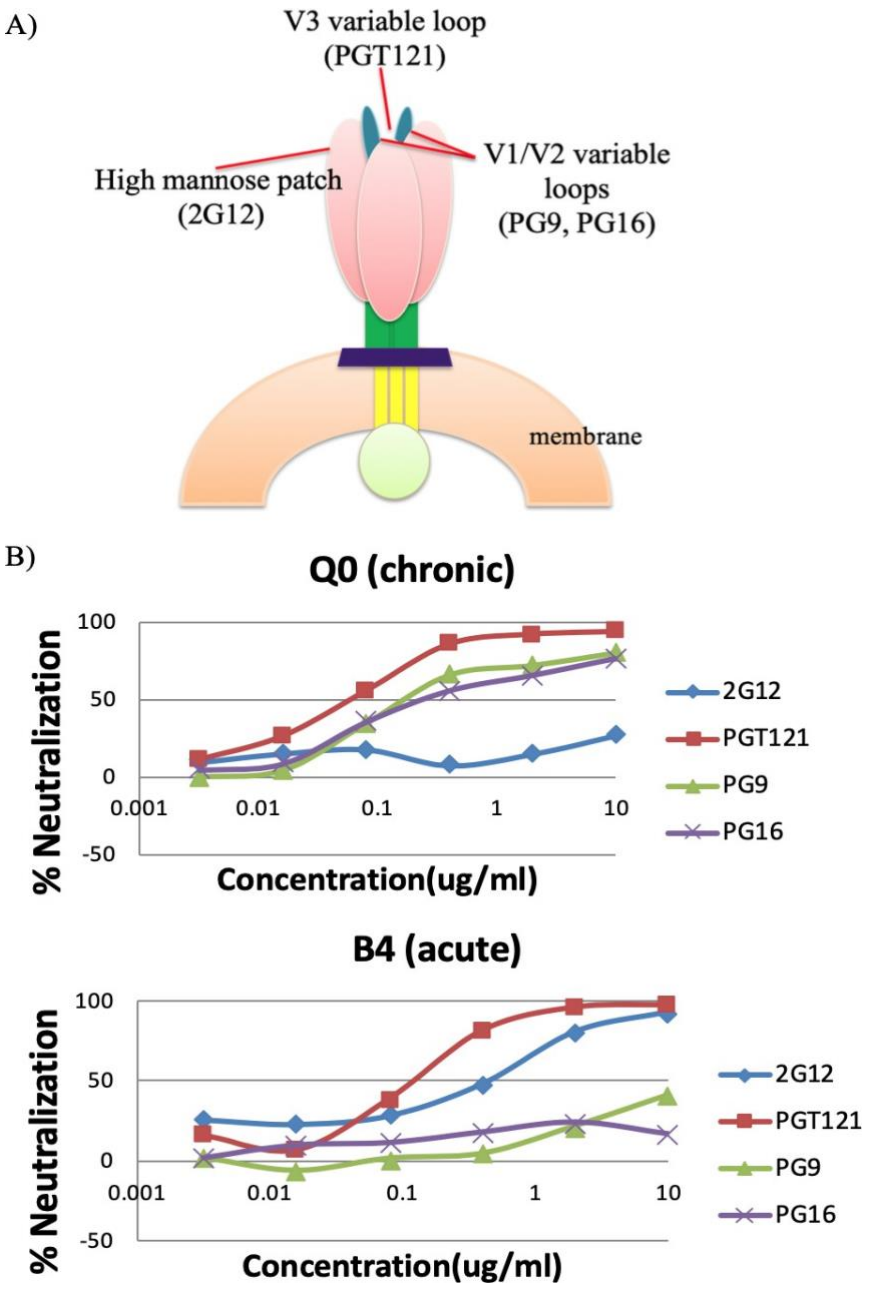


Figure 6. Neutralization sensitivity of acute and chronic subtype B HIV-1 to specific glycan targeting antibodies on gp120.

(A) Depiction of HIV-1 envelope polyprotein gp160 and the specific regions targeted by glycan targeting antibodies PG9, PG16, PGT121, and 2G12. Percent neutralization of chronic Q0 (B) and acute B4 (C) chimeric HIV-1 isolates against glycan targeting antibodies PG9, PG16, PGT121, 2G12.

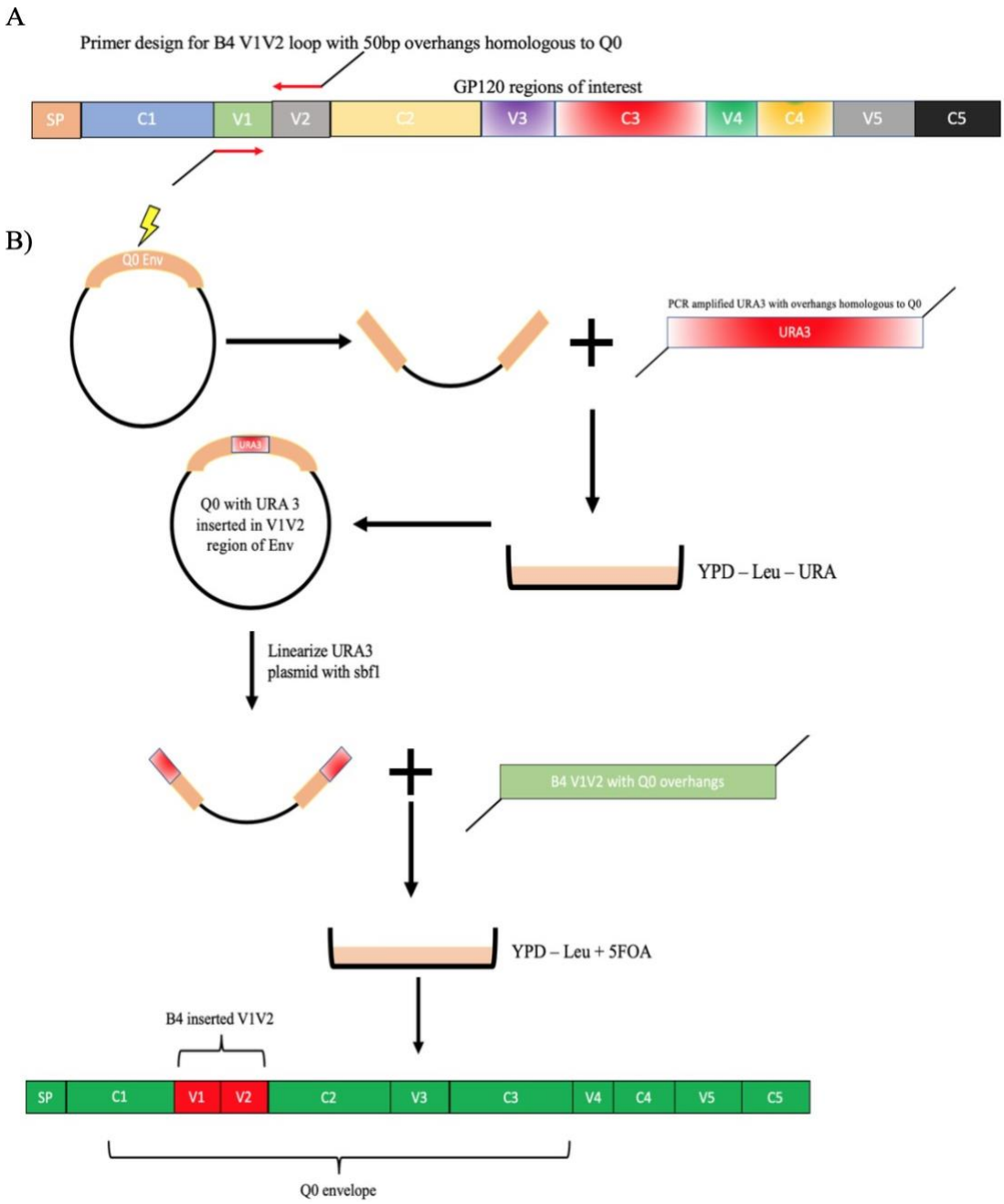


Figure 7. Schematic of yeast recombination strategy for replacement of V1V2 loop of chronic Q0 HIV-1 with V1V2 loop of acute high transmission fitness B4 HIV-1.
(A) Brief depiction of primer design of B4 V1V2 loop sequence with overhangs homologous to Q0 envelope. (B) Two-step yeast recombination system involving the insertion of URA3 into the region of interest in Q0 envelope followed by the insertion of the acute B4 HIV-1 V1V2 loop in place of URA3.

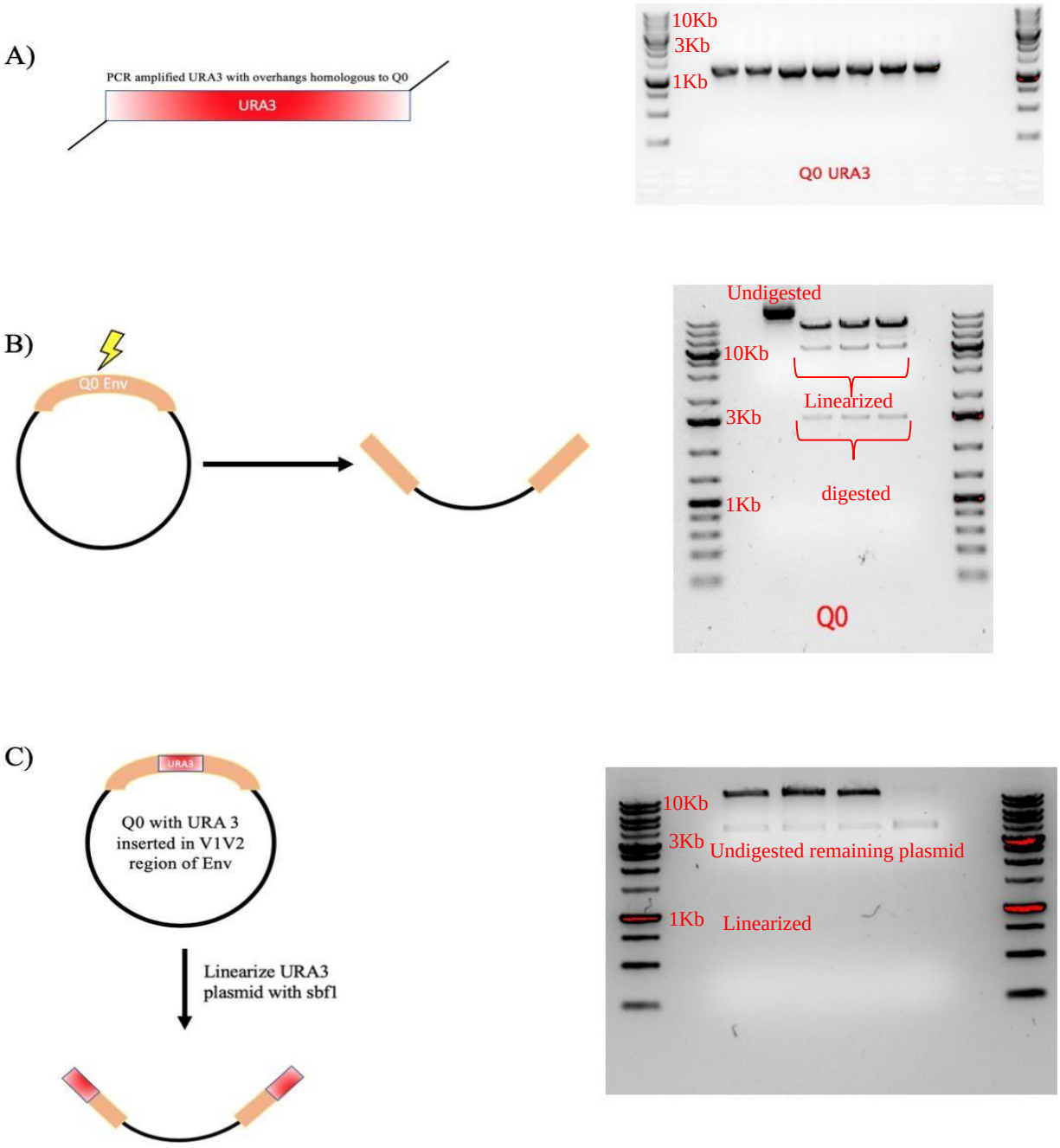


Figure 8. Stepwise gel images of homologous yeast-based recombination of acute virus B4 V1V2 loop inserted into chronic virus Q0 envelope.

(A) PCR amplified products of URA3 gene with Q0 50bp regions of homology surrounding the V1/V2 loop. All lanes are the same product. (B) Partial digest of the Q0 Env gene using Spe1. Left lane is undigested whole plasmid, and right three lanes are partially digested plasmids. (C) Diagnostic digest confirming the insert of URA3 into the Q0 V1/V2 loop using Sbf1.

5.4 Digestion of whole viral particles using the glycosidase PNGase F reduces glycan binding affinity to bNAb 2G12.

To determine if modifications of the glycan content or composition influence HIV transmission, B4 and Q0 were treated with PNGaseF to remove N-linked glycans (Fig 8A). Changes in glycan binding were measured using a GNA ELISA. At each dilution, a marked reduction in binding affinity was seen in the digested B4 compared to the undigested particle. Specifically, a 4.97, 10.57, 14.06 -fold reduction at 1:10, 1:30, 1:90 was determined, respectively (Fig.9.B) No binding was detected at the 1:270 dilution for the digested viral particle (Fig.9.B). To assess if the removal of glycans from the HIV envelope influences the infectivity of the viral particles, PNGAse F treated and wild type B4 and Q0 were titrated in a TZM-bl cell infection assay (Fig.9.C). Interestingly, PNGase F digested viruses were no longer infectious while B4 and Q0 wild type viruses showed substantial infectivity (Fig.9.C). This suggests that treatment with the glycosidase PNGase F removes the majority of glycans on HIV-1 env which might reduce their ability to attach and bind to receptors on target cells necessary for infection.

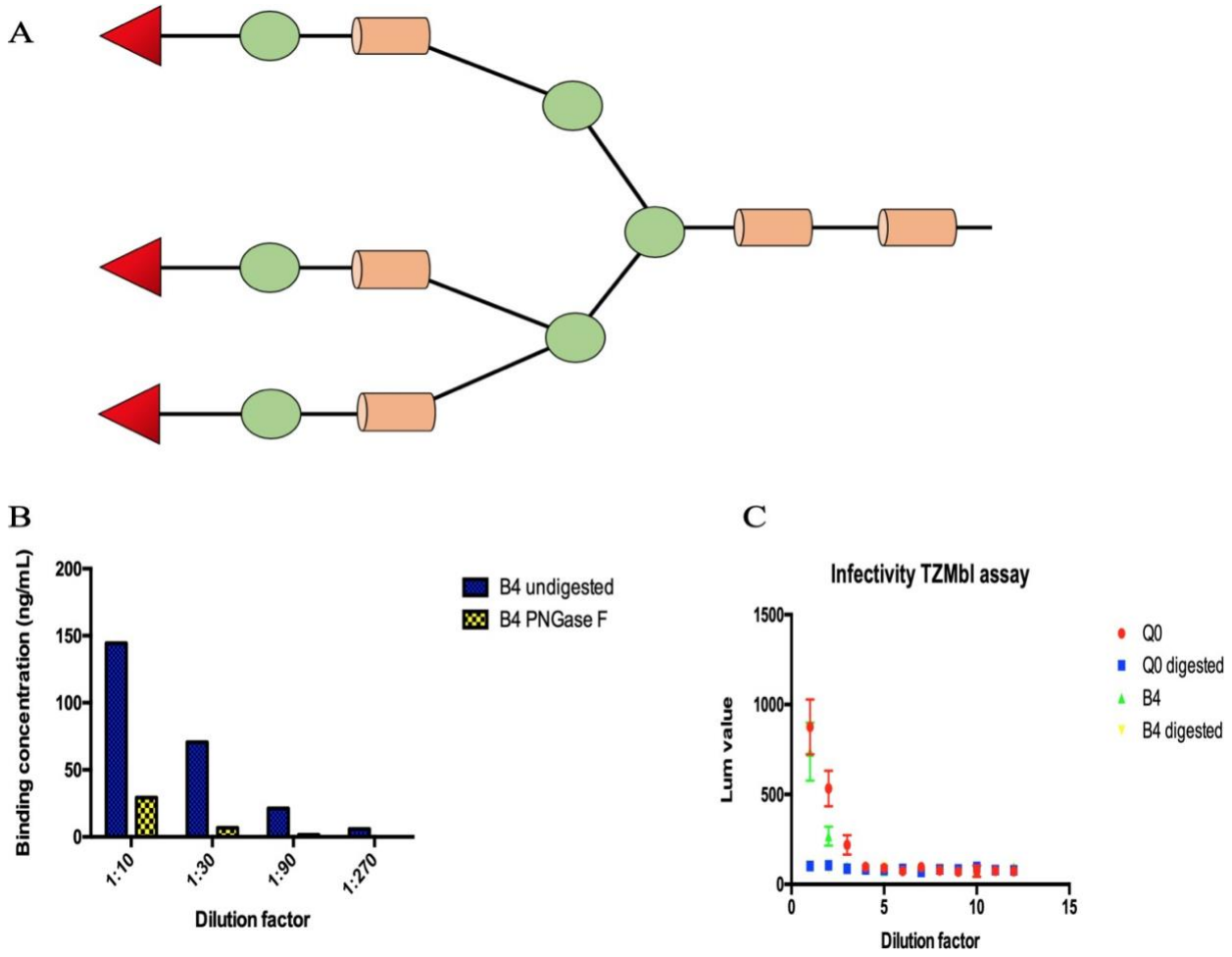


Figure 9. Digestion of whole infectious viral particles using PNGase F causes reduced glycan binding to bNAb 2G12.

(A) Schematic of the whole glycan which is removed using PNGase F digestion of whole viral particles. (B) GNA ELISA measuring binding affinity of acute subtype B B4 whole virus with and without PNGase F digestion treatment. (C) Measuring infectivity of B4 and Q0 viruses with and without PNGase F digestion treatment.

5.5 Propagation in the presence of alpha-glucosidase castanospermine reduces glycan binding affinity to bNAb 2G12.

Castanospermine is an alpha-glucosidase inhibitor which has been used to inhibit N-linked glycosylation in cultured cells. To determine the effect of alpha-glucosidase inhibitors on HIV-1 N-linked glycosylation and transmission, viruses were propagated in U87.CD4.CCR5 cells in the presence of castanospermine. Briefly, 12 hours prior to infection with chronic or acute viruses, castanospermine was added to U87.CD4.CCR5 cells at a final concentration of 50uM. Each of the two viruses was then propagated for 6 days under the propagation conditions described above. 50mL of the whole viral particles were purified and their binding affinity of gp120 to the bNAB 2G12 was measured using a GNA ELISA described above as a measure of reduction of total glycan content on Env. A 4.73 and 85.80-fold reduction in binding affinity to the glycan binding bNAb was observed at the 1:10 and 1:30 dilutions in the Q0 castanospermine propagated virus compared to the untreated propagated Q0 virus, respectively (Fig.10.A). To determine if the treated virus remained viable relative to its untreated counterpart, an RT assay was performed (Fig.10.B). According to the RT assay, it was determined that the propagation of both the Q0 and B4 viruses in the presence of the alpha-glucosidase inhibitor castanospermine had no effect on the reverse transcriptase activity of the viruses compared to the same viruses grown in the absence of this same inhibitor as was shown in the number of positive ‘spots’ at the various dilution factors (Fig.10.B).

5.6 PCR amplification from DNA extractions of proviral DNA following cervical tissue explant assay

Following the 10-day culture of the infected tissue explants and infected migratory cell-T cell co-culture, proviral DNA was isolated according to the DNeasy Blood and Tissue kit protocol (Qiagen). The C2-V3-C3 region of Env was PCR amplified from the Extracted proviral DNA samples using two rounds of PCR with the primers described in Table 1. Following the second round of PCR amplification a ~500bp fragment was visualized by gel electrophoresis (Fig.11). In general, the PCR success rate (ie. Positive band visualization by gel electrophoresis when loading 5uL of sample) was approximately 60-80% depending on the explant assay. Bands that were smeared or contained double bands were gel extracted and ethanol precipitated prior to Next generation sequencing library preparation.

5.7 C-type lectins inhibit subtype B HIV-1 from transmitting through penile and cervical tissue ex vivo

To evaluate the effect of C-type lectin binding to sugars in the recipient mucosa on transmission fitness, penile and cervical explants were pretreated/soaked with 1 mM mannan for 12 hrs and washed prior to addition of the acute B4 and the chronic Q0 virus. N linkage-derived mannan from *S. cerevisiae*, has a conserved [mannose]x8 [N-acetylglucosamine]x2 core structure with high affinity for all C-type lectins. Viral replication in both tissue and MC + PM1 was evaluated by a gag-specific qPCR as described above. Analysis of the total viral copy number demonstrated that penile and cervical tissue soaked in mannan prior to virus exposure resulted in reduced total viral replication compared to tissue that was untreated (Fig.12). Further, an increased HIV-1 copy number was found in MC + PM1 co-cultures when tissue was treated with mannan (Fig.12). Specifically, mannan-treated tissue resulted in 6.6-fold reduction in total DNA

copy number and the corresponding MC+T cell co-culture had a 4-fold increased viral copy number compared to untreated tissue (Fig.12). These findings suggest with the addition of saturating levels of mannan, viruses were no longer being trapped by factors in the mucosal tissue such as C-type lectins and were able to cross the mucosa with MCs to infect T cells.

5.8 Competing acute B4 against chronic Q0 in cervical tissue reveals greater B4 replication in tissue.

To determine the transmission fitness of an acute env chimeric B4 virus compared to the chronic env chimeric Q0 virus in cervical tissue, a explant assay was performed. Briefly, 750 infectious units of both acute virus B4 and chronic virus Q0 were added to 3 tissue donors for 6hrs. The tissue was subsequently washed with warm PBS and following 24hrs, migratory cells with bound virus was washed and added to cultured PM1 CD4 T cells. The infected tissue and cultured cells were incubated for 10 days. Following these 10 days, proviral DNA was extracted, and the C2-V3 region was amplified. Samples were then barcoded and Illumina Miseq next generation sequencing was performed. Following sequence read analysis by Dr. Hathaway using the SeekDeep Pipeline software, greater B4 replication was observed. In the tissue, of the total viral replication, B4 consisted of 76.56% and Q0 consisted of 15.93%. However, both viruses had no replication in the migratory cell +T cell coculture.

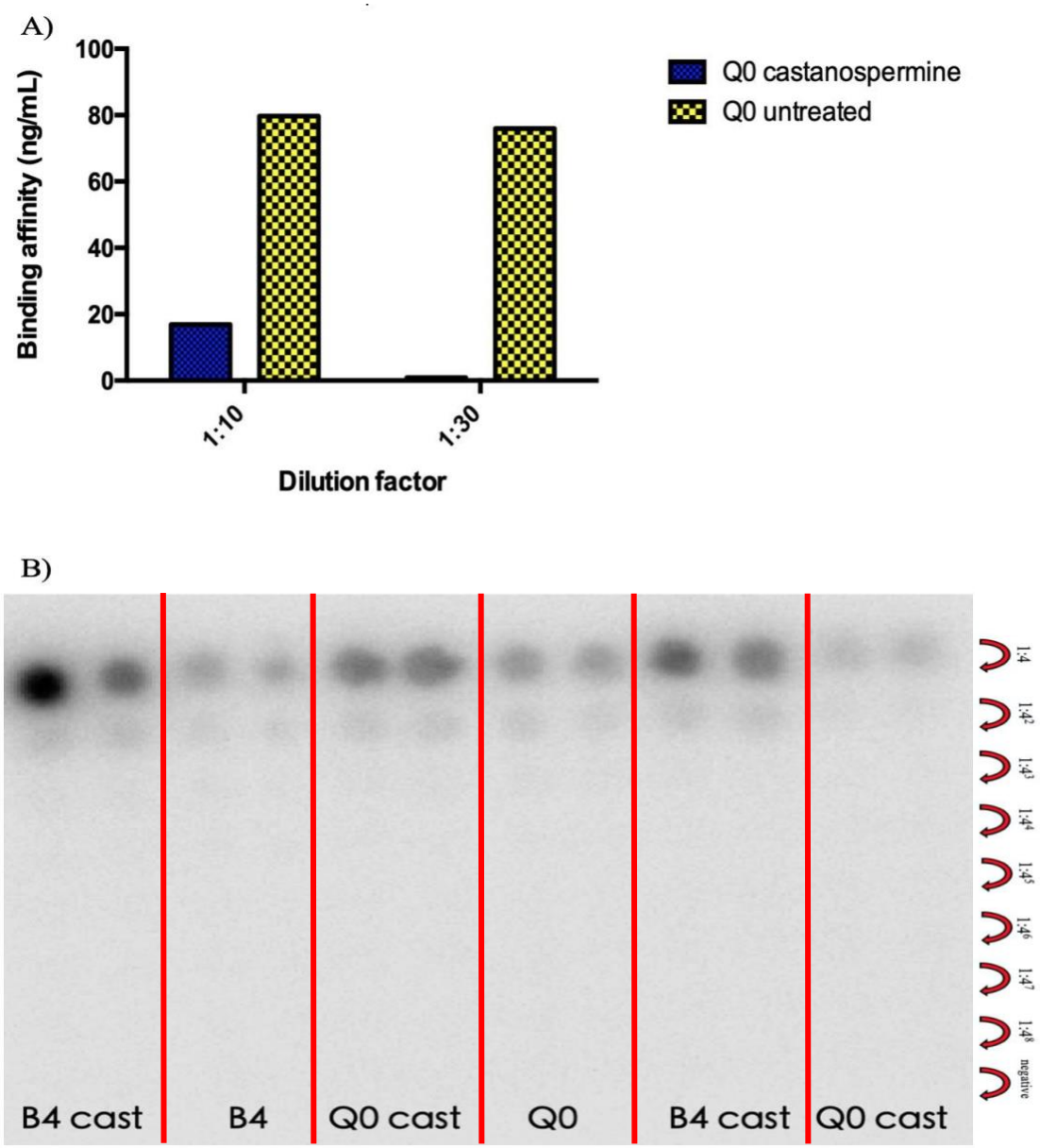


Figure 10. Glycan binding affinity and viral reverse transcriptase activity following cellular treatment with alpha-glucosidase castanospermine.

(A) Binding affinity of whole Q0 chronic HIV-1 particles propagated in the presence of alpha-glucosidase inhibitor castanospermine to glycan binding broadly neutralizing antibody 2G12. (B) Reverse transcriptase activity of castanospermine treated and untreated Q0 chronic and B4 acute HIV-1.

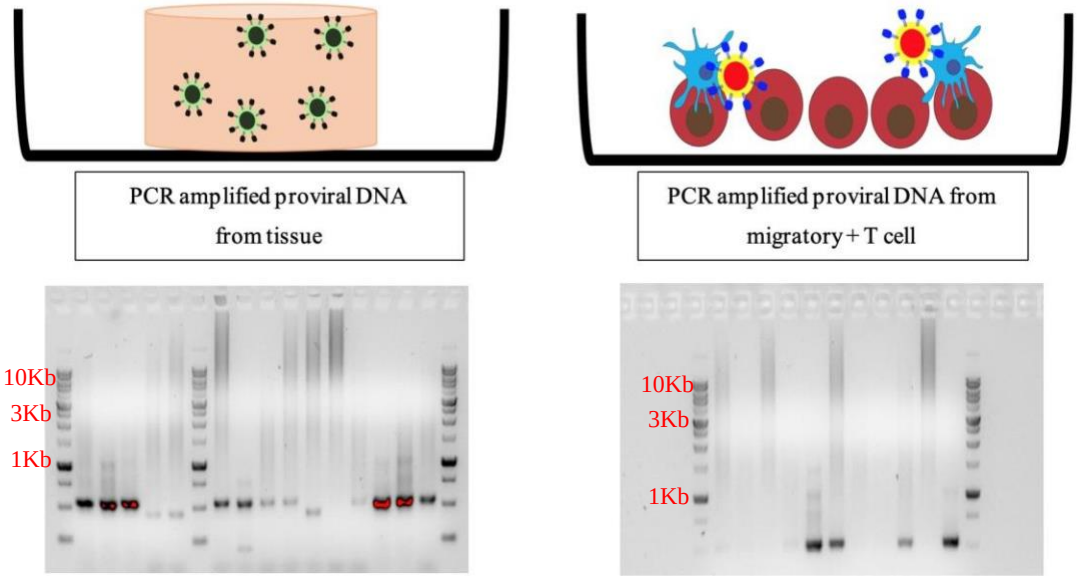


Figure 11. Sample PCR amplification of C2-V3 region of envelope from Proviral DNA in tissue and migratory cell samples.

All lanes represent separate replicates of a particular competition between Q0 and B4 HIV-1 env chimeric viruses. E80 and E125 primers were used to amplify the 480bp region to be sent for NGS.

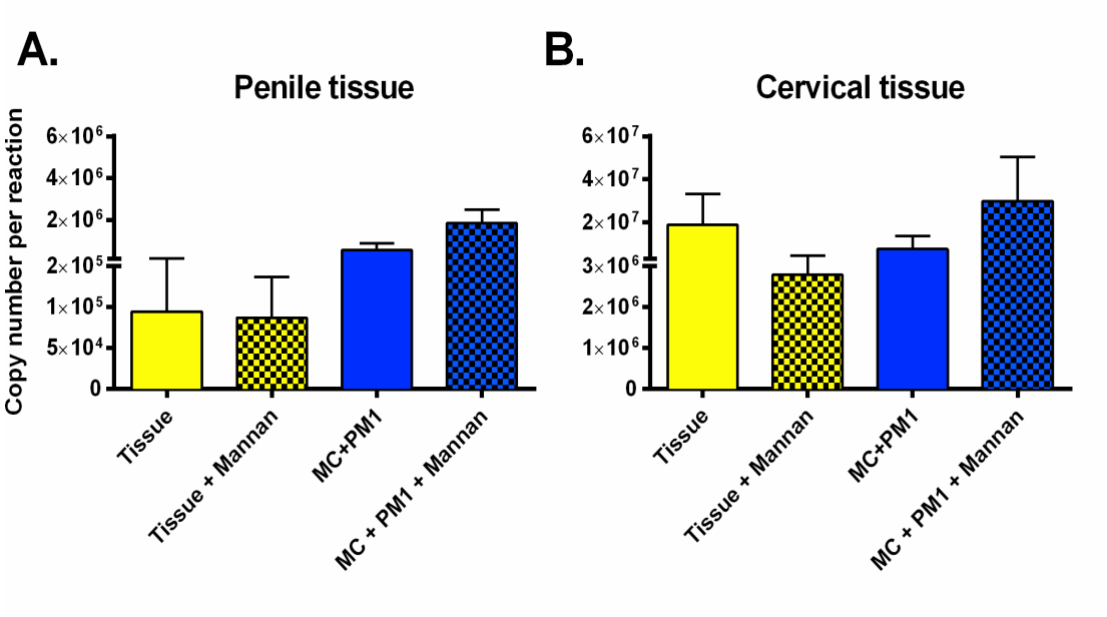


Figure 12. Replication of subtype B HIV-1 in tissue and T cells in the presence and absence of mannan.

Quantitative PCR of HIV-1 DNA copy number as a measure of DNA replication in tissue and migratory cells following an ex vivo tissue explant assay. Comparing subtype B HIV-1 in the presence or absence of mannan as a measure of lectins inhibition of transmission in (A) penile tissue and (B) cervical tissue. The bars represent standard error.

5.9 Measuring purified gp120 binding affinity to C-type lectins using Surface Plasmon Resonance (SPR)

Purified gp120 from acute B4 HIV-1 and chronic Q0 viruses was quantified and resuspended in running buffer (Table 3). Recombinant HIV-1 gp140 protein known as CN54 was obtained as a control protein and resuspended in running buffer prior to sensor chip immobilization. To assess the binding affinity of our purified gp120 molecules from our acute and chronic viruses, conditions were first optimized on SPR (Nicoya lifesciences). Briefly, nitriloacetic acid (NTA) coated sensor chips were activated using an Imidazole solution followed by a NiCl2 solution. Following this priming procedure, 300µl of mannose binding lectin (diluted to 10uM in running buffer) was added to the sensor chip at the lowest flow rate for 5 minutes of interaction time. Following this time frame, the CN54 gp140 protein was injected on the sensor chip now immobilized with mannose binding lectin (Fig.14.A).

Two different concentrations were injected on the chip and several regeneration steps were required to fully remove each subsequent CN54 injection prior to injecting the next (Fig.14.B). The chip was determined to contain little to no prior CN54 injection when the SPR signal baseline prior to injection was equal to the SPR signal baseline following injection of a particular dilution of CN54 (Fig.14.B). Peaks were measured in wavelength (nm) and a general representative trend was seen with decreasing concentrations of injected control CN54 (Fig.14.C). Sudden blips in the signal are common using the Nicoya lifesciences instrument when an air bubble is injected in the instrument in a sample injection (Fig.14.B, Fig.14.C). This control protein establishes the necessary procedures to perform methods on purified B4 and Q0 gp120 in future experiments.

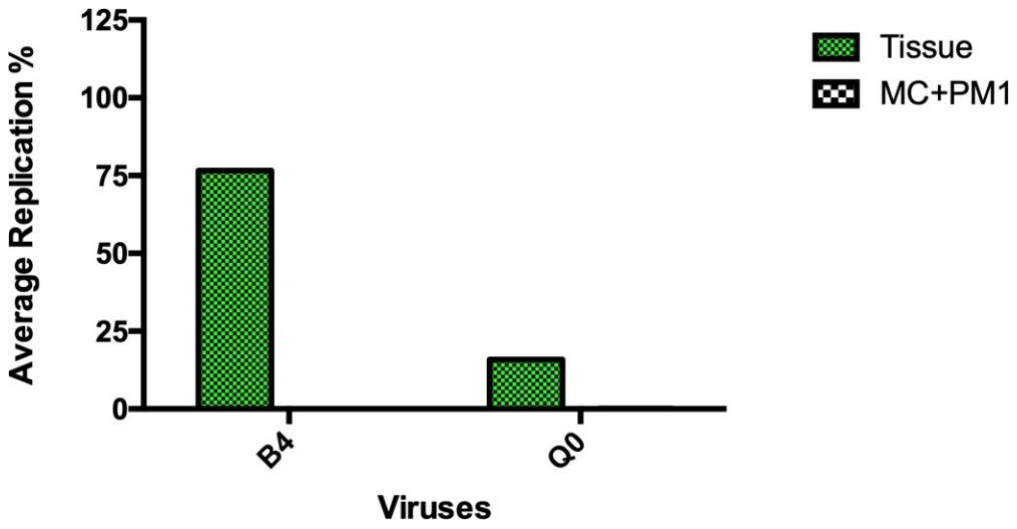


Figure 13. Transmission fitness of acute subtype B B4 and B7 HIV-1 compared to chronic subtype B Q0 HIV-1 in a cervical tissue explant model.

Summary of transmission fitness data comparing the average replication in tissue vs migratory cells in two subtype B acute env chimeric viruses (B4) and one chronic subtype B env chimeric virus (Q0).

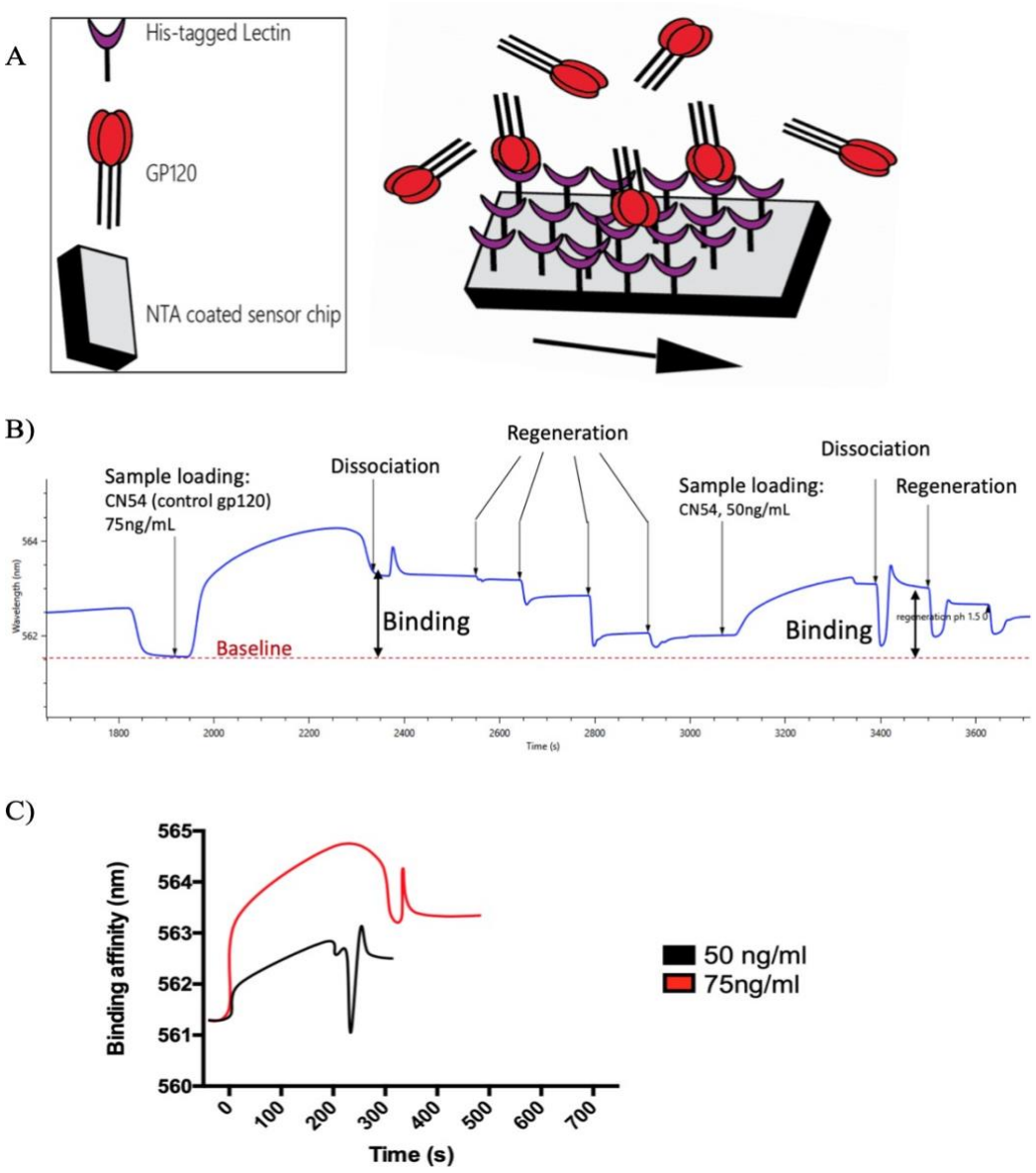


Figure 14. Representative SPR plots demonstrating binding and dissociation of purified whole HIV-1 envelope to mannose-binding-lectin on a nickel coated sensor chip.

(A) Schematic of experimental basis of SPR measuring binding affinity to recombinant his-tagged MBL by subtype B HIV-1 purified gp120. (B) Sample experimental output displaying sample loading and binding, removal of sample by regeneration, and re-addition of samples at various concentrations. (C) Binding curve of human CN54 gp140 optimized at two concentrations.

Chapter 4

6 Discussion and Conclusion

This study began with two preliminary studies performed by Drs. Eric Arts and Robin Shattock at Case Western Reserve and Imperial College London UK, respectively. A global analysis of gp120 and gp41 potential N-linked sites of 20 acute subtype B viruses revealed that compared to conserved sequences of HIV-1 group M and subtypes A, B, C, and D, they had as many as 8 fewer sites. This suggested that in subtype B HIV-1, TF virus envelopes appear to have fewer N-linked sites compared to chronic viruses of the same and other subtypes.

The development of a model that could give a quantitative measure of 'transmission fitness' was necessary for this study. Drs. Eric Arts and Robin Shattock developed a model which uses human tissue explants *ex vivo* to determine how well a virus can penetrate human mucosal tissue, bind to resident migratory DC's, and be carried to susceptible target T cells, similarly as a transmission occurs *in vivo*. Using this model, 20 acute and 3 chronic subtype B env chimeric viruses were determined to differ significantly in their transmission fitness in penile tissue (Fig.5.B). Acute virus B4 and chronic virus Q0 had high and low transmission fitness, respectively, and were chosen as model viruses for this study. These viruses were now known to differ in total N-linked sites, and transmission fitness, and subsequently a Western blot measuring global glycan content was performed on each virus. The presence of a lower molecular weight band (~55-60kDA) was observed on the B4 virus env and was absent on the Q0 env. Knowing that these viruses differ in their transmission fitness, and global glycosylation of env, specific regions of env which differed between the chronic and acute virus were investigated.

Results from the neutralization assay outlined differences between the viruses in the V1/V2 loop and the high mannose patch. Specifically, the B4 virus was highly sensitive to neutralization by the high mannose patch targeting antibody 2G12 (92.98% neutralization), whereas chronic Q0 HIV-1 was largely resistant to neutralization by the same antibody with only a 26.9% neutralization. An opposite trend was seen with the V1/V2 loop targeting antibodies PG9 and PG16. While the chronic Q0 virus showed high neutralization sensitivity for both antibodies, the acute B4 virus was more resistant to these same antibodies. These results led us to create our first mutant virus containing an altered env protein whereby the V1/V2 loop from a high transmission fitness B4 virus was swapped into the V1/V2 loop of the low transmission fitness Q0 virus using yeast- based recombination. The V1/V2 loop has been heavily implicated in being targeted by bNAbs and thus contains high diversity. For instance, it has been shown in women infected with subtype A HIV-1 that sequences isolated early in infection had shorter V1/V2 loops compared to sequences obtained during chronic infection[112]. Since these glycans tend to be more exposed on the gp120 protein, they are under immense selective pressure to avoid immune interaction. For these reasons, I believe this region may also be important in transmission, perhaps in avoiding binding to host lectins. Further, a study demonstrated that glycan deletions in the V1/V2 loop caused sensitized binding to carbohydrate binding agents (CBA) suggesting that these deletions may cause some sort of unraveling of specific sugar motifs[120]. This is interesting because the same study noted how viruses propagated in the presence of these CBAs select for glycan deletions on gp120 except for the V1V2 loop. The ability of deletions to cause exposure of sugars to preventative pressures in the recipient serve as important clues for better understanding of how this virus transmits. Knowing based on neutralization

sensitivity that our B4 and Q0 viruses likely differ in some way in this region, it would be interesting to elucidate the specific glycans present which results in this effect. Similar studies will need to be carried out swapping the high mannose patch between the two viruses to determine its role in transmission as it has been shown that lectins such as MBL bind to the high mannose regions of gp120 on HIV-1[121].

Specific regions of env including specific N-linked sites will likely be important in determining sites which may be selected for during a transmission event. However, it is important to note that a single site or even a particular region of Env will not likely be the entire answer of what distinguishes a TF virus compared to the other majority of viruses which do not make it through the transmission bottlenecks. In other words, our approach to determining the glycan 'footprint' of the TF virus involves looking at both specific sites as well as the global glycosylation of the entire gp120 polyprotein as many factors that were mentioned earlier such as steric hindrance of certain glycans could affect access of certain enzymes which process them. It is therefore important to also look at the overall sugar content as a whole to see which causes a virus to transmit better over another. For this reason, whole viral particles were digested with glycosidases to modify the glycans on an infectious virus, which could then be used to determine if cleaving glycans at certain points is more favourable for transmission. To my knowledge, this work had never been done with PNGase F on native, infectious HIV-1 in a way that was meant to maintain the infectivity of the virus for future use. Several optimization steps needed to be carried out to ensure the proper amount of enzyme was used for the total amount of protein to be digested and a GNA ELISA was performed to measure the amount of glycans which were shed in the process as a measure of loss of binding affinity. Overall, the treatment of the virus with PNGase F for 24hrs led to as high as 14- fold reduction in binding affinity to the bNAb 2G12,

suggesting a significant amount of the overall gp120 glycans were shed in the process. The viral infectivity was then measured on TZM-bl cells as previously described and a loss of infectivity following this treatment was observed in both viruses, suggesting the PNGase F was too harsh to be treating whole viral particles for that amount of time. The experiments need to be repeated, but in the process of digesting these viruses, they were subjected to as many

as 3 freeze-thaws, which may have caused the viruses to lyse and thus lose their infectivity. To determine what caused this loss of infectivity, the viruses will be subjected to the same conditions while only being subjected to one freeze-thaw, and if infectivity is still compromised, less enzyme and shorter digestion times will be trialed.

Further to the idea of modifying viruses in order to determine a global glycosylation 'footprint' for transmission, an alternate approach to virus digestion with glycosidases was taken in that viruses were propagated in the presence of alpha-glucosidase inhibitors to produce infectious virus in the presence of altered N-linked glycosylation machinery in cultured cells. Several of these inhibitors have been used in the context of HIV-1 in vitro to propagate virus and thus viruses were propagated in the same base cell line used in previous propagations but 12 hrs prior to infection, 50mM of castanospermine was added to culture media. Following 6 days, viruses which propagated in the presence of castanospermine were harvested and their altered glycosylation was measured as a loss of binding affinity to bNAb 2G12 using an ELISA. As high as an 85-fold reduction in binding to the bNAb 2G12 was observed following castanospermine treatment. Since the glycosylation of the viruses were clearly being altered using these methods, the next step was to determine if these viruses remained infectious following this propagation using an RT assay as described earlier. The RT assay revealed approximately equal RT activity in the presence or

absence of the alpha-glucosidase inhibitor castanospermine. These methods are now optimized to be used with other alpha-glucosidase inhibitors and these modified viruses can then be run through our ex vivo tissue explant assay to determine their transmission fitness. If modified viruses display a marked increase in transmission fitness, their glycan content can then be analyzed by mass spectrometry to outline which glycan 'footprint' is most favourable for transmission.

Based on the idea that glycan content may be the driving factor that is selected for in a TF virus, the idea that C-type lectins could be the major bottleneck which prevents viruses from traversing the mucosa was proposed. Since C-type lectins bind to carbohydrates and have been shown to bind the high mannose content of gp120, the tissue explant assay was altered to determine if C-type lectins play a role in the virus' ability to transmit. If C-type lectins are a major factor for determining how well a virus penetrates our mucosal tissue, binds to resident migratory cells, and is transferred to T cells, then by removing them, or inhibiting them in some way, you would see a marked increase in viral transmission based on viruses which normally get trapped, can now transmit efficiently. To observe the effects of C-type lectins on HIV-1 transmission, a tissue explant assay with cervical and penile tissue was performed but prior to infection, the tissue was soaked in Mannan from *Saccharomyces cerevisiae* which is a common carbohydrate bound by lectins. Following 12hrs of soaking the tissue with a high concentration of mannan, the tissue was infected as described above. Replication was measured by performing a qPCR on proviral DNA of the gag region in the tissue explants and migratory cell+ T cell coculture. In cervical tissue, there was 7-fold reduced replication in the tissue when pre-treated with mannan and 4-fold increased replication in migratory cells +T cells. A similar trend was seen in penile tissue. These numbers seem promising in terms of the impact C-type lectins may have on transmission. In theory, if a 7-fold difference in

replication can be attributed to the trapping ability of lectins in tissue, this could represent a significant bottleneck that is present at the site of the mucosa during a transmission event. This experiment preliminarily outlines the importance C-type lectins may have in selecting for the TF virus *in vivo*. This experiment may be further implicated if NGS was performed on the mannan and untreated tissue experiments. In this way, it could be determined which viruses were primarily being 'trapped' by C-type lectins over others. If the effect of C-type lectin trapping on chronic, low transmission viruses was greater than TF viruses, it would implicate that C-type lectins may be a significant reason why the chronic virus glycosylation pattern is the reason it does not transmit, and why TF glycosylation patterns allow them to transmit.

To repeat the experiments that were performed by Dr. Eric Arts in penile tissue, they were repeated in cervical tissue in three donors using the high transmission fitness acute B4 virus and the low transmission fitness Q0 virus. Following these competitions in cervical tissue, the PCR efficiency ranged from as low as 40% to as high as 80% depending on the experiment. The majority of the experiments fell in the lower end of this range. The NGS results in which B4 and Q0 viruses were added in equal infectious units based on RT activity were inconclusive. Both viruses appeared to have the majority of their replication in the tissue, with very little virus being transferred to T cells by the migratory cells. Several factors could have led to these results. One being that the point where migratory cell+virus was washed and transferred to cultured PM1 CD4 T cells results in a loss of cells just by nature of the process. These experiments may have allowed B4 and Q0 viruses to infect tissue, but the resulting T cells could not be infected due to the low amount of total virus being added to them. Further and more likely the cause of the fact that the high transmission fitness B4 virus actually seemed to be trapped more than the low transmission fitness Q0 virus, is likely due to the fact that too little

total virus was added to the overall tissue experiment. The previous experiments involved 5-10 viruses being added to the same 3mmx3mmx3mm tissue pieces performed by Dr Eric Arts. This meant that these viruses being added at the same MOI resulted in the tissue being infected by 5 times less total virus based on RT activity. I postulate that in order for a representative transmission event to occur, it may be a requirement that there is a certain amount of virus to saturate the host immune mechanisms such as C-type lectins to allow for the higher transmission fitness TF viruses to be selected for. In other words, if for instance a piece of mucosal tissue contains a certain amount of C-type lectins that bind with high affinity to the gp120 envelope, and you infect this tissue with fewer viral particles than there are C-type lectins present, it is likely that even viruses with a glycosylation profile favourable for transmission will get 'trapped' due to the ratio of defense mechanisms to virus. However, if you added 5 times the amount of virus to C-type lectins in a given mucosal tissue piece, the viruses with low transmission fitness and perhaps a higher glycosylation of their envelope, will now be more preferentially trapped and saturate all of the defense mechanisms present, allowing the high transmission fitness TF viruses to be selected for and traverse the mucosa. In the future, these experiments need to be repeated to confirm or deny this hypothesis, as to my knowledge, there is no evidence suggesting this to be necessary for transmission to occur.

In order to determine if the Q0 viral envelope glycosylation binds to human C-type lectins with greater affinity than the B4 viral envelope glycosylation, surface plasmon resonance was used with his-tagged C-type lectin MBL bound to an NTA coated sensor chip. Prior to using samples, the protocol needed to be optimized using human recombinant CN54 gp140 to the same lectin. Briefly, 3 replicates of 75ng/mL and

50ng/mL were injected and flown over human recombinant MBL that had saturated the NTA coated sensor chip. Binding affinity curves were generated on the TraceDrawer software by Nicoya lifesciences. Specifically, measuring binding affinity and removing previous samples was optimized until successive samples at the same concentration yielded approximately equal binding curves. Future experiments may now be performed with the optimized conditions to determine the binding affinities of B4 and Q0 viruses to MBL as well as other lectins found in the mucosa such as DC-SIGN and Dectin-1.

Overall, this thesis lays the groundwork for a bottom-up approach to determining the best glycosylation profile for transmission. Several glycan modified viruses as well as a region-swapped virus have been generated and can now be used in transmission fitness experiments to see how specific regions as well as global glycosylation differences affect transmission. From that point, mass spectrometry can be performed on the envelopes of high transmission fitness variants to eventually lead us toward an overall glycosylation profile which is selected for in a transmission event. Once a ‘best’ TF glycosylation pattern has been determined, a prophylactic vaccine targeting specific motifs on the TF may be used as a potential therapy. Further, knowledge of what is selected for during a transmission event can lead to other avenues of research in other enveloped viruses as well as HIV, in order to know how virus’ have evolved to transmit more efficiently.

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