

**To compare the expression, processing, incorporation  
and function of pseudoviruses and infectious  
molecular clones using different cell types and HIV  
backbones**

by

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## Declaration

I, Bianca Abrahams, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I have used the referencing style from the journal Nature. Each significant contribution to, and quotation in, this dissertation from the work(s) of other people has been attributed, cited and referenced.

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Signed by candidate

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## Abstract

Understanding HIV transmission mechanisms is essential for the design and development of an efficacious, broadly acting vaccine that targets features common to transmitted viruses. However, there is a lack of consensus amongst current HIV studies characterising transmitted founders (TFs). When investigating the methods employed across studies, it becomes clear that methodologies are highly variable and thus, could be impacting research outcomes. This study therefore aimed to determine whether Envelope (Env) expression and processing affects function and whether cell type and/or expression system were responsible for these differences. Our data suggest that even though we did not observe differential expression of recombinant Env clones across cell types, when pseudovirus and infectious molecular clone (IMC) backbones were introduced, expression of Env decreased. We also found differences in processing in the form of cleavage, *N*-glycosylation and incorporation of Env across cell types. We conclude from this that methods used to study Env characteristics are highly sensitive to cell type and HIV backbone which suggests that a more standardised system is required to make meaningful comparisons between studies. The results of our functional Env analysis revealed high variation depending on the methodology used. We found that entry of TZM-bl cells by pseudovirus (PSV) is dependent on the cell line used to produce the viral particles. Unfortunately, due to low IMC titre, we had to expand the virus in PBMCs, negating the effect that cell type might have had on IMC expression. We could thus not directly compare PSVs to IMCs. However, PSV and IMC entry as well as IMC replication in PBMCs suggested that CHO cells were not suitable for robust viral production and better suited for recombinant Env expression. Overall, the findings in this project support previous findings that PSVs and IMCs are not directly comparable due to multiple factors that influence Env expression and virus production. We suggest that researchers who focus on HIV functional analysis, particularly Env, with the end-point of informing vaccine design, need regulated methods across laboratories, similar to the way that neutralisation assays were standardised.

## List of abbreviations

<b>Full Name</b>	<b>Abbreviation</b>
50 % tissue culture infective dose	TCID50
5-fluoro-1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid	FOA
Approximately	~
Base pairs	bp
Broadly neutralising antibodies	bnAbs
Calcium	Ca
Carbon dioxide	CO <sub>2</sub>
C-C chemokine receptor type 5 CD4/8 Cluster of differentiation 4/8	CCR5
Chinese hamster ovary	CHO
Chronic control	CC
Circulating recombinant forms	CRFs
Cluster of differentiation 4	CD4
Colony forming units	cfu
C-X-C chemokine receptor type 4	CXCR4
Cytomegalovirus	CMV
Cytotoxic T-lymphocyte	CTL
Degrees Celsius	°C
Denritic cell specific ICAM3-grabbing non-integrin	DC-SIGN
Denritic cells	DCs
Deoxynucleoside triphosphate	dNTP
Deoxyribonucleic acid	DNA
Dimethyl sulfoxide	DMSO

Distilled water	dH <sub>2</sub> O
Dulbecco's modified eagle medium	DMEM
Endoglycosidase H	Endo H
Endoplasmic reticulum	ER
Envelope	Env
Envelope signal peptide	Env-SP
Enzyme-linked immunosorbent assay	ELISA
Escherischia coli	E. coli
Ethylenediaminetetraacetic acid	EDTA
Female genital tract	FGT
Fetal bovine serum	FBS
Gut-associated lymphoid tissue	GALT
Henrietta Lacks	HeLa
Human embryonic kidney 293T	HEK293T
Human immunodeficiency virus	HIV
Infectious molecular clone	IMC
Interleukin-2	IL-2
Kilobases	kb
Kilodalton	kDa
Litres	L
Long-terminal repeat	LTR
Luria agar	LA
Luria broth	LB
Magnesium	Mg
Maraviroc	MVC

Membrane proximal region	MPER
Micrograms	μg
Microliters	μl
Micromolar	μM
Milliamps	mA
Millilitres	ml
Molar	M
Molecular weight	MW
Monoclonal antibodies	mAbs
N-acetyl glucosamine	GlcNAc
Nanograms	ng
Nanometer	nm
Neutralising antibodies	nAbs
Optical density	OD
Peptide: <i>N</i> -glycosidase F	PNGase F
Peripheral blood mononuclear cells	PBMCs
Phenylmethylsulfonyl fluoride	PMSF
Phosphate buffered saline	PBS
Phytohaemagglutinin-P	PHA-P
Picograms	pg
Polymerase chain reaction	PCR
Polyvinylidene difluoride	PVDF
Potential <i>N</i> -linked glycosylation site	PNGS
Preintegration complex	PIC
Pseudovirus	PSV

Recombinant forms	RFs
Relative light units	RLU
Revolutions per minute	rpm
Roswell park memorial institute	RPMI
Saccharomyces cerevisiae	<i>S. cerevisiae</i>
Simian immunodeficiency virus	SIV
Single genome amplification	SGA
Sodium bicarbonate	NaHCO <sub>3</sub>
Sodium chloride	NaCl
Sodium dodecyl sulfate	SDS
Transmitted founder	TF
Tris buffered saline	TBS
Tris EDTA	TE
Units	U
Virus-like particles	VLPs
Yeast extract peptone dextrose	YEPD

# Chapter 1

## Literature review

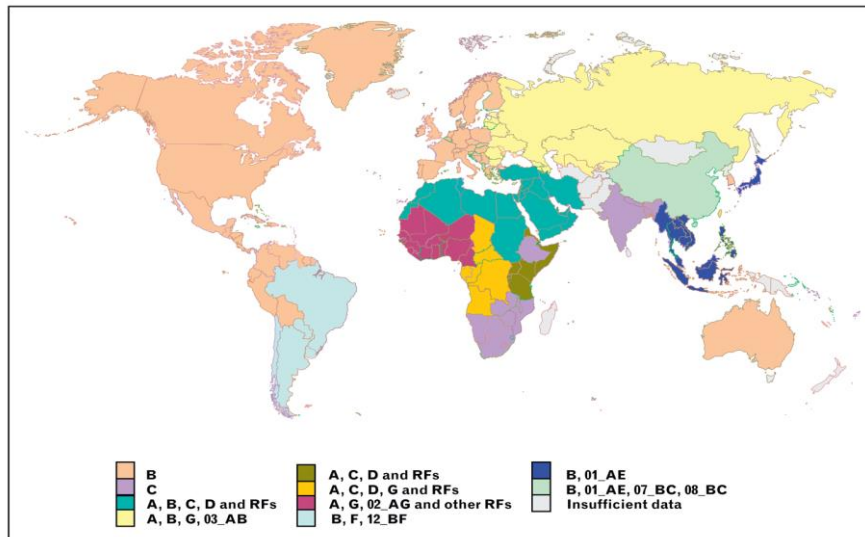
### 1.1 Introduction

The Envelope (Env) of HIV-1 is first synthesized as a glycoprotein, gp160 which is then cleaved into gp120 and gp41<sup>8</sup>. The two glycoproteins associate and form a trimer of gp120-gp41 heterodimers<sup>9</sup>. The gp120 subunit binds sequentially to CD4 and CCR5 receptors on the surface of T cells while the membrane-bound gp41 mediates fusion between the viral and host membranes enabling viral entry and replication in the host cell<sup>8</sup>. As this step mediates the first contact with host cells, inhibition of Env-host interactions would prevent transmission and is thus an important target in the development of novel drugs and vaccines against HIV.

To understand the mechanism of HIV transmission, PSVs or IMCs are generated to mimic *in vivo* HIV-1 infection. However, previous studies on HIV-1 transmission have reported conflicting results, which could, in part, be due to differences between PSV and IMC methodologies<sup>10-12</sup>. This review aims to discuss studies that have utilised PSV and IMC assay systems, within the context of HIV-1 transmission, to evaluate the relevance of these findings to vaccine development.

### 1.2 HIV diversity

High HIV diversity is a major challenge to the development of an effective vaccine noting that Env from different subtypes can vary by 30 %<sup>13</sup>. HIV is grouped into two genetically distinct types, type 1 (HIV-1) and type 2 (HIV-2)<sup>14</sup>, with the former subdivided into four groups (M, major; O, outlier; N, non-M, non-O; and P) and Group M further divided into 9 subtypes (A1, A2, B, C, D, F1, G, H, J, and K) plus 43 circulating recombinant forms (CRFs) (see Los Alamos National Lab (LANL)-database; <http://www.hiv.lanl.gov>). CRFs are recombinant viruses that arise due to co-infection of two or more subtypes and circulate within a certain region. Unique recombinant viruses on the other hand have unique breakpoints and are poorly transmitted in the population<sup>15,16</sup>. HIV-1 subtype B is the dominant variant in North America, Western Europe, and Australia, whereas subtype C viruses are found mainly in Southern Africa and India. Subtype C accounts for more than half of all HIV infections worldwide<sup>1,17</sup> (Figure 1.1), emphasising the need for further research on this subtype as a vaccine raised against subtype B might not protect against other subtypes.

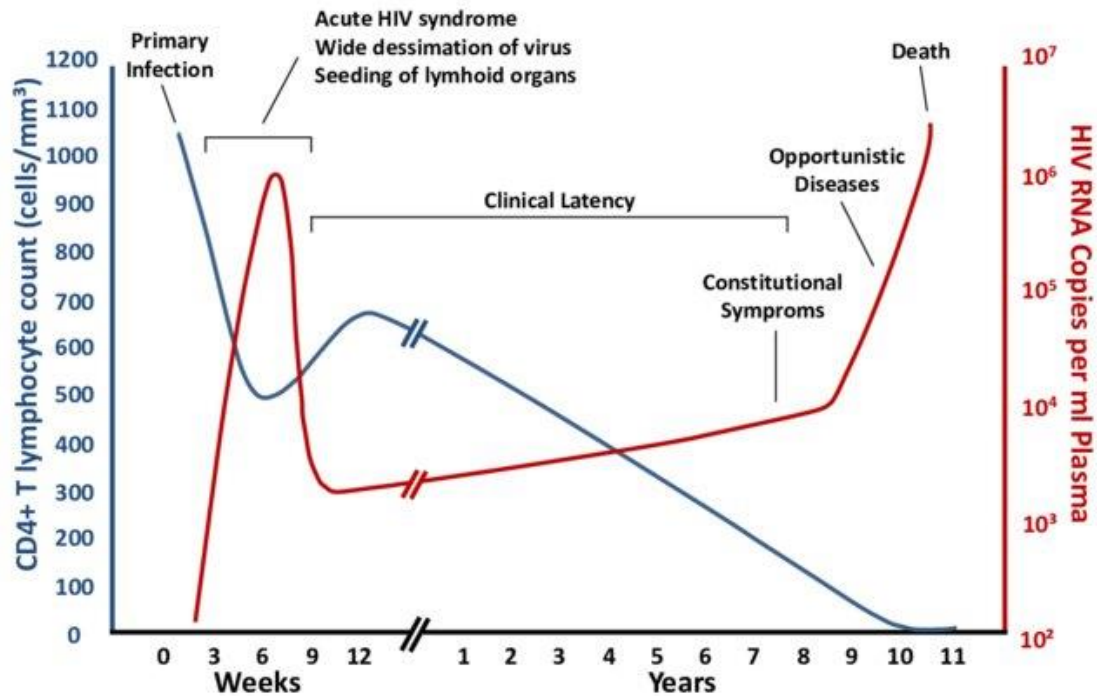


Modified and updated from Hemelaar *et al.* [6]. 03\_AB=CRF03\_AB; 12\_BF=CRF12\_BF; 01\_AE=CRF01\_AE; 07\_BC=CRF07\_BC; 08\_BC=CRF08\_BC; RF=unique recombinant form.

**Figure 1.1: HIV-1 subtypes and recombinants forms (RFs) circulate within specific regions globally <sup>1,2</sup>.**

### 1.3 HIV-1 immune response

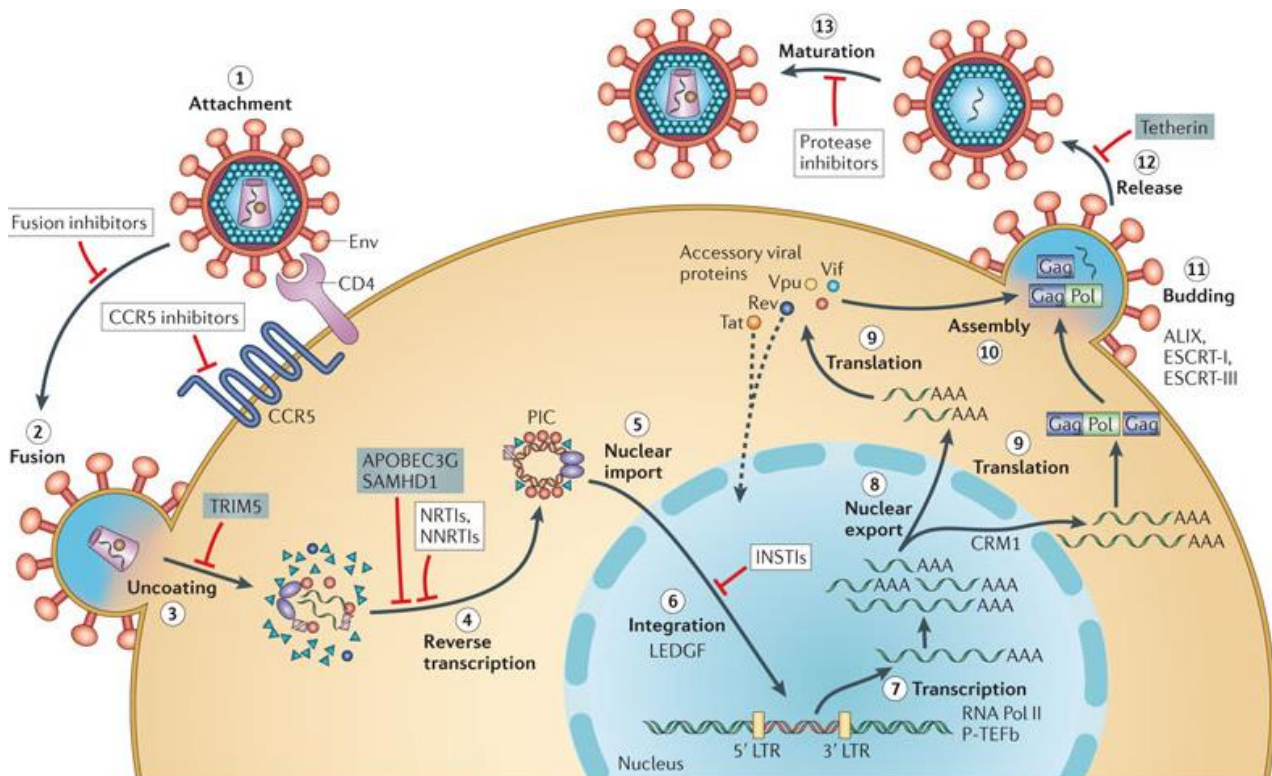
HIV-1 infection can be divided into three stages – acute, early and chronic (Figure 1.2) <sup>4</sup>. During early stages of infection, neutralisation antibodies to HIV-1 are not produced, leading to chronic infection <sup>11</sup>. HIV-1 is known to elicit humoral and cytotoxic T-lymphocyte (CTL) immune responses. A robust CTL response is essential to control viral infections <sup>18</sup>. In the case of HIV-1, long-term non-progressors have higher amounts of HIV-1-specific CTL precursor cells compared to those who have progressed to AIDS <sup>18</sup>. The increased level of CTL response is correlated with a decrease in plasma viremia. These results indicate that CTL response to HIV-1 infection is essential for controlling virus replication and is therefore a potentially important aspect for vaccine development <sup>19,20</sup>. Acute and early HIV-1 infection is defined by the presence of specific viral markers and antibodies in the blood <sup>21</sup>. The first antibody response to HIV-1 infection is a non-neutralising response and the first neutralising response can only be detected three months after infection <sup>21</sup>. Acute HIV-1 infection leads to the reduction of CD4<sup>+</sup> T lymphocytes in lymphoid tissues, which cannot be replaced once destroyed. This is a possible explanation for the weak CD4<sup>+</sup> T cell response to acute infection <sup>21</sup>. Broadly neutralising antibodies (bnAbs) could be the key to an effective vaccine against HIV-1 <sup>22,23</sup> but the generation thereof has proven to be very complex. The high variation of Env present in the early infection virus population is likely the result of immune pressure exerted by nAbs (neutralising antibodies) <sup>24</sup>. The pressure exerted by nAbs, which target Env, results in escape variants <sup>24</sup>. A study by Wei, et al. <sup>25</sup>, showed that escape variants presented with mutations that were linked to changes in *N*-glycosylation sites. Studying immune responses to HIV is of great importance as these studies will inform vaccine design.



**Figure 1.2:** Changes in viral load and CD4<sup>+</sup> T cell counts over the course of HIV infection. HIV infection is characterised by an increase in viral load (red) during acute infection, with a decrease in CD4<sup>+</sup> T cell count (blue). The immune responses then reduce viral loads until viral setpoint and clinical latency which can last for many years before the onset of AIDS-related infections (reproduced from <sup>4</sup>).

#### 1.4 HIV-1 replication

HIV-1 infection begins with the attachment of Env to the CD4 receptor and co-receptors on the cell surface of helper-T cells, followed by fusion between viral and host cell membranes (Figure 1.3). Upon entry into the cell, the viral RNA and the content of the viral core are released into the host cell (Figure 1.3). This is followed by reverse transcription of the viral RNA into a double-stranded DNA molecule by the enzyme, reverse transcriptase. This DNA molecule is transported to the nucleus where the viral DNA is integrated into the host's genome. Upon activation of the LTR promoter, other viral proteins – Gag, Nef, Tat and Rev are produced <sup>8</sup>. Once the viral proteins required for new virions are made, viral particles containing all of these necessary proteins are formed and the particles bud off from the host cell after acquiring new Env proteins (Figure 1.3). The Env proteins on the surface of the new viral particles are free to attach to other CD4<sup>+</sup> cells to begin a new round of replication.

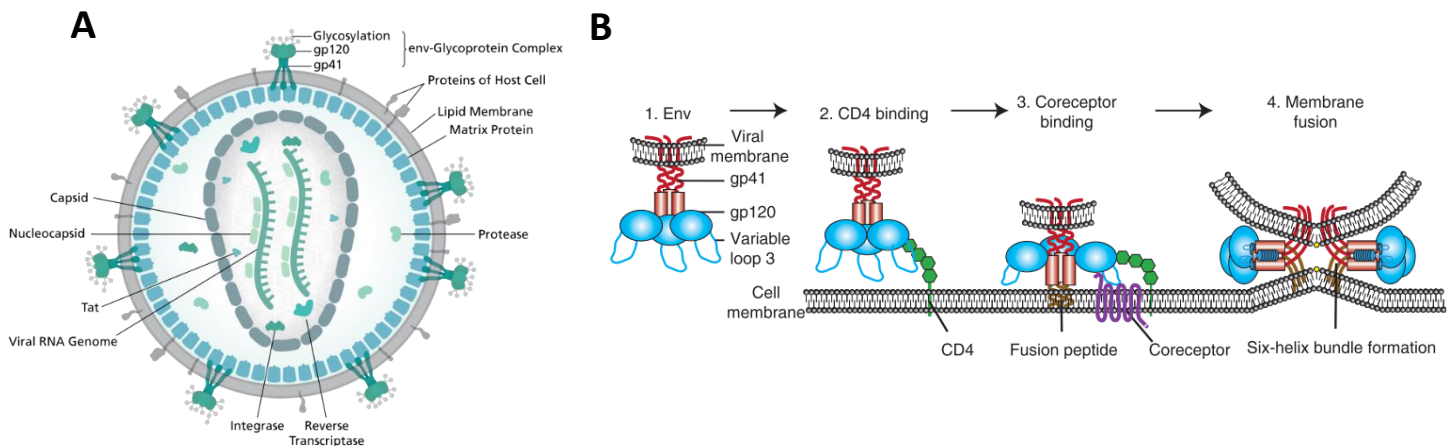


**Figure 1.3: Overview of the replication cycle of HIV-1.** 1) Contact and attachment of the HIV-1 virion with the host cell CD4 receptor. 2) Fusion of the viral and host cell membranes. 3) Release of the viral RNA and viral core into the host cell. 4) Reverse transcription of the viral RNA into viral DNA. 5) Formation of the preintegration complex (PIC) and import into the nucleus. 6) Integration of the PIC into the host DNA. 7) Transcription into the viral mRNA. 8) Export of the viral mRNA into the cytoplasm. 9) Translation into viral proteins. 10) Assembly of proteins into the viral genome. 11) Budding of the immature virion from surface of the host cell membrane. 12) Release of the immature viral particle. 13) Maturation of the viral particle. (6)

## 1.5 Envelope processing and function

The production of functional Env proteins is reliant on the correct processing thereof, which consists of synthesis, trimerization, *N*-glycosylation and cleavage. Moreover, the interactions between gp120 and gp41 with host and viral proteins are vital for proper functioning and processing. For example, the interaction between Gag and the cytoplasmic tail of gp41 is crucial for incorporation of Env as it mediates the formation of Gag-Env particles<sup>26</sup>. *N*-glycosylation of Env is a crucial step of processing in which *N*-linked glycans are enzymatically added to the protein at specific amino acid motifs, called potential *N*-glycosylation sites (PNGS)<sup>27,28</sup>. The addition of these glycans affect structure and can affect function of Env. Expression of Env also influences *N*-glycosylation as Bonomelli, et al.<sup>29</sup> showed that when Env is overexpressed, the *N*-glycosylation profile is altered. Another important step in Env processing is the cleavage of gp160 into gp120 and gp41. Cleavage is essential for Env functionality as only incorporated gp120-gp41 trimers allow for viral infection. The incorporation of uncleaved gp160 into viral particles will render the virus non-infectious<sup>30</sup>. The effect of processing, specifically cleavage and *N*-glycosylation, on Env function will be investigated in detail in this study.

### 1.5.1 Structure



**Figure 1.4:** A) Schematic diagram of the HIV-1 virion structure. Figure taken from: [www.scistyle.com](http://www.scistyle.com). B) Process of Env binding to CD4 receptor and co-receptors followed by membrane fusion and formation of a six-helix bundle <sup>3</sup>.

The glycoproteins gp120 and gp41 are non-covalently associated and form trimers of heterodimers <sup>9</sup> on the surface of HIV-1 particles (Figure 1.4 A). The gp120 surface subunit is made up of variable (V1-V5) and constant (C1-C5) regions demarcated by disulphide bonds <sup>31,32</sup>. The C1 and C5 regions contain residues essential for gp41 binding while C4 has been implicated in membrane fusion <sup>33</sup>. The V1-V5 regions contain conserved PNGS that aid in shielding Env from immune recognition although V2 and V3 are also known targets for nAbs <sup>33 34</sup>. V2 and V3 are important for membrane fusion and for determining HIV-1 tropism <sup>33</sup>.

Gp41 is comprised of a cytoplasmic tail, transmembrane region and a highly conserved hydrophobic membrane proximal external region, known as the MPER. The MPER is believed to be involved in trimer self-assembly, <sup>35</sup> whereas the C-terminal tail is essential for anchorage into the viral lipid membrane <sup>35</sup>. Truncation of the C-terminus leads to a soluble form of the glycoprotein, termed gp140, due to its approximate molecular weight of 140 kDa <sup>36,37</sup>.

### 1.5.2 Function

The gp120 subunit is responsible for binding to the CD4 receptor and CCR5 co-receptors on the surface of macrophages and helper T cells <sup>38</sup> and to CD4 and CXCR4 on the surface of T cells <sup>39</sup> while gp41 mediates the fusion of the viral membrane with the host cell membrane <sup>40</sup>. Upon gp120 binding to CD4, a conformational change is induced which allows binding to co-receptors CCR5 or CXCR4 <sup>41</sup>. Binding to co-receptors brings about further conformational changes which ultimately result in the exposure of the hydrophobic fusion peptide of gp41 <sup>42</sup>. This then leads to further conformational changes which allow gp41 to form a six-helix bundle (Figure 1.4 B) that fuses the viral and host cell membranes together <sup>42</sup> for delivery of the vRNA to the host cell <sup>40</sup>.

Gp41 has also been shown to play a role in the formation of syncytia <sup>43</sup>. Initially viruses were designated as M-tropic and T-tropic depending on whether they infected macrophages or T cells, respectively <sup>44</sup>. However, subsequent studies identified viruses as R5- and X4-tropic based on receptor binding and M- or T-tropic depending on the cell type infected <sup>45</sup>. Transmitted founders (TFs) have been identified as R5-tropic and T-tropic as they bind to the CCR5 co-receptors on T cells <sup>45</sup>. Given that gp120 and gp41 mediate the first interaction between the virus and the host cell, Env is thus a crucial element in HIV-1 transmission, infection and vaccine design.

### **1.5.3 Cleavage**

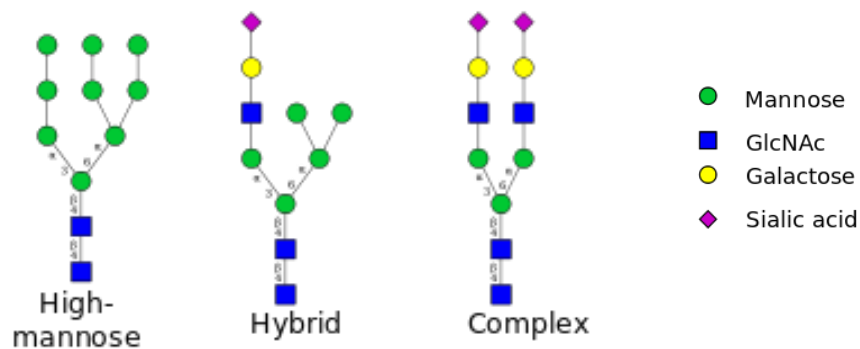
Like other retroviruses, the Env protein is synthesized as a polyprotein which is then proteolytically processed into the mature glycoprotein <sup>46</sup>. Cleavage is carried out at a conserved tetrapeptide sequence, Arg-X-Arg/Lys-Arg, by host cell proteases <sup>30,46</sup>. There are two potential cleavage sites located directly upstream of the gp41 *N*-terminus. When either of these cleavage sites were mutated on their own, cleavage still took place at the intact site but when both were mutated together, cleavage was completely abrogated <sup>30</sup>. The uncleaved mutants were still transported efficiently to the cell surface however, the viruses containing these proteins were non-infectious <sup>30</sup>. Although the viruses containing mutations at only one of the cleavage sites were still cleaved, they were inefficiently incorporated, thereby demonstrating that these two sites are crucial for generating fully infectious viruses <sup>30</sup>.

The cleavage of gp160 into gp120 and gp41 is essential for their respective functions. *N*-glycosylation of Env has also been shown to affect cleavage of the polyprotein into its subunits <sup>47</sup>. Dash, et al. <sup>47</sup> showed that when certain PNGS are mutated cleavage does not occur, resulting in non-functional Env proteins. Taken together, these results imply that incorrect processing of Env results in non-infectious virus particles. Incomplete processing of Env can have various effects on the resulting virus and consequently on immune responses. Crooks, et al. <sup>48</sup> found that HIV-1 virus-like particles (VLPs) carried a monomeric, uncleaved form of Env that appeared to bypass the normal protein maturation steps <sup>48</sup>. Not only was the Env uncleaved and not trimerized, the *N*-glycosylation profile was also altered leading to changes in antibody recognition <sup>48</sup>.

### **1.5.4 N-glycosylation**

Gp120 and gp41 are both highly *N*-glycosylated, have highly variable glycosylation patterns, with *N*-glycans making up roughly half of the total mass of gp120. Gp120 has a variable number and positioning of PNGS <sup>28</sup>, while gp41 is less extensively glycosylated with most PNGS conserved across isolates <sup>49</sup>. The extensive *N*-glycosylation of Env shields it from immune recognition however, it is not

yet understood how the variability in glycosylation affects antibody reactivity <sup>28,50</sup>. Contrary to shielding Env, some *N*-glycans provide epitopes for bnAbs <sup>22,50-52</sup>, further emphasising the importance of *N*-glycosylation patterns. Due to differences in *N*-glycosylation machinery, cell types differentially *N*-glycosylate Env <sup>28</sup> so that expression in one cell line can drastically affect *N*-glycan patterns and thus immunogenicity, posing a huge problem for vaccine design.



**Figure 1.5: Three major types of *N*-glycans.** Envelope *N*-glycans can comprise of High mannose, hybrid and complex carbohydrates. Figure taken from: [www.scistyle.com](http://www.scistyle.com).

In the endoplasmic reticulum (ER) *N*-linked glycans are attached co-translationally to the protein at specific amino acid motifs, N-X-S/T, called PNGS. *N*-glycans have a common trimannosyl core structure which is composed of pentasaccharides. They can further be classified into three different types based on their structure. The three types are referred to as high mannose, complex or hybrid (Figure 1.5). High mannose *N*-glycans have only mannose bound to the core, the complex type has more than two *N*-acetyl glucosamine residues (GlcNAc) with variable numbers of galactose and sialic acids and the hybrid form is a mixture of the complex and high mannose types <sup>27</sup>. These structures are highly variable and their synthesis has been shown to be cell-specific <sup>28</sup>.

After mannosylation, gp160 trimerises and *N*-glycans are further modified to complex or hybrid carbohydrate structures in the Golgi, before cleavage into gp120 and gp41 and the incorporation of functional trimers into viral particles <sup>52</sup>.

It has been suggested that there is rapid turnover of Env at the plasma membrane due to endocytosis and shedding of gp120. Therefore, it has been suggested that approximately ten Env trimers are incorporated per virus <sup>53</sup>. Increased expression of Env did not lead to increased infectivity, and it was thus hypothesised that low numbers of Env on the virion surface was an advantage to the virus as it limited recognition by the immune responses <sup>54</sup>.

#### 1.5.4.1 *N*-glycosylation in various cell types

Although PNGS are determined by the viral genome, the biosynthetic machinery of the host cell is responsible for the addition and composition of *N*-glycans. Therefore, *N*-glycan profiles are highly variable between isolates with some PNGS being unoccupied <sup>28</sup>. When recombinant gp120 was produced in CHO and Dakiki cells, the molecular mass was revealed to be approximately 125 kDa while in HepG2 cells the mass was approximately 145 kDa. This difference could be explained by the differential ratio of high mannose relative to complex *N*-glycans. Gp120 produced by T cells and CHO cells were more heavily mannosylated than gp120 produced in HepG2 cells which had high levels of heterogeneous complex *N*-glycans <sup>28</sup>.

Recombinant subtype C TF gp120 produced in Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293T) cells differed in that the former cell line produced more sialylated, fucosylated and phosphorylated *N*-glycans. There were also differences at specific PNGS where CHO cells added high mannose while HEK293T cells added more complex residues and vice versa <sup>55</sup>. Interestingly the gp140 construct of the same TF Env used in the 2013 study had a similar *N*-glycosylation profile as the gp120 monomers analysed in 2011, suggesting that gp140 and gp120 *N*-glycosylation might be similar irrespective of the presence of additional gp41 sequence <sup>56</sup>. Glycosylation of Env is essential to its folding in the ER, suggesting that expression in different cells will produce gp120 with variable function and structure.

#### 1.6 HIV-1 transmission

Despite the genetic variation, and most likely phenotypic variation of the donor virus population, transmission and clinical infection of HIV-1 is as a result of a single variant, deemed the TF virus <sup>57 58</sup>. If a single variant is usually selected, that TF virus is likely to carry a selective advantage over the rest of the variants <sup>59</sup>. The high mutation rate of HIV-1 coupled with the selection pressure from the host's immune system results in rapid evolution so that the viruses at chronic stages of infection are no longer similar to the TF. Characterisation of variants sampled at late stages of infection will thus, most likely, not represent the founder population. Single genome amplification (SGA) enabled the identification of the TF and a number of studies have focused on understanding how the phenotype of the TF contributes to HIV transmission <sup>57,60</sup>. The research community rationalised that a TF-specific phenotype or transmission motif(s) would make ideal targets for vaccine design as these would be functionally constrained and thus conserved across the transmitted population.

## **1.6.1 HIV-1 Transmitted founder selection mechanisms**

### **1.6.1.1 R5 tropism**

Thus far, the only common feature of all TFs is that they are all R5-tropic which suggests that either some barrier prevents transmission via CXCR4 or that CCR5 receptors possess some selective advantage over CXCR4<sup>61</sup>. This is evident in studies showing that those failing to express a functional copy of CCR5 are generally protected from HIV-1 infection even when a functional CXCR4 is present, thus demonstrating a strong positive selection towards CCR5. Deciphering the mechanism acting to prevent infection via CXCR4 or the mechanism acting to promote infection of CCR5+ cells is important for vaccine development<sup>62</sup>.

The V3 loop that interacts with CXCR4 has a higher level of exposed cationic charge compared to R5-tropic gp120 which could lead to more interaction between CXCR4 and mucin and thus, more efficient clearance and impairment of infectivity<sup>61</sup>. Furthermore, in the lower female genital tract (FGT), levels of CCR5 mRNA are up to ten fold higher than levels of CXCR4 suggesting that cells expressing CCR5 are more common in the FGT than those carrying CXCR4 cells<sup>61</sup>. However, McClure, et al.<sup>63</sup> found conflicting evidence as FACS analysis showed that CXCR4 was increased on the cell surface in the FGT<sup>63</sup>. R5-tropic variants might have an advantage during penile transmission, because although the levels of CCR5 and CXCR4 are equivalent in the urethral tissue, Langerhans cells and CD4<sup>+</sup> T cells express more CCR5. Another study reported that X4 viruses may be more sensitive to inactivation by proteins found in the vaginal mucus<sup>64</sup>, however, this was refuted by Sun, et al.<sup>65</sup> who found that there was no difference in sensitivity of X4 and R5 variants to inactivation.

Parker, et al.<sup>66</sup> reported that subtype B and C chronic control (CC) Envs were better able to utilise a maraviroc (MVC) (CCR5 antagonist)-bound CCR5, while Wilen, et al.<sup>59</sup> found no difference. The findings by Parker, et al.<sup>66</sup> suggest that CC Envs are more likely to infect cells in the presence of MVC which in turn implies that CC Envs are selected against during transmission, but selected for during chronic infection, due to their ability to utilise different conformations of CCR5. How this will provide a selective advantage during transmission is still unclear.

Given that one single natural barrier cannot fully explain the strong selection of R5 tropic TFs, it is therefore possible that a combination of mechanisms act in concert to favour R5-tropic variants during transmission<sup>62</sup>.

### **1.6.1.2 N-glycosylation in transmission**

Derdeyn, et al.<sup>67</sup>, and Chohan, et al.<sup>68</sup> found that subtype C TFs tended to have shorter variable loops with fewer PNGS<sup>68,69</sup>. The authors suggested TF Env were less N-glycosylated and that this might

provide a selective advantage. In contrast, Frost, et al. <sup>70</sup> investigated the variable loop length and number of PNGS in subtype B variants and found no difference between TF and Envs from variants during chronic stages of infection <sup>70</sup>. It was suggested that subtype B and C TFs might have evolved along alternative paths resulting in different transmission motifs. When Go, et al. <sup>56</sup> compared the *N*-glycosylation of HEK293T cell-derived recombinant TF and CC Envs from subtype C and subtype B, they found that the two TF Env's *N*-glycosylation was more similar to each other than to the Envs from chronic stages of infection with the most marked difference being the abundance of complex sugars <sup>56</sup>. This suggests that there is a difference *N*-glycosylation between TF and CC Envs, although the reason remains unknown.

#### **1.6.1.3 Trans-infection of HIV-1 by dendritic cells**

Although a controversial topic, it has been suggested that dendritic cells (DCs) are potentially involved in the selection of TFs based on their Env *N*-glycosylation. It has been shown that DCs are responsible for the *trans*-infection of T cells during HIV transmission where DCs capture HIV in the FGT, migrate to the lymphoid nodes where CD4<sup>+</sup> T cells become infected, fuelling rapid HIV replication. DCs express a C-type lectin called DC-SIGN (dendritic cell specific ICAM3-grabbing non-integrin) that binds to gp120 high mannose residues <sup>71</sup>. Endoglycosidase H (Endo H) treatment abrogated gp120 binding to DCs and DC-SIGN<sup>+</sup> cells <sup>72</sup>. Furthermore, by producing gp120 in different cell lines, it was demonstrated that DC-SIGN did not bind to macrophage-generated gp120 due to its high complex sugar glycosylation <sup>73</sup>. This suggests that Env *N*-glycosylation might be important for HIV transmission. <sup>74</sup>. Parrish, et al. <sup>75</sup> suggested that TF viruses were more susceptible to being captured by DC-SIGN and thus more easily transferred to CD4<sup>+</sup> T cells <sup>75</sup>. However, when investigating the susceptibility of pseudotyped TF viruses and CC viruses to being captured by DC-SIGN, Wilen, et al. <sup>59</sup> failed to find any significant differences (Table 1.1).

#### **1.6.1.4 The role of $\alpha_4\beta_7$ integrin in HIV transmission**

Dissemination of the virus into the gut-associated lymphoid tissue (GALT) results in rapid depletion of CD4<sup>+</sup> cells, a contributing factor to the loss of immune responses during progression to AIDS-defining illness. The integrin, alpha-4-beta-7 ( $\alpha_4\beta_7$ ), is highly expressed on CD4<sup>+</sup> T cells of the FGT <sup>76</sup> and gut that preferentially express the CCR5 co-receptor <sup>77</sup>. It has been suggested that  $\alpha_4\beta_7$  is responsible for the dissemination of HIV to the GALT by transporting infected T cells. The homing integrin binds to the V2 loop of gp120 via a binding motif and when the site was mutated the efficiency at which the virus replicated decreased <sup>78</sup>. The reactivity of  $\alpha_4\beta_7$  increased when PNGS were deleted in the V1-V2 loop and it was proposed that TF variants with fewer *N*-glycans in this region might bind  $\alpha_4\beta_7$  with higher affinity and thus have an advantage that facilitates their transmission <sup>79</sup>. CD4, CCR5 and  $\alpha_4\beta_7$  form

complexes on the surface of T cells <sup>77</sup> and therefore, binding to  $\alpha_4\beta_7$  might promote interactions with CD4 and CCR5, enhancing HIV infection of host cells <sup>78</sup>. More recently it was shown that gp120- $\alpha_4\beta_7$  interaction is involved in synapse formation and cell-cell transfer of HIV <sup>78</sup>. Parrish, et al. <sup>80</sup> however, reported that blocking the  $\alpha_4\beta_7$  receptor with Act-1 (an mAb against  $\alpha_4\beta_7$ ) had no effect on viral infectivity. The same research group also showed that TFs did not bind  $\alpha_4\beta_7$ , CD4 or CCR5 better than variants from chronic stages of infection, suggesting that the interaction between gp120 and  $\alpha_4\beta_7$  was not essential for transmission <sup>80</sup>. Therefore, it has yet to be proven that  $\alpha_4\beta_7$  is involved in HIV transmission due to Env *N*-glycans.

#### **1.6.1.5 Neutralisation sensitivity**

One early study suggested that variants circulating soon after transmission would be more sensitive to neutralisation <sup>69</sup>. The premise of their hypothesis was that escape from neutralisation during chronic infection came with a fitness cost and thus, most variants at later time points had low infectivity. Therefore, the variants transmitted had not escaped immune responses and had high viral fitness. In support of this suggestion, Wilen, et al. <sup>59</sup> found that the clade B TF Envs exhibited increased sensitivity to mAbs b12 and VRC01 and clade B HIV Ig. The increased sensitivity was not seen with bnAbs PG9 and PG16 that recognise Env *N*-glycans. Go, et al. <sup>55</sup> showed that TF gp120, despite having differences in *N*-glycans, did not differ in antigenicity when treated with a range of antibodies: C1 (A32), conformational V2 (697D, 2158), CD4 binding site (VRC01), and the carbohydrate binding bnAb 2G12. Furthermore, PG9, PG16, and CH01 did not even bind to gp120 <sup>55</sup>. Studies, thus far, have not shown consistent findings on the antigenicity of TF Env which has direct implications for vaccine design.

#### **1.6.1.6 Transmission motifs**

When Env sequences representing TF and CC were compared, two transmission motifs were identified: His12 in the signal peptide and a PNGS at position 413 within gp120 <sup>81</sup>. Asmal, et al. <sup>82</sup> suggested that these motifs provided TFs with the advantage of increased Env processing and incorporation into HIV particles thus enhancing viral replication <sup>82</sup>. On the other hand, Wilen, et al. <sup>59</sup> suggested that the transmission of HIV-1 requires only a fit R5-tropic virus and that no single genetic or phenotypic characteristic enabled transmission. They concluded that any small changes in Env function that increased viral fitness could provide the necessary selective advantage for infection via CCR5 <sup>59</sup>. When Parrish, et al. <sup>83</sup> compared the characteristics of TF and CC Envs they suggested that TF viruses have higher levels of incorporated Env and are more infectious than CC viruses <sup>83</sup>. It is possible that any one of these factors - binding DC-SIGN, binding  $\alpha_4\beta_7$ , incorporation, antigenicity etc -might randomly provide an advantage to transmitted variants by enhancing viral fitness and that not all TFs

have the same advantageous phenotype. This might explain the lack of consensus between studies with regard to the selection mechanism of TFs. However, it is also possible that the lack of standardised methodology to study HIV transmission might have affected the outcome of the experiments, so we cannot compare one finding to another (Table 1.1).

**Table 1.1: Variation in study methodology that compared IMCs with PSVs.**

Paper	PSVs/IMCs	Env clone origin	Number of clones	HIV backbone	Host cell line
Parker et al.	PSVs and chimeric IMCs	Clade B/C	40	pNL43- $\square$ Env- vpr $\leq$ -luc $\leq$ or pNL43- $\square$ Env- vpr $\leq$ -eGFP	293T17, 293T-derived Affinofile, NP2/CD4/CCR5, NP2/CD4/CXCR4 & U87/CD4
Parrish et al.	Full-length IMCs	Clade B/C	TF; n=27 CC; n=14	NA	293T
Wilén et al.	PSVs	Clade B T/F Envs were selected from single-variant transmission. CC Envs amplified from 14-83 months post infection. Southeastern and Northwestern US.	TF; n=24 CC; n=17	pNL43- $\square$ Env- vpr $\leq$ -luc $\leq$ or pNL43- $\square$ Env- vpr $\leq$ -eGFP	293T17, NP2 cells

## 1.7 Methodology used to characterise transmitted founders

### 1.7.1 Cell lines

According to tables 1.1 and 1.2, a number of different cell lines have been used to investigate TF characteristics, although HEK293T cells and their derivatives are the most common. Vaccine production on the other hand, usually utilises CHO cells. The selection of cells will be affected by the expression of HIV restriction factors such as APOBEC3G, TRIM5 $\alpha$ , and tetherin<sup>84</sup>. PBMCs have been shown to express all these factors and replication of HIV is dependent on the expression of viral proteins that negate restriction<sup>85</sup>. These viral proteins include Vif, Nef, Vpu, and Vpr<sup>86</sup>. Vpu overcomes the effect of tetherin by sequestering the restriction factor in the trans golgi network. Cell lines are termed “restrictive or Vpu-responsive cells” if they require Vpu for HIV infection such as CHO, HeLa, T cells (Jurkat, CEM) and primary T lymphocytes. HEK293T, HOS and HR1080 cells on the other hand are termed “permissive or Vpu-unresponsive” as they do not produce tetherin<sup>87</sup>.

Although CHO and HeLa cells require the presence of Vpu to produce virus particles they are used extensively in HIV-1 research and we thus included them in this study.

### **1.7.2 Purified recombinant Envelope**

A number of studies have looked at *N*-glycosylation and antigenicity using purified recombinant gp120 or gp140<sup>55,56,78,88</sup>. This involves the over expression of recombinant proteins in cell lines, usually CHO and HEK293T cells, and purification using *N.galanthus* lectin affinity chromatography. Soluble forms of Env, gp140 and gp120, are used as these are secreted directly into the culture supernatant, circumventing the plasma membrane and making purification easier. It has been raised that monomeric Env does not represent the trimeric form and that using recombinant proteins might lead to results that do not represent native protein found *in vivo*<sup>89,90</sup>. This problem has been circumvented by the use of disulphide linked trimers<sup>89</sup>. Purifying Env trimers from PSVs, IMCs or primary isolates have a number of challenges, most notably the low levels of Env incorporation. Therefore, phenotypic characterisation of Env has utilised whole virus in the form of PSVs and IMCs and not purified protein. As most studies on TF phenotype have utilised PSVs and chimeric IMCs, this review will focus on these methods.

### **1.7.3 Pseudoviruses and infectious molecular clones**

PSVs and IMCs are two common systems used to investigate and characterise HIV. PSVs are produced by expressing the HIV genome, with a deletion in the Env gene, using an exogenous promoter such as that of the cytomegalovirus (CMV) to ensure high expression of all viral proteins or the LTR, to maintain physiological levels of expression. The *env* gene is cloned into an expression vector under the control of a constitutive promoter. When vectors are co-transfected into cell lines, PSVs are produced carrying the HIV-1 RNA genome without a functional *env*. These PSVs are replication-incompetent and are capable of only a single round of infection<sup>12</sup>. PSVs are commonly used to characterise and compare Env function as a common HIV genome can be pseudotyped with a number of different Env variants. A reporter cell line, such as TZM-bl cells, is then used to measure the ability of the Env clones to mediate efficient entry.

Infectious molecular clones are used to study viral replication. These clones carry the full proviral genome of HIV. Transcription is driven by the LTR which means that mRNA splicing and rate of production are the same as native virions<sup>12</sup>. Usually, Envs are cloned into a common backbone to generate chimeric IMCs in order to compare the impact of different Env clones on viral replication. Contrary to PSVs, IMCs are fully infectious. However, when Provine, et al.<sup>12</sup> compared Env cleavage

and incorporation between PSVs and IMCs, they found that IMCs exhibited significantly higher levels of cleavage and incorporation but showed no significant differences in neutralisation sensitivity.

#### **1.7.4 Comparison between PSV and IMC results**

In order to directly compare the results obtained from the PSV and IMC systems, Provine, et al. <sup>12</sup> utilised the 6 matched PSVs and chimeric IMCs. They observed four factors that are known to influence HIV-1 function and infection; cleavage of Env into gp120 and gp41, the amount of Env incorporated into the virion, viral infectivity and sensitivity to the inhibitors that target different steps in viral entry. As determined by p24 levels, PSVs and IMCs produced similar amounts of virions. By using the relative amounts of gp160 and gp120, Provine, et al. <sup>12</sup> calculated that there were significant differences in Env incorporation for 3 out of the 6 pairs tested, suggesting that the assays influence experimental outcome in a clone-specific manner. Across all 6 pairs, most showed less incorporation in PSVs. The gp160 and gp120 levels were also used to determine the percentage cleavage. This showed that IMCs always had a higher percentage gp160 cleavage than did PSVs <sup>12</sup>.

To determine whether these differences had any biological significance, each pair was assayed for neutralisation and infectivity. Differences were observed in infectivity in all 6 pairs. However, there was no consensus as to whether IMCs or PSVs were more infectious. Neither incorporation nor cleavage alone could explain the differences in infectivity <sup>12</sup>.

To test the sensitivity to neutralisation, two matched pairs with marked differences in cleavage, incorporation and infectivity were chosen. These pairs were exposed to antibodies and inhibitors that targeted different stages of viral fusion <sup>12</sup>. The results showed that neutralisation/inhibition sensitivity to mAbs was similar for both pairs. In another study conducted by Louder, et al. <sup>10</sup>, identical Env genes were inserted into both PSVs and IMCs. When compared, the PSVs showed similar neutralisation sensitivities to IMCs for a number of antibodies. However, after one passage in HEK293T cells, IMCs differed in neutralisation sensitivity in comparison to PSVs. This appears to be a host cell effect, as no genetic changes in Env were observed. Long-term culture of HIV-1 may cause genetic changes which can result in changes in neutralisation, incorporation and infectivity <sup>10</sup>. This has major implications on vaccination research.

In another study done by Miglietta, et al. <sup>11</sup>, three PSVs were compared to three TF IMCs with almost identical Env sequences (Table 2). The PSVs and IMCs were produced in HEK293T cells to eliminate the effect of host cell on neutralisation sensitivity. They also used the TZM-bl assay as a standardised method to eliminate any other sources of variability. Their results showed that PSVs were as sensitive

to neutralisation as IMCs but that IMCs had higher IC50s. The difference in IC50 could not be fully explained by Env sequence, nor by the use of PSVs versus IMCs. This result was corroborated by Provine, et al. <sup>12</sup>.

Any differences in neutralisation sensitivity may be explained by the method of Env expression which differs between PSVs and IMCs. In PSVs the Env gene is expressed on a separate plasmid, while in IMCs the full proviral genome is usually expressed by the LTR <sup>11</sup>. When using PSVs, it is possible that certain properties of the Env protein are altered due to Env being expressed by a separate plasmid. Another important factor that could influence neutralisation sensitivity is the Env:backbone ratio <sup>12</sup>. Varying this ratio resulted in PSVs with different levels of Env cleavage, as well as different levels of gp120 expressed on the cell surface. These alterations have been shown to affect the infectivity and possibly antibody reactivity, which has implications for vaccine development <sup>11</sup>.

Based on these results, the virions produced by PSVs and IMCs are not identical. They differ in Env processing, such that IMCs exhibit higher levels of Env processing. This is demonstrated by cleavage of gp160 into gp120 and gp41. Despite the difference in Env processing, no differences were observed in neutralisation sensitivity and incorporation was isolate-dependent. Therefore, both systems might be acceptable when the differences in processing and cleavage of Env are considered in interpreting neutralisation assays. Overall these findings suggest that when comparing the PSV and IMC systems, great care must be taken to standardise the two methods.

**Table 1.2: Variation in study methodology that compared IMCs with PSVs.**

Paper	Env clone origin	Number of clones	HIV backbone	Host cell line
Louder et al.	Full-length env genes of HIV-1 BL01 and BR07 were cloned from blood and brain tissue.	2 pairs	pNL4-3env	293T and PBMC
Provine et al.	Maternal primary isolates, clade A	6 pairs	Q23 $\Delta$ env	293T
Miglietta et al.	Clade B, from sexually acquired, acute/early infections	11 (5 PSVs, 6 IMCs)	pSG3 $\Delta$ env	293T

## 1.8 Vaccine development

Due to variation in findings of Env phenotype between studies, we next discuss the efficacy of vaccines within the context of methodology employed to produce the immunogen. One of the reasons for the

identification of a phenotype common to all TFs was that these motifs could be targeted during the rational design of vaccines. Generating an immune response specifically against TFs might prevent infection. However, in the absence of this information, immunogen design has relied on a shotgun approach where a number of methods and Envs have been utilised. Effective vaccines require virus-specific CD8<sup>+</sup> T cells as well as neutralisation antibodies<sup>91</sup>. A number of different strategies have been employed over the years including DNA and protein-based vaccines<sup>92</sup>. The AIDSVAX B/E is a vaccine against HIV/AIDS that is based on the Env protein gp120<sup>92</sup>. After clinical trials by Gilbert, et al.<sup>93</sup> and Pitisuttithum, et al.<sup>94</sup> this vaccine was shown to be ineffective in protection against HIV. Thus, a new combination method was devised. The combination of AIDSVAX B/E with ALVAC canarypox vaccine led to the ALVAC-HIV/AIDSVAX B/E vaccine<sup>92</sup>. This vaccine presented a 31 % protection against HIV-1 infection and is the first to have demonstrated any level of efficacy. Future vaccines may be able to draw from the basis of the ALVAC-HIV/AIDSVAX B/E vaccine in order to improve the efficacy in prevention of HIV acquisition (Table 3).

**Table 1.3: Summary of vaccine trials, outcomes and approaches used to date.**

Name (Year)	Country	Type	Result
AIDSVAX B/B and AIDSVAX B/E (2002)	Canada, USA, Netherlands and Puerto Rico	recombinant gp120 surface proteins from different HIV-1 strains	No protection
STEP (2007)	North America/ Australia	adenovirus with synthetically produced HIV genes: gag, pol and nef	Increased infection by 48%
RV144 (2009)	Thailand	Canary pox vector containing genetically engineered Env, gag and pol. Boost was composed of genetically engineered gp120	Lowered infection by 31%

In the study done by Miglietta, et al.<sup>11</sup> as previously described, a number of antibodies were tested against PSVs and IMCs. This showed that no single antibody was able to neutralise all isolates whether PSVs or IMCs. Based on the results discussed, it is probable that when using methods that rely on 293T-derived viruses, the potency of the antibodies being tested is overestimated<sup>95</sup>. This would result in ineffective vaccines. Furthermore, the fact that no single antibody is able to neutralise the virus completely suggests that combinations of current vaccines, much like the ALVAC-HIV/AIDSVAX B/E vaccine described above, might prove to be the best way to develop a highly effective vaccine. Alternatively, we still have hope that identifying a phenotype common to all TFs might provide the direction needed to design an efficacious vaccine.

## 1.9 Conclusion

Rational vaccine design depends on identifying features of HIV that, when targeted by the immune response, elicits a robust CTL and/or humoral response. These features should be common to the majority of circulating isolates so that immune responses are broadly neutralising. The discovery of TFs led to a number of studies that identified transmission motifs using a number of different approaches. These included PSVs, IMCs, and purified protein produced under different conditions. However, the structure and function of Env, the main target for vaccine design, was shown to vary depending on whether produced as PSV or IMC. This has important ramifications for, not only vaccine design, but also strategies employed to understand HIV-1 transmission. The accuracy of research findings is only as strong as the method used and without careful consideration, and standardisation of assays, we run the risk of testing vaccine candidates that will not protect against HIV-1 infection in highly vulnerable populations. However, if selection of TFs is not due to a common feature, then a multi-pronged approach will be required where rational design of vaccines is coupled to random selection of variants and epitopes that prove to be highly immunogenic using cohort studies.

## **1.10 Aim**

Understand how experimental methodology might have contributed to inconsistent findings related to the role of Env in HIV-1 transmission

### **1.11 Objectives**

1. Determine whether the expression, cleavage and incorporation of different Env clones into viral particles are influenced by the cell line used to produce pseudovirus and infectious molecular clones with different HIV backbones.
2. Understand how entry of TZM-bl cells and replication kinetics of infectious molecular clones in PBMCs are affected by the cell line used to produce the infectious molecular clones with different HIV backbones.
3. Compare entry of TZM-bl cells with infectious molecular clones to that of matched pseudovirus to identify whether cell line, Env clone and/or backbone is a factor that contributes to potential differences in outcome of the two assays

## Chapter 2

### Methods and materials

#### 2.1 Transforming *E. coli*

Plasmid DNA was added to competent *E. coli* JM109 cells. The volume of DNA used was 1/10<sup>th</sup> the volume of the cells used. The DNA and cells were allowed to bind on ice for 25 – 30 minutes and subsequently heat shocked at 42 °C for 45 seconds and placed on ice again for 2 minutes. Luria broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl in distilled water) was added up to 1 ml and the culture was allowed to grow at 37 °C, with shaking for 1 hour. After 1 hour, the cells were pelleted by centrifugation at 5 000 rpm for 5 minutes. The supernatant was removed, the pellet was resuspended in the remaining supernatant and then spread onto Luria agar (LA) (LB, 1.5% w/v agar and 100 µg/mL carbenicillin disodium salt) plates which were incubated at 37 °C overnight.

#### 2.2 Bacterial cultures

LB was prepared in 5 ml and 50 ml aliquots. Plasmid glycerol stocks were used to inoculate LB supplemented with carbenicillin (Sigma) (100 µg/ml). Starter cultures (5 ml) were grown at 30 °C for approximately 17 hours, with shaking. The starter cultures were then transferred to 50 ml LB, supplemented with carbenicillin (100 µg/ml) and the same procedure was followed.

##### 2.2.1 Plasmid DNA extraction

DNA was extracted from 50 ml cultures using a Midiprep Kit (Promega) or the Plasmid DNA Purification kit (Qiagen) according to the manufacturer's specifications. DNA was then stored at -20 °C until required.

#### 2.3 Tissue culture

All cell lines (HEK293T, HeLa, CHO-K1 and TZM-bl cells) were grown in Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 10% Fetal Bovine Serum (FBS) and 1 U/ml penicillin and 1 µg/ml streptomycin. Cells were incubated at 37 °C and 5% CO<sub>2</sub> in a water-jacket incubator.

### **2.3.1 Transient transfections**

For each cell line, cells were plated at a density of  $2 \times 10^6$  cells per well in a 6-well plate, in a total volume of 2 ml full DMEM. Cells were incubated overnight to allow growth to reach approximately 40-60% confluency.

#### **2.3.1.1 Transfection of HEK293T cells**

Transfection mixes were made in 400  $\mu$ l serum-free DMEM with Polyethylenimine (PEI) and plasmid DNA in a ratio of 3:1, PEI: DNA. pGL4-luc at a concentration of 0.5  $\mu$ g was added as a measure of transfection efficiency. Transfection mixes were vortexed for 15 seconds and incubated at room temperature for 10 minutes to allow formation of complexes. Thereafter, growth medium was removed from the 6-well plate, replaced with 1.5 ml fresh medium and transfection mixes were added in a drop-wise fashion. Cells were incubated at 37 °C for six hours before medium was removed and replaced with 2 ml fresh medium, 1 ml for gp140 samples, and then incubated for a further 48 hours before cell lysis.

#### **2.3.1.2 Transfection of HeLa and CHO cells**

Transfection mixes were made in 200  $\mu$ l serum-free DMEM with Genecellin transfection reagent (Celtic) and plasmid DNA in a ratio of 3:1, Genecellin: DNA. As mentioned in section 2.3.1.1, 0.5  $\mu$ g of pGL4-luc was added as a measure of transfection efficiency. Transfection mixes were vortexed for 3 seconds and incubated at room temperature for 15 minutes. DMEM was removed from each of the wells in the 6-well transfection plate and replaced with 2 ml fresh DMEM. Transfection mixes were added in a drop-wise fashion and then incubated at 37 °C for 48 hours before cell lysis.

### **2.3.2 Endoglycosidase treatments**

One ml of medium was harvested from the wells of the gp140 and pcDNA transfections and placed into 2 ml Eppendorf tubes. One ml of MES buffer (pH 6: 20 mM MES monohydrate, 130mM NaCl, 10mM  $\text{CaCl}_2$ ) and 30  $\mu$ l of *Galanthus nivalis* beads was added to the medium and the mixtures were incubated at 4°C overnight, with rolling. The following day, the 2 ml tubes were centrifuged at 14 000 rpm for 3 minutes after which the supernatant was discarded without disturbing the pelleted beads. The beads were washed with 1 ml of cold phosphate buffered saline (PBS), supplemented with  $\text{Ca}_2^+$  and  $\text{Mg}_2^+$ , by centrifugation at 14 000 rpm for 3 minutes. The PBS was removed without disturbing the beads. This was repeated thrice. The pelleted beads were resuspended in 300  $\mu$ l of PBS ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) and divided equally into three 1.5 ml eppendorfs labelled “untreated”, “Endo-H” and “PNGase F”. The

tubes were centrifuged at 14 000 rpm for 3 minutes and the supernatant was carefully removed. To the three tubes, 9  $\mu$ l of dH<sub>2</sub>O and 1  $\mu$ l 10x glycoprotein denaturing buffer (NEB) was added. The tubes were then boiled at 90°C for 10 minutes. To the “untreated” samples, 5  $\mu$ l of dH<sub>2</sub>O, 2  $\mu$ l of 10Xx buffer G7 (NEB) and 2  $\mu$ l 10% NP40 was added. To the “Endo H” tube, 7  $\mu$ l of dH<sub>2</sub>O, 2  $\mu$ l of 10x buffer G5 and 1  $\mu$ l (1:3) Endo H was added. To the “PNGase F” sample, 5  $\mu$ l of dH<sub>2</sub>O, 2  $\mu$ l of 10x buffer G7, 2  $\mu$ l of 10% NP40 and 1  $\mu$ l (1:3) PNGase F was added. All tubes were incubated at 37 °C overnight. The reactions were stopped by adding 5  $\mu$ l of loading dye and boiling for 10 minutes at 90 °C. The total volume of each tube was loaded to an SDS-PAGE gel (see section 2.3.6 (for method)) for visualisation of proteins.

### **2.3.3 Cell lysis**

After 48 hours of incubation, DMEM was removed and 300  $\mu$ l of RIPA buffer (10mM Tris, pH 7.2, 2mM EDTA, pH 8.0, 150mM NaCl<sub>2</sub>, 1% Triton X-100 (Sigma) and 0.1% phenylmethylsulfonyl fluoride (PMSF)) was added to each well. The 6-well plate was placed on ice for 5 minutes, during cell lysis. Lysates were collected in cold 1.5 ml Eppendorf tubes and left to lyse for a further 5 minutes on ice, then centrifuged at 4°C, at 14 000 rpm for three minutes. Cell debris was pelleted and the supernatants were transferred to fresh Eppendorf tubes. The samples were then stored at -20 °C until protein quantification and Western blot (see section 2.3.7) analysis.

### **2.3.4 Bradford assay**

Proteins in lysates were quantified using a Bradford assay. Bovine Serum Albumin (BSA) was serially diluted in RIPA buffer (1:10) to generate a standard curve which was used to quantify the unknown protein concentrations of the cell lysates. The 96-well plates were read at 595 nm using the Gen5 plate reader.

### **2.3.5 Transfection efficiencies**

To measure transfection efficiencies, 30  $\mu$ l of lysates was mixed with 30  $\mu$ l of BrightGlo (Promega). Luminescence was measured with a luminometer (Glomax 96 microplate luminometer) and relative light units (RLU) values were used to quantify entry of pGL4-luc into cells (a measure of transfection efficiency).

### **2.3.6 SDS-PAGE**

An amount of 100 µg protein was added to protein loading buffer (1% SDS, 4% glycerol, 1% β-mercaptoethanol and 0.01% bromophenol blue) and then boiled at 100°C for 10 minutes in order to denature the proteins. The protein samples were loaded into a 5% acrylamide stacking gel and a 10% acrylamide resolving gel and run in running buffer (25mM Tris, 190 mM glycine, 0.1 % SDS) under 0.2 mA for small gels and 0.6 mA for large gels. Proteins were transferred to pre-wet polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using the Bio-Rad Mini Trans-Blot® Cell according to the manufacturer's instructions.

### **2.3.7 Western blots**

After protein transfer, PVDF membranes were incubated at 4°C overnight in blocking buffer (5% fat free skim milk, 0.5% Tween-20 in tris buffered saline (TBS)). These membranes were cut into segments in order to separate the low molecular weight proteins from the high molecular weight proteins. Each membrane was incubated at room temperature for one hour, with shaking, in the corresponding primary antibodies. The low molecular weight membranes were incubated in mouse anti-β-actin antibody while the high molecular weight membranes were incubated in sheep anti-gp120 antiserum (ARP Cat #288). Membranes were washed thrice with TBS-T (50 mM Tris, 150 mM NaCl, pH 7.5, 0.5 % Tween-20), in 15 minute intervals, with shaking at room temperature. Secondary antibodies were added to their respective membranes and incubated for one hour at room temperature, with shaking. Low molecular weight membranes were incubated in horse-radish peroxidase conjugated goat anti-sheep IgG and high molecular weight membranes were incubated in horse-radish peroxidase conjugated goat anti-mouse IgG. Once again, membranes were washed thrice in TBS-T in 15 minute intervals. A fourth wash was done in TBS. In order to visualise the proteins on autoradiographic film (Santa Cruz Biotechnology®), Lumino Glo® substrate kit was added to each membrane.

### **2.3.8 Pseudovirus production**

#### **2.3.8.1 Pseudovirus production in HEK293T cells**

HEK293T cells were plated at a density of  $2 \times 10^6$  cells per ml in 2 ml in a 6-well plate, then left to incubate overnight at 37°C. Cells were ~40 – 60 % confluent prior to transfection. Transfection complexes were generated by adding DNA and PEI in a 3:1 ratio (2.5 µg gp160 + 5 µg of pSG3Δenv + 22.5 µl PEI), to 400 µl of serum-free medium. Transfection mixes were vortexed for 15 seconds and incubated at room temperature for 10 minutes. Medium was removed from each well and replaced with 1.5 ml of fresh medium before adding complexes in a drop-wise fashion. Cells were incubated for 48 hours before harvesting PSVs. 2 ml of medium was collected from each well and placed into 2 ml

syringes. Culture medium was then filtered into 15 ml tubes through 0.2 µm filters. 200 µl of FBS was added to each sample to obtain a final concentration of 10 % FBS. The medium was mixed thoroughly and aliquoted into Eppendorfs (50 µl) for quantification via p24 ELISA and cryovials for long-term storage. All samples were stored at -80 °C.

### **2.3.8.2 Pseudovirus production in HeLa cells**

HeLa cells were seeded and incubated in the same way as described above for HEK293T cells. Transfection complexes were generated by adding DNA and Genecellin transfection reagent (Bulldog Bio) in a 1:6.7 ratio (0.9 µg gp160 + 1.8 µg pSG3Δenv + 18 µl Genecellin), to 200 µl of serum-free medium. Transfection mixes were vortexed for 3 seconds and incubated at room temperature for 15 minutes. Medium was removed from each well and replaced with 2 ml of fresh medium before adding complexes in a drop-wise fashion. Cells were incubated for 48 hours at 37 °C before harvesting PSVs. Growth medium was collected from each well and placed into 2 ml syringes. Medium was then filtered into 15 ml tubes through 0.2 µm filters. 200 µl of FBS was added to obtain a final concentration of 10% FBS. The medium was mixed thoroughly and aliquoted into Eppendorf tubes (50 µl) for p24 ELISA and the remainder into cryovials for long-term storage at -80 °C. The cells left in the plate were then lysed using RIPA buffer (see section 2.3.3 on cell lysis) for Western blot analysis.

### **2.3.8.3 Pseudovirus production in CHO cells**

CHO cells were seeded and incubated as described above for HeLa cells with the following changes; transfection complexes were generated by adding DNA and Genecellin transfection reagent (Bulldog Bio) in a 1:6.7 ratio (1.1 µg gp160 + 2.2 µg pSG3Δenv + 22 µl Genecellin), to 200 µl of serum-free medium. PSVs were stored at -80 °C and cells were lysed as described above (section 2.3.3).

## **2.3.9 p24 ELISA**

### **2.3.9.1 Coating p24 ELISA plates**

High binding 96-well plates (Sigma, # CLS3922-100EA) were coated with lyophilised sheep anti-HIV p24 Gag, affinity purified coating antibody (5.5 ng/ml) diluted 1:600 with 1x NaHCO<sub>3</sub> (pH 8.5). 100 µl of the diluent was added to each well and left at room temperature overnight. The next day, plates were washed twice with 1x TBS, then left for one hour at room temperature with 100 µl of 5% BSA in each well. Plates were stored at -20 °C until needed.

### **2.3.9.2 p24 ELISA assay**

Before performing this assay, viruses were inactivated with 200 µl of 1.25% Empigen per 50 µl of virus sample, for one hour at room temperature. Standards were prepared with lyophilised recombinant HIV-p24 (Aalto #AG6054) at concentrations; 32, 16, 8, 4, 2, 1, 0.5 and 0 ng/ml. Virus samples were diluted with 1% Empigen (in 1x TBS) between 10 and 800 x depending on the sample. 100 µl of each standard was added to the plate in duplicate. 100 µl of each sample at each concentration was added to the plate in duplicate or triplicate. The plate was left at room temperature for three hours. TROPIX buffer (Applied Biosystems) was diluted to 1x with dH<sub>2</sub>O. Secondary conjugate was prepared by diluting conjugate (EH12AP (Aalto Bioreagents # BC-1071-AP)) in 1x TBS, 20% sheep serum (Lasec), 0.05% Tween (Sigma), and 8% BSA. After three hours of incubation, plates were washed four times with 1x TBS. 100 µl of conjugate was added to each well and left to incubate for one hour at room temperature. Thereafter, plates were washed eight times with 1x TBS and twice with 1x TROPIX buffer. 50 µl of ELISA CPD Star/Sapphire II (Applied Biosystems, # T1025), diluted 1:4 with 1x TROPIX buffer, was added to each well and left to incubate at room temperature for two minutes before reading in the luminometer.

### **2.3.9.3 Ultracentrifugation of Pseudoviruses**

After generated PSVs were shown to have very low p24 concentrations, ultracentrifugation was used to concentrate the PSVs. Transfections were performed as described above (section 2.3.8) for each cell type and 6 wells were pooled for each sample in order to concentrate the samples. The samples were layered onto a 1 ml, 20 % glycerol cushion and ultracentrifuged at 26 000 rpm for two hours at 4 °C. The supernatant was carefully poured off and the pellets were resuspended in the remaining supernatant. The samples were stored at -80 °C until needed.

### **2.3.10 Titration of Pseudoviruses in TZM-bl**

TZM-bl were plated in 96-well plates at a density of  $1 \times 10^4$  cell per well and left to incubate overnight at 37 °C. PSVs were allowed to thaw at room temperature and then serially diluted in a deep-well plate. Concentrations of p24 were as follows: 100 ng/ml, 50 ng/ml, 25 ng/ml and 12.5 ng/ml. 100 µl of medium was removed from the cell culture and 100 µl of each virus dilution was added to the 96-well plate in triplicate. The cells were incubated for 48 hours before lysis. For cell lysis, 150 µl of medium was removed from each well, such that 50 µl remained. 50 µl of BrightGlo was then added,

mixed thoroughly and then transferred to an opaque 96-well plate and left to lyse for 2 minutes before reading in the luminometer.

### **2.3.11 Generating infectious molecular clones**

#### **2.3.11.1 Amplification of the env gene**

The *env* gene was polymerase chain reaction (PCR) amplified using Platinum Taq High Fidelity Polymerase (Invitrogen®), from the CAP210 E8 plasmid with the following primers:

Forward primer: 5'- AGAAAGAGCAGAAGACAGTGGCAATGA – 3'

Reverse primer: 5' – TTTTGACCACTTGCCACCCAT – 3'

The reaction components and PCR cycling conditions can be seen in Table 2.1 and Table 2.2 below.

Table 2.1: Components and final concentrations for reaction mixes.

Reaction component	Concentration
Buffer	1 x
MgSO <sub>4</sub>	1.5 mM
dNTPs	0.2 mM
Forward primer	0.2 μM
Reverse primer	0.2 μM
Q5 Taq polymerase	1 ng
H <sub>2</sub> O	up to 50 μl
DNA template	1 U

Table 2.2: Cycling conditions for PCR to amplify env gene.

Cycle	Temperature (°C)	Time (minutes)
Initial denaturation	94	2
Denaturation	94	0.5
Annealing	55	0.5
Extension	72	2.5

Amplification of the desired product was confirmed by agarose gel electrophoresis. 5 μl of the PCR product was loaded to a 0.8 % agarose gel with ethidium bromide and run for an hour at 80 V. Once the presence of the 3 kb product was confirmed, the remaining PCR products were purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega®), following the manufacturer's instructions.

### 2.3.11.2 Linearising the Q23 vector

For homologous recombination to take place between the env gene and the Q23 vector, the vector is required in a linear conformation. To generate linear Q23, the DNA was digested with the Sall restriction enzyme (NEB) for one hour at 37 °C. See table 2.3 for reaction components.

Table 2.3: Components added to the digestion reaction of Q23.

Component	Concentration
Buffer	1 x
Sall enzyme	1 U
DNA	250 ng
H <sub>2</sub> O	up to 50 µl

To confirm that the Q23 vector was linearised in the digestion reaction, the product was run on a 0.8 % agarose gel and visualised. After confirming digestion, the remainder of the products was run on a 0.8 % agarose gel and the linear band was excised from the gel. The gel slice was purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega®), according to the manufacturer's instructions.

### 2.3.11.3 Generating competent yeast cells

To generate competent yeast cells, 10 ml yeast extract peptone dextrose (YEPD) medium (1% yeast extract, 2% peptone, 2% dextrose) was inoculated with 100 µl of S288C yeast cells and incubated at 30 °C overnight, with shaking. OD readings were taken at 600 nm by diluting the overnight culture 1:10 in YEPD. Once the absorbance was established, the cultures were diluted to OD<sub>600</sub> = 0.3. The diluted cultures were then incubated again for 1 – 2 hours until the OD<sub>600</sub> readings reached 1.2. The cultures were then centrifuged at 3 000 rpm for 5 minutes. The pellet was resuspended in dH<sub>2</sub>O in half of the volume of the original yeast culture. The supernatant was discarded, and the pellet was resuspended in 1 ml 10 % glycerol per 50 ml of culture medium. Cells were then divided into 100 µl aliquots and stored at -80 °C until needed.

### 2.3.11.4 Transforming competent yeast cells

Salmon sperm DNA (Invitrogen®) was placed in the heating block at 95 °C for 3 minutes and then on ice for 2 minutes. Competent S228C cells were thawed on ice and centrifuged at 3 000 rpm for 5 minutes and the supernatant was discarded. To transform the yeast cells, the following reagents were added as seen in Table 2.4 below:

Table 2.4: Components making up the transformation reaction.

Component	Concentration/Volume
Insert (env PCR product)	1 µg
Vector (linearised Q23)	0.2 µg
Salmon sperm DNA	10 µl
50 % Polyethylene glycol	240 µl
1 M lithium acetate	36 µl
dH <sub>2</sub> O	up to 370 µl

The mixture was incubated at 30 °C for 30 minutes, then transferred to 42 °C for 15 minutes. Tubes were centrifuged for 30 seconds at maximum speed, the supernatant was discarded, and the pellets were resuspended in 100 µl of Tris-EDTA (TE) buffer. The total volume was spread onto yeast amino acid dropout plates + 5-fluoro-1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid (FOA), 2% peptone, 1% yeast extract, 2% w/v agar, 0.67% w/v complete supplement mixture minus leucine (CSM-leu), 2% dextrose, 0.1% w/v FOA). The plates were then incubated at 30 °C for 2 – 3 days.

After incubation, positive colonies were screened by colony PCR using the following primers:

Forward yeast primer: 5' AAT GTC AGC ACA GTA CAA TGT ACA CAT GG 3' and Reverse yeast primer: 5' GGA GCT GTT GAT TTA GGT ACT TTT 3'. The reaction was set up and performed as seen in Table 2.5 and Table 2.6 below.

Table 2.5: Components for yeast colony PCR.

Reaction Component	Concentration (final)
Buffer	1 x
dNTPs	0.2 mM
Forward primer	0.2 µM
Reverse primer	0.2 µM
DNA	Tip full
Taq	1 U
dH <sub>2</sub> O	up to 25 µl

Table 2.6: Cycling conditions for yeast colony PCR.

Cycle	Temperature (°C)	Time (minutes)
Initial denaturation	95	0.5
Denaturation	95	0.5
Annealing	55	1
Extension	68	1
Final extension	68	5

The PCR was carried out using Q5 High Fidelity Polymerase kit (Inqaba Biosystems®). The presence of a ~3 kb band was confirmed by gel electrophoresis on a 0.8 % agarose gel. Positive colonies were selected and YEPD was inoculated with each positive sample. The cultures were incubated with shaking at 30 °C overnight. The DNA was extracted from the yeast cells using the Zymoprep™

Yeast Plasmid Miniprep Kit (Zymo Research Corporation®) and sent for sequencing by Central Analytical Facility (CAF). Sequencing results were then analysed using Chromas Pro to confirm the product.

#### **2.3.11.5 Electroporation of electrocompetent *E. coli***

Extracted DNA was electroporated into electrocompetent *E. coli* β-10 cells (NEB) following the manufacturer's instructions. 100 µl of the cells was plated onto LA + 100 µg/ml carbenicillin disodium salt and the plates were incubated at 30 °C overnight. After incubation, colonies were selected and screened by PCR as described in section 2.3.11.1. DNA was sent for sequencing by CAF. The sample that returned the best sequencing results was taken forward for generating IMCs, Q23.6.

#### **2.3.11.6 Transfections to generate infectious molecular clones in HEK293T cells**

HEK293T CHO cells were seeded in 6-well plates at a density of  $2 \times 10^6$  cells per ml in 2 ml final volume and left to incubate overnight at 37 °C, 5 % CO<sub>2</sub> in a water-jacket incubator. Cells were allowed to reach ~40 – 60 % confluency prior to transfection. To form transfection complexes, 1 µg of Q23 helper plasmid DNA, 1 µg of Q23.6 DNA and 0.2 µg of pGL4-luc was mixed in 400 µl of serum-free medium. Thereafter, 6 µg of PEI was added, and samples were vortexed for 15 seconds before incubating at room temperature for 10 minutes. The complexes were then added to the cells after replacing the cells with fresh medium. The cells were left to incubate at 37 °C, 5 % CO<sub>2</sub> in a water-jacket incubator for 48 hours. After 48 hours, the cells were lysed as described in section 2.3.3.

#### **2.3.11.7 Transfections to generate infectious molecular clones in HeLa and CHO cells**

The same procedure as described in section 2.3.11.6 was followed using 6 µl of Genecellin instead of PEI to form transfection complexes. All IMCs were ultracentrifuged following the same procedure described in section 2.3.10. After ultracentrifugation, IMCs were quantified by p24 ELISA as outlined in section 2.3.9.2.

## 2.3.12 Replication Kinetics

### 2.3.12.1 Isolation of peripheral blood mononuclear cells

Whole blood buffy packs were collected from the Western Province Blood Transfusion Service (WPBTS). Donors were all confirmed to be healthy after being tested for Hepatitis B and C, HIV-1 and syphilis. For the isolation of peripheral blood mononuclear cells (PBMCs), 15 ml of Histopaque (Sigma-Aldrich®) was added to Leucosep tubes (Greiner Bio-One®) and centrifuged at 2 500 rpm for 1 minute. Whole blood samples were diluted in a 1:1:1 ratio with Roswell Park Memorial Institute (RPMI) medium (Lonza®) and PBS (Lonza®). The total volume was added to the pre-centrifuged Leucosep tube which were centrifuged again at 2 500 rpm for 15 minutes. The centrifugation led to the separation of the blood into three layers. The top layer contained the plasma, the middle layer contained the PBMCs and the bottom layer contained the erythrocytes (Figure 2.1).

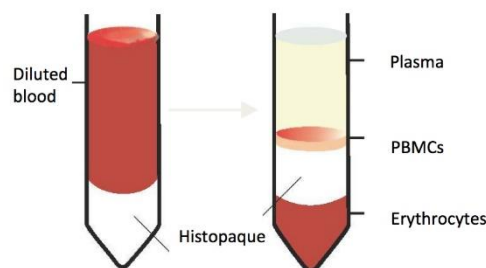


Figure 2.1: Diagram showing the separation of blood samples after centrifugation in Leucosep tubes with Histopaque Image taken from <https://bio-protocol.org/e1300>.

A Pasteur pipette was used to remove the PBMCs without disrupting the other layers to ensure that only PBMCs were collected. The PBMCs were collected into a clean 50 ml tube with 30 ml of PBS supplemented with 1 % FBS to wash the cells. The cells were pelleted by centrifuging at 1 200 rpm for 5 minutes. This wash step was repeated two to three times and after the final wash, 10 µl of cell resuspension was added to 90 µl of Trypan Blue (Lonza®) for counting on a haemocytometer. To make cell stocks, cells were centrifuged at 1 500 rpm for 8 minutes and stored in 10 % DMSO in FBS at a concentration of 20 – 50 million cells per ml, in 1 ml aliquots at -80 °C until required.

### 2.3.12.2 Activation of peripheral blood mononuclear cells

PBMC stocks were thawed, counted and seeded at a density of 1 million cells per ml in a final volume of 10 ml in a T25 flask (NEST®). The RPMI medium was supplemented with 10 % FBS, 200U/ml Interleukin-2 (IL-2) (Gentaur®) and 0.5 µg/ml phytohemagglutinin-P lectin (PHA-P)

(ThermoScientific™) to activate replication. These activated PBMCs were left to incubate at 37 °C 5% CO<sub>2</sub> in a water-jacket incubator for 72 hours.

#### **2.3.12.3 Testing donors for ability to support replication**

To ensure that PBMCs isolated from each donor are able to support replication, cells were tested by infection with IMCs. After activation, the cells were counted and plated at a density of 10<sup>6</sup> cells per ml in a 96-well plate and infected with 40 000 ng p24 IMCs. To enhance infection of PBMCs, the 96-well plates were spinoculated by centrifugation at 1 500 x g for 2 hours at room temperature. The plates were incubated at 37 °C in 5 % CO<sub>2</sub> in a water-jacket incubator for a total of 14 days. 50 µl of culture medium was harvested on days 4, 7, 10 and 14 and cells were replenished with 50 µl of fresh medium after each harvest. The harvests were stored at -80 °C until required. To determine whether the PBMCs were able to support replication of the IMCs, the 50 µl harvests were tested by a p24 ELISA assay as described in section 2.3.9.2.

#### **2.3.12.4 Expansion of infectious molecular clones**

Due to low virus yields of IMCs as determined by p24 ELISA, IMCs were expanded in PBMCs to obtain higher virus titres. PBMCs were isolated and activated, as described above (section 2.3.12.1), and seeded in 6-well plates at a concentration of 5 x 10<sup>6</sup> cells per well in 2 ml RPMI supplemented with IL-2 and PHA-P. Each well was infected with a different virus using the total available virus, due to low yields. The cells were then spinoculated as mentioned above (section 2.3.12.3). After spinoculation, cells were removed from the 6-well plate and cultured upright in T25 flasks in a final volume of 5 ml supplemented RPMI medium. Cells were incubated at 37 °C in 5 % CO<sub>2</sub> for 4 days. After 4 days the infected cells were harvested, counted and replenished with fresh supplemented RPMI to obtain a concentration of 1 million cells per ml. The cells were incubated at 37 °C in 5 % CO<sub>2</sub> for another 3 days. PBMCs were activated as described above -in preparation for the next step of expansion. After 3 days, the infected cells were harvested, counted and resuspended in supplemented RPMI to obtain a final concentration of 1 million cells per ml. The supernatants, containing virus, from each sample was collected and ultracentrifuged as described in section 2.3.9.3 to concentrate the virus and determine p24 concentration. The activated PBMCs were also harvested, counted and resuspended in supplemented RPMI to a final concentration of 1 million cells per ml. Thereafter, 5 ml of activated PBMCs was added to each of the expanding virus samples and left to incubate for another 3 days. Once again, fresh PBMCs were activated. The same procedure was repeated until the viruses had been

expanded for a total of 17 days. After p24 ELISAs were performed, many samples were too low to quantify. Thus, day 17 samples were assumed to have the highest viral titres and were carried forward.

#### **2.3.12.5 Entry efficiency of infectious molecular clones**

Since only approximately 100 µl of concentrated virus was obtained per sample, non-concentrated viruses were used to infect TZM-bls. TZM-bls were seeded as described in section 2.3.10. Each virus was used to infect TZM-bls in duplicate with 11 serial dilutions, beginning with undiluted supernatants. The infected TZM-bls were incubated at 37 °C in 5 % CO<sub>2</sub> for 72 hours. Cells were then lysed and quantified as described in section 2.3.10.

#### **2.3.12.6 Replication of infectious molecular clones in peripheral blood mononuclear cells**

To test replicative fitness of each virus, PBMC stocks, which had previously been determined to be capable of supporting viral replication, were activated and seeded as described in section 2.3.12.3. To normalise the amount of viruses added, the entry efficiency data was used. All samples were normalised to 15 x background luminescence obtained by the infection of TZM-bl cells. CHO cells produced substantially lower readings, therefore the total volume of virus was used for the infection. After normalising, 100 µl of each sample was used to infect the activated PBMCs. The infected cells were spinoculated as described previously (section 2.3.12.3) and incubated at 37 °C in 5 % CO<sub>2</sub>. 50 µl of supernatant was harvested on days 4, 7, 10, 14 and 17 and quantified by p24 ELISA. On each harvest day, the cells were replenished with 50 µl of fresh supplemented RPMI.

### **2.4 Statistical analysis**

All statistical tests were conducted using Prism 5.0 and graphs presented in this thesis were generated using Microsoft Excel.

## Chapter 3

Determining whether pseudovirus and infectious molecular clones differ in HIV-1 Envelope expression, processing and incorporation into viral particles

### 3.1 Introduction

Thus far, there have been conflicting reports on whether pseudovirus (PSV) and infectious molecular clones (IMC) are directly comparable (Table 1.2) due to differences in Env expression and processing when produced within the context of these two experimental approaches<sup>10-12</sup>. Variation in Env expression can affect incorporation into budding virus as shown by Bachrach, et al.<sup>54</sup> who differentially expressed Env using an inducible vector and found that changes in expression directly influenced the number of Env proteins incorporated into viral particles<sup>54</sup>. Another study showed that there was a direct link between Env expression, incorporation and PSV infectivity<sup>96</sup>. Furthermore, changes in Env expression has been indirectly linked to changes in *N*-glycosylation based on the impact of the Env signal peptide on expression and *N*-glycosylation<sup>97</sup>, suggesting that factors that influence Env expression will impact overall Env processing and viral infectivity. As vaccine design requires that Env elicits a broadly neutralising immune response, factors that alter Env processing will most likely interfere with antigenicity and thus expression systems need to be carefully considered when generating vaccine candidates<sup>98</sup>.

Env clones are expressed differently even when cloned into the same mammalian vector<sup>99</sup>, and differentially glycosylated depending on the cell type used for expression<sup>28</sup>. Therefore, in this study HEK293T, CHO and HeLa cells were compared because they are most commonly used in studies that investigate HIV-1 Env expression, *N*-glycosylation, function, production of PSVs and IMCs as well as production of vaccine candidates<sup>100 101</sup>. As different Env clones might have varied expression due to genotypic and/or phenotypic differences, we tested two subtype C clones, CAP210 E8 and Du151a. Previous studies in our lab have shown that cell-associated Env usually represents under processed protein (unpublished data). To compare *N*-glycosylation of mature Env, CAP210 E8 was truncated to form gp140, by removing the transmembrane region and generating a soluble construct that is released into the culture medium once trafficked to the plasma membrane<sup>102</sup>. Furthermore, as Env expression could be influenced by the presence of other viral proteins, we investigated expression in the presence of PSV backbones pSG3Δenv (pSG3)<sup>103</sup> and DHIV-pNL4-3-E-R (pNL4.3)<sup>104</sup>. Similarly, for IMC production, the backbones pNL4.3 and Q23<sup>5</sup> were used. Therefore, this chapter will report on how Env expression, incorporation and *N*-glycosylation are impacted by cell-type, HIV backbones and systems for generating PSVs and IMCs.

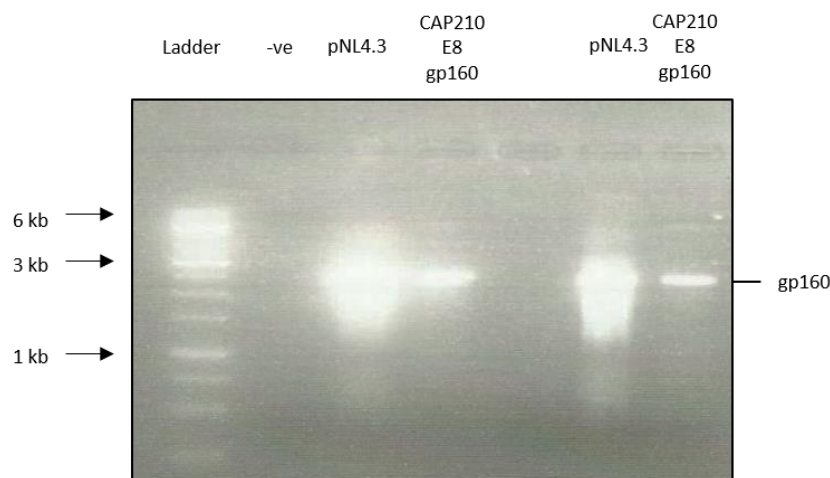
## 3.2 Results

### 3.2.1 Generating infectious molecular clones

In order to determine whether IMC backbones impact Env expression, processing and viral replication, CAP210 E8 was inserted into the pNL4.3 (Bahiah Meyer, MSc student in our lab) and Q23 backbones by homologous yeast recombination.

#### 3.2.1.1 PCR amplification of *env*

CAP210 E8 was cloned into the mammalian expression vector, pCDNA3.1\_TOPO (Invitrogen) (kind gift from Prof Carolyn Williamson) using single-genome amplification and primers complimentary to *vpu* and *nef*. To generate IMCs, CAP210 E8 was amplified from pCDNA3.1\_TOPO using primers designed to regions that flanked *env*. (Forward primer, Env F: 5'- AGAAAGAGCAGAAGACAGTGGCAATGA – 3' Reverse primer, Env R: 5' – TTTTGACCACTTGCCACCCAT – 3'). The PCR was carried out in duplicate to obtain sufficient product following purification. The presence of a ~ 3 kb band, the same size as the positive control (amplified *env* of the pN4.3 provirus) indicated that the correct product had been generated (Figure 3.1). This band was subsequently excised from the agarose gel and purified for ligation into the linearised Q23 vector.

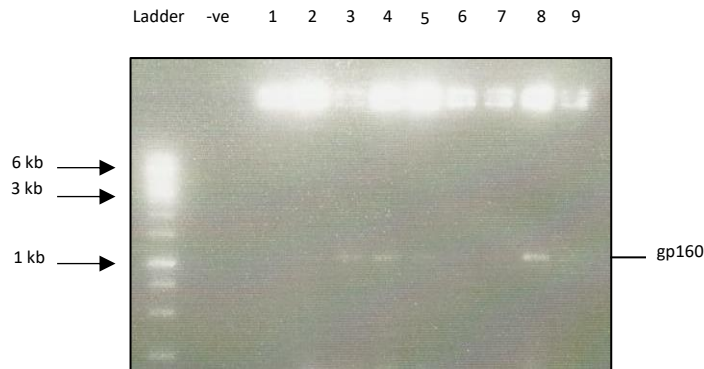


**Figure 3.1: PCR amplification of CAP210 E8.** Primers flanking the *env* gene of CAP210 E8 were used to amplify the gene in preparation for the homologous recombination assay. -ve indicates the negative control, pNL4.3 was used as a positive control, CAP210 E8 gp160 is the plasmid containing the *env* gene of interest. The reaction was performed in duplicate. The gene of interest is present at ~ 3 kb which is the same size as the positive control and therefore verified as the correct product.

#### 3.2.1.2 Screening of transformed yeast colonies

Q23 was digested with *Sall* located within the URA gene that neutralises the activity of 5-fluoro-1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid (FOA) a compound toxic to yeast cells. Thus, when transformed cells were grown on FOA<sup>+</sup> plates, only those colony forming units (cfu) with a URA gene disrupted by the insertion of *env* would grow. Yeast cells were transformed with *env* PCR product

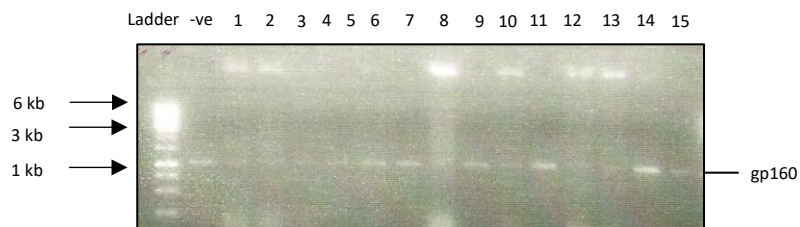
and linear Q23 for homologous recombination. Following transformation, the yeast cfu were screened by PCR to determine which colonies contained the *env* gene. Colony PCR was carried out using primers located within *env* so that a PCR product of 1 kb indicated the presence of *env* within Q23. The agarose gel revealed that cfu 2, 3, 4, 5, 6, 7 and 8 were positive for the presence of the *env* (Figure 3.2). These colonies were thus carried forward for plasmid extraction and subsequent electroporation into *E. coli*.



**Figure 3.2: Colony PCR of transformed yeast cells.** Following transformation of yeast cells with the *env* PCR product and linear Q23 HIV-1 IMC backbone, 9 colonies were selected for colony PCR to determine which colonies contained the *env* gene. -ve indicates the negative control: water only PCR, 1-9 indicates the colonies that were chosen for screening. The molecular weight marker (Biorad) is indicated.

### 3.2.1.3 Electroporation of *E. coli*

The plasmid DNA from the positive yeast cfu identified in section 3.2.1.2 was extracted and electroporated into electrocompetent *E. coli* cells. The colonies were screened by PCR to identify which colonies contained the *env* gene. A band was detected at the expected size of ~ 1 kb, however, due to the presence of a band in the negative control, we could not be sure that the samples expressing a band at 1 kb were true positive colonies (Figure 3.3). To confirm which colonies contained the *env* gene and to check for any mutations which many have occurred during the cloning process, these samples were all sent for sequencing.



**Figure 3.3: Colony PCR of electroporated *E. coli* cells.** After electroporation of *E. coli* cells with the plasmid DNA extracted from the yeast cfu, PCR was used to screen 15 *E. coli* cfu for the presence of *env*. -ve indicates the negative control: water only PCR, 1-15 indicate the colonies that were selected for screening. The arrow indicating gp160 refers to the 1kb band expected for positive colonies

#### **3.2.1.4 Sequencing results of *E. coli* colonies**

Sequences were aligned in BioEdit using the CAP210 E8 sequence as a reference. The colony chosen for subsequent experiments was shown to align to the reference sequence, indicating that a chimeric IMC – Q23\_CAP210 E8 – which contained the *env* gene had been constructed (See appendix Figure A1 for full sequence alignment).

#### **3.2.2 Cell types influence expression and processing of Env**

##### **3.2.2.1 Optimising transfections in HeLa and CHO cells**

The protocol for transfection of HEK293T cells has been optimised in our laboratory. However, this method did not yield positive results for HeLa and CHO cells with regard to Env expression and PSV and IMC production, and thus extensive optimisation was carried out. Table 3.1 shows the parameters that were modified to optimise transfections using various commercial transfection reagents. In addition, we also attempted to optimise DNA complex formation and cell lysis by varying the duration of these crucial steps. We identified that Genecellin with a ratio of 1:3 DNA:transfection reagent yielded the best results and this protocol was used for all subsequent experiments.

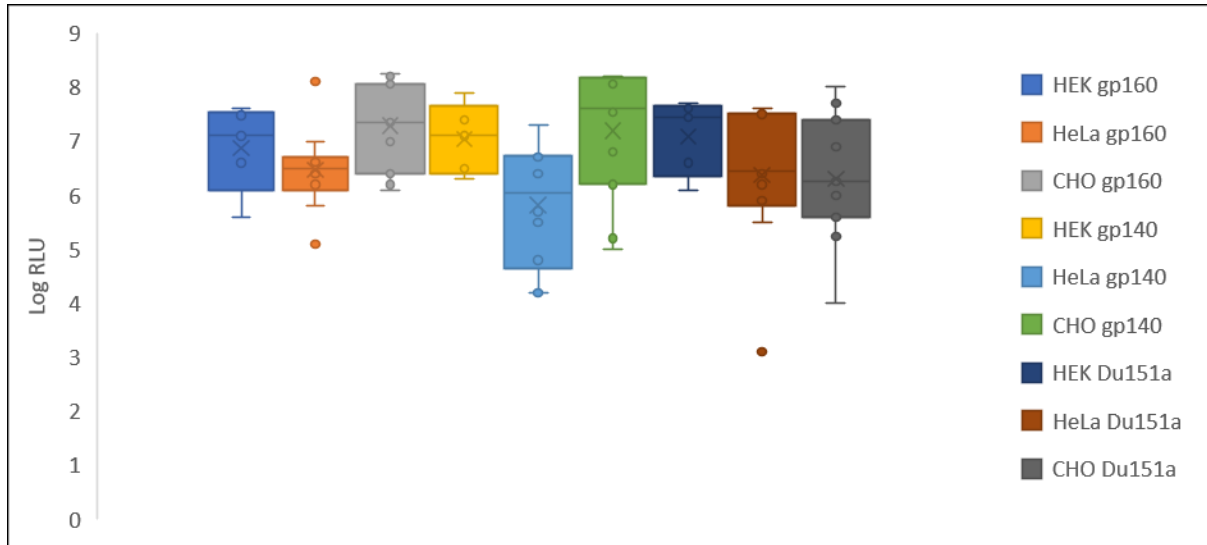
**Table 3.1: Optimisation of transfection of pGL4-luc in HeLa and CHO cells using luciferase assay.**

Transfection reagent	Ratio of DNA:transfection reagent	Expression levels	
		HeLa	CHO
PEI	1:3	+	-
	1:4	-	-
	1:5	-	-
	1:6	-	+
X-treme Gene HP	1:3.4	-	-
Lipofect amine	1:1.6	-	-
ViaFect	1.5:1	++	+
	2:1	++	+
	3:1	+++	+
	4:1	++	+
	6:1	++++	-
GeneCellin	1:2	+	+
	1:2.5	+	+
	1:3	++++	++++
	1:3.3	++++	++++
	1:3.75	+++	+++
	1:4	+	++
	1:5	+	++
	1:10	+	-

- indicates that there was no expression, +, ++, +++, +++++ indicates the level of expression with + showing low levels and +++++ showing high levels relative to each other.

### 3.2.2.2 Transfection efficiency

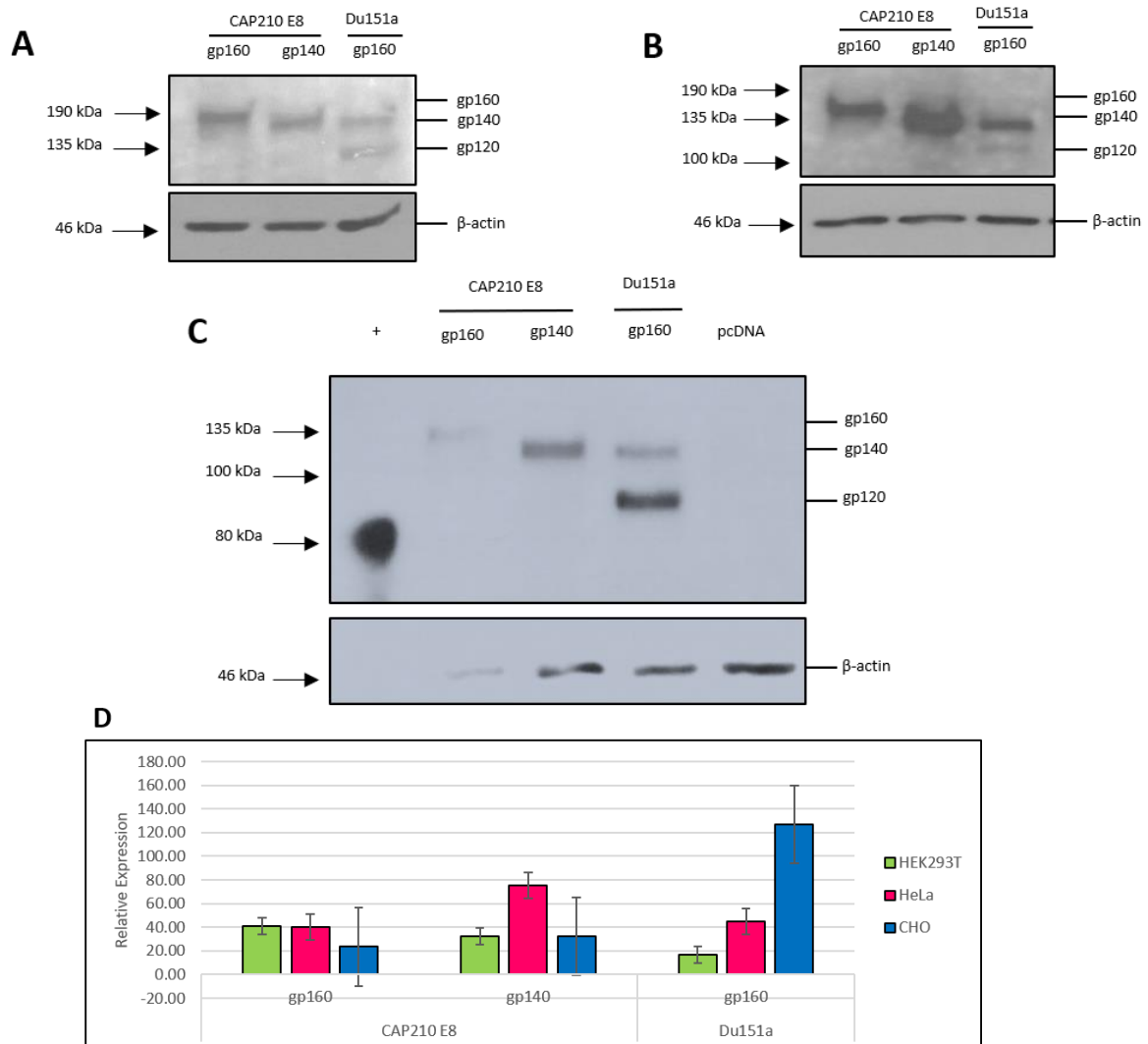
HEK293T, HeLa and CHO cells were co-transfected with either CAP210 E8 or Du151a and pGL4-luc, with the latter controlling for transfection efficiency via expression of the firefly luciferase gene. Cell types take up plasmid DNA at different efficiencies due to cell-specific expression of factors that facilitate uptake. For example, endocytosis and transport into the nucleus<sup>105</sup>. Therefore, differences in expression of Env might be due to variable transfection efficiency, both within and between experiments, and not intrinsic differences in gene-specific expression. After co-transfections of all samples and cell lysis, the intracellular expression of pGL4-luc was quantified using a luminometer. Figure 3.4 represents the transfection efficiency of 5-10 independent transfection experiments and statistical analysis indicated that there was no difference in expression between samples, suggesting that efficiency of the transfections should not influence the comparison of Env expression between cell types. However, to ensure accuracy, all expression, and incorporation data were normalised to the respective transfection efficiencies.



**Figure 3.4: Transfection efficiency in HEK, HeLa and CHO cells.** Cells were co-transfected with either CAP210 E8 (gp160, gp140), Du151a or pcDNA and pGL4-luc to measure efficiency at which plasmids were taken up and expressed by the different cells. The box and whisker plot depicts the efficiencies for samples transfected into three cell lines. Each box represents the distribution of RLU for 5-10 experiments which each had duplicate measurements. The duplicates were averaged and plotted above. Column statistics analyses was carried out using Prism 5.0 and showed no significant differences between transfection efficiencies between any of the samples.

### 3.2.2.3 Comparison of Env expression in different cell lines

Comparison of CAP210 E8 gp160, CAP210 E8 gp140 and Du151a gp160 levels, normalised to their respective  $\beta$ -actin loading controls and transfection efficiencies, indicated that HEK293T, HeLa and CHO cells expressed recombinant CAP210 E8 to similar degrees (Figure 3.5). Although not significant, CAP210 E8 gp140 expression was about 2-fold higher in HeLa cells compared to HEK293T and CHO cells. Du151a however was differentially expressed in CHO cells as gp160 levels were 16- and 15-fold higher than when produced in HEK293T and HeLa cells, respectively (Figure 3.6 D). This suggested that differences in expression of Env clones can vary depending on the cell type. For example, Du151a and CAP210 E8 gp160 are expressed similarly in HEK293T cells but differently in CHO cells. Therefore, the selection of cell type to compare the expression of different recombinant Envs will influence experimental outcome.



**Figure 3.5: Expression of CAP210 E8 gp160, CAP210 E8 gp140 & Du151a gp160 in HEK293T, HeLa & CHO cells.** A) Representative of 3 Western blots showing expression of gp160, gp140 & gp120 in CAP210 E8 and Du151a in CHO cells. B) Representative of 3 Western blots for HEK293T cells. C) Representative of 3 Western blots for HeLa cells. 100  $\mu$ g total protein was run on an SDS-PAGE gel and visualized via Western blot. Anti-gp120 antibody was used to detect gp120, gp160 and gp140 while anti- $\beta$ -actin was used to detect  $\beta$ -actin. '+' indicates gp120 positive control, pcDNA was used as a negative control.  $\beta$ -actin was used as the loading control. D) Densitometry (ImageJ) was used to determine the relative intensities of bands representing Env and  $\beta$ -actin for three independent Western blots. The average densitometry of Env of the three independent Western blots were normalised to that of  $\beta$ -actin as well as the respective transfection efficiency for each transfection experiment. Bars represent average relative expression and error bars represent standard deviation of the three Western blots for each cell line. Statistical analysis by one-way ANOVA conducted in Prism 5.0 showed no significance.

### 3.2.2.4 Effect of cell line on apparent molecular weight of Env

Western blotting indicated that molecular weights (MW) of Env varied across cell lines (Figure 3.5) (Table 3.2). When CAP210 E8 was expressed in HEK293T cells, there was only a very faint band corresponding to gp120 (approximately 150 kDa). The band representing CAP210 E8 gp160 corresponded to approximately 170 kDa, 187 kDa and 211 kDa in HEK293T, HeLa and CHO cells, respectively. Du151a gp160 and gp120 was approximately 189 kDa and 161 kDa in CHO cells, respectively; 192 kDa and 166 kDa in HEK293T cells, respectively; and 151 kDa and 132 kDa in HeLa

cells, respectively. There was a similar difference in MW between gp160 Du151a and gp160 CAP210 E8 in HEK293T and CHO cells (22 kDa) although CAP210 E8 was larger than Du151a when expressed in HEK293T cells but smaller when expressed in CHO cells. On the other hand, expression of gp160 CAP210 E8 was 36 kDa larger than Du151a when both clones were expressed in HeLa cells. As Env is highly *N*-glycosylated and glycosidase expression is highly dependent on cell type<sup>28</sup>, variation in MW is likely due to different cellular glycosylation machinery. When the sizes of CAP210 E8 gp160 and gp140 were compared across all cell types, gp160 MW varied from 211 – 170 kDa and gp140 ranged from 189 – 151 kDa. Similarly, Du151a also showed varying MW with gp160 ranging from 192 – 151 kDa and 166 – 132 kDa for gp120. Although we must take into account the limitation of SDS-PAGE to determine MW, overall, this suggests that cell types differentially glycosylated Env.

**Table 3.2: Molecular weight of CAP210 E8 and Du151a expressed in different cell lines.**

Cell line	CAP210 E8 *				Du151a #	
	gp160		gp140		gp160	
	gp160 (kDa)	gp120 (kDa)	gp140 (kDa)	gp120 (kDa)	gp160 (kDa)	gp120 (kDa)
HEK293T	170	150	159	134	192	166
HeLa	187	169	151	Not detected	151	132
CHO	211	192	189	Not detected	189	161

\* The MW of CAP210 E8 gp160, gp120 and gp140 were determined using the molecular weight marker of one Western blot used to measure Env cleavage (Figure 3.6).

# The MW of Du151a gp160 and gp120 were determined using the molecular weight marker of three independent Western blots used to measure Env expression (Figure 3.5)

### 3.2.2.5 Cleavage of Env in different cell lines

Cleavage of gp160 and gp140 is influenced by folding, trimerisation and *N*-glycosylation of Env as these factors influence the ability of furin to gain access to the cleavage site<sup>106</sup>. Therefore, one of the aims of this study was to compare the level of cleavage of Env in different cell lines noting that they express different levels of furin<sup>107</sup>.

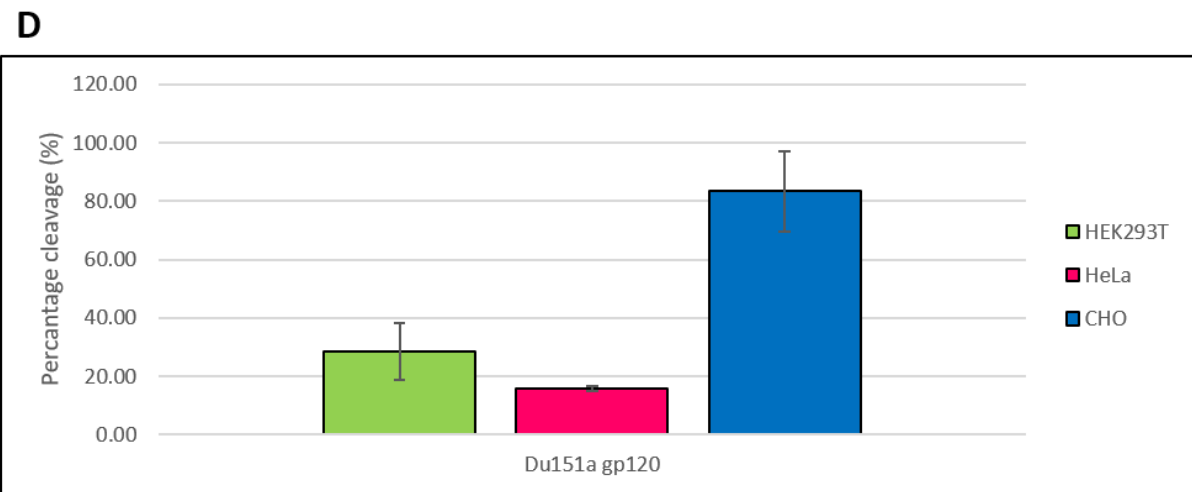
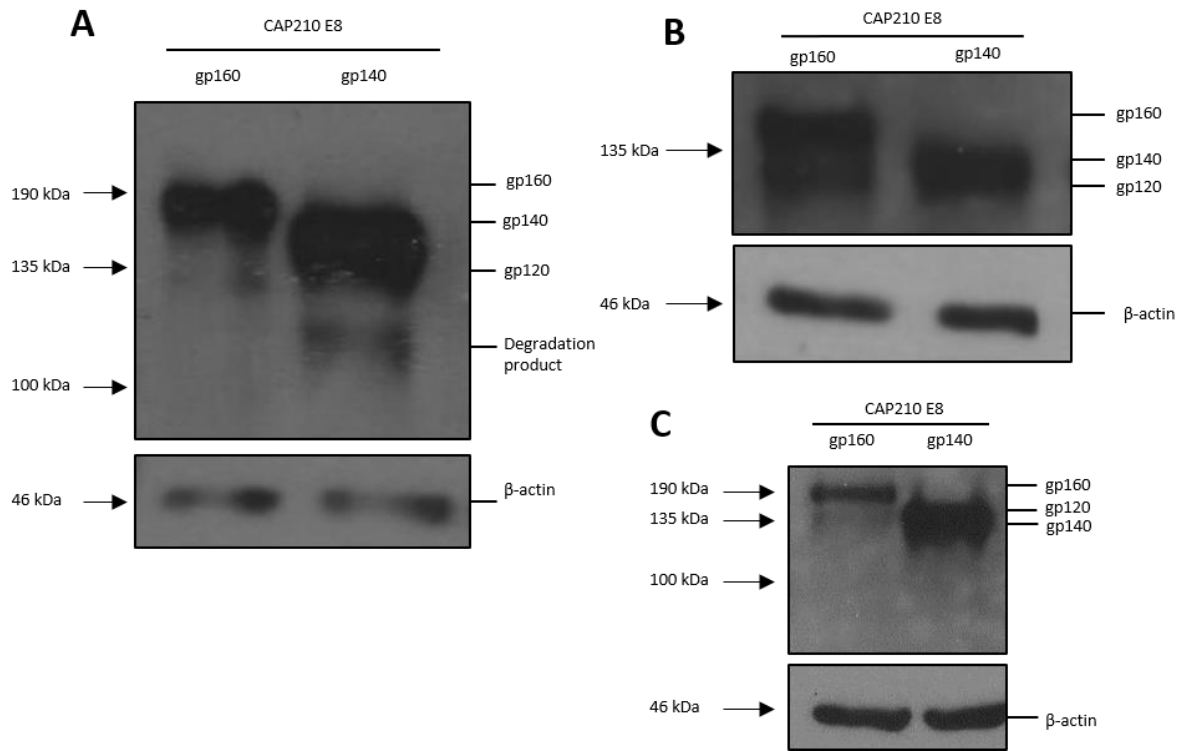
Uncleaved cell-associated gp160 can occur as a doublet with the higher MW of the two bands representing heavily sialylated Env. Furthermore, sialylated uncleaved gp140 occurs as a smear which might mask gp120 leading to underestimation of cleavage<sup>108</sup>. It was thus a challenge to determine cleavage of CAP210 E8 gp160 and gp140 using cell-associated samples. The use of a gp41-specific antibody would have distinguished gp120 from gp160, but our laboratory was unable to generate Western blots using anti-gp41 antibodies.

When CAP210 E8 was expressed in the various cell types, cleavage was not consistently detected (Figure 3.5). Figure 3.6 shows an example Western blot of an instance when gp160 cleavage was apparent. CAP210 E8 gp160 cleavage in HEK293T cells resulted in a weak gp120 band (Figure 3.6 A) suggesting that HEK293T cell proteases are inefficient at CAP210 E8 cleavage. This finding supports previous data which showed that Env cleavage occurs poorly in Env-transfected cells resulting in an accumulation of intracellular gp160<sup>106,109</sup> and that HEK293T cells produce low levels of furin<sup>107</sup>.

CHO cell proteases cleaved Du151a gp160 3-fold higher than HEK293T and 5-fold higher than HeLa cells. As we were unable to consistently show cleavage of CAP210 E8, we could not directly compare it to that of Du151a, but apparently, Du151a is more susceptible to cleavage than CAP210 E8. CHO cells might be better at Env cleavage as it was previously shown to produce a number of proteases, including furin that are able to cleave gp160<sup>110</sup>. Overall, this would suggest that like expression, cleavage is also cell type-specific and dependent on the Env clone analysed.

When gp140 was expressed in HEK293T cells a band corresponding to approximately 120 kDa was detected (Figure 3.5 A). However, this band did not represent gp120 because it did not correspond to the size of gp120 (150 kDa) when gp140 was expressed in HEK293T cells (Figure 3.5 A) and was most likely a degradation product. We were thus unable to determine the cleavage efficiency of CAP210 E8 gp140 (Table 3.2) possibly because the gp140 and gp120 bands were not resolved due to similar MW and extensive *N*-glycosylation.

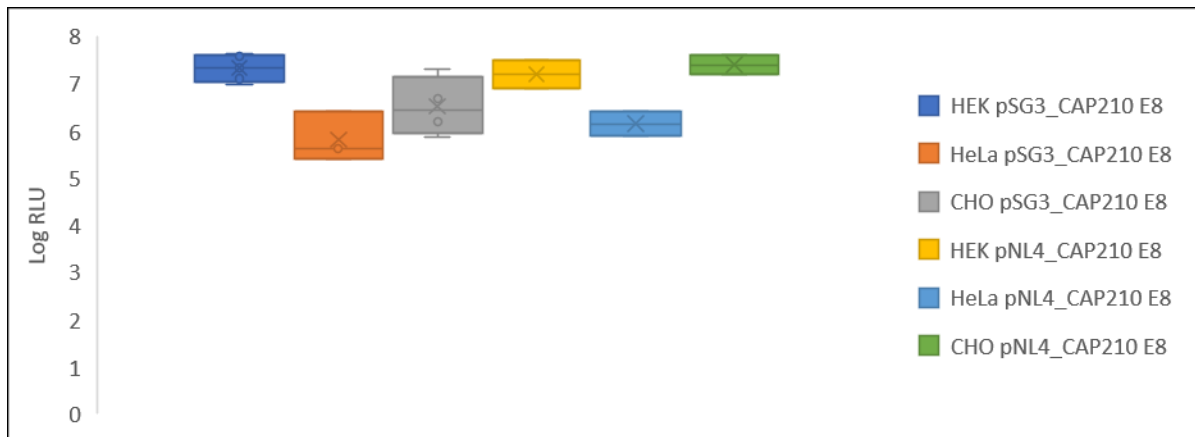
Previous studies have shown that HeLa cells express higher levels of furin than HEK293T cells and that in CHO cells furin is not the only protease responsible for Env cleavage<sup>107,110</sup>. Our results support these findings as CHO apparently cleaved Du151a and CAP210 E8 better than HeLa cells and both cell lines cleaved Env better than HEK293T cells. It has been reported that furin cleaves gp160 very inefficiently and some Envs are resistance to cleavage even when co-expressed with high levels of furin. Moreover, over-expression of furin resulted in a decrease in Env expression suggesting that this could be one reason why some cell lines express low levels of Env but exhibit high cleavage efficiency<sup>106</sup>.



**Figure 3.6: Expression of CAP210 E8 gp160, CAP210 E8 gp140 & Du151a gp160 in HEK293T, HeLa & CHO cells.** A) Representative of 3 Western blots showing expression of gp160, gp140 & gp120 in CAP210 E8 and Du151a in HEK293T cells. B) Representative of 3 Western blots for HeLa cells. C) Representative of 3 Western blots for CHO cells. 100  $\mu$ g total protein was run on an SDS-PAGE gel and visualized via Western blot. Anti-gp120 antibody was used to detect gp120, gp160 and gp140 while anti- $\beta$ -actin was used to detect  $\beta$ -actin.  $\beta$ -actin was used as the loading control. D) Representative of 3 Western blots showing Du151a cleavage. Expression was normalised to  $\beta$ -actin and transfection efficiencies to compare across cell types.

### 3.2.2.6 Transfection efficiency of pseudovirus

As in section 3.2.2.1, transfections to produce pseudovirus included co-transfection of pGL4-luc to determine the variability in transfection efficiency. Figure 3.7 shows that there was no significant difference in transfection efficiencies across Env constructs but there did seem to be a trend where HeLa cells were less easily transfected than the other cell types. We therefore normalised all expression data to transfection efficiency.



**Figure 3.7: Transfection efficiency in HEK, HeLa and CHO cells to produce pseudoviruses.** Cells were co-transfected with either CAP210 E8 and pSG3 $\Delta$ env pNL4.3-R-E-luc<sup>+</sup> and pGL4-luc to measure efficiency at which plasmids were taken up and expressed by the different cells. The box and whisker plot depicts the efficiencies for samples transfected into three cell lines. Each box represents the distribution of RLU for 5-10 experiments which each had duplicate measurements. The duplicates were averaged and plotted above. Column statistics analyses was carried out using Prism 5.0 and showed no significant differences between transfection efficiencies between any of the samples.

### 3.2.2.7 Determining the effect of viral proteins on Env expression

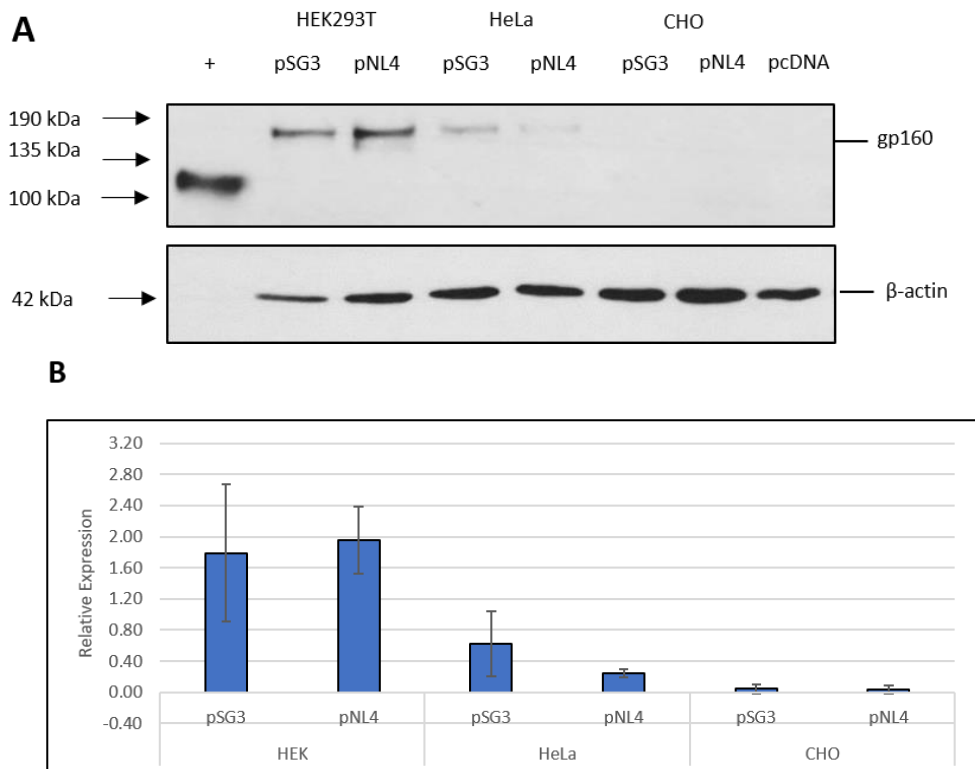
Despite high transfection efficiency of pGL4-luc, the expression of cell-, PSV- and IMC-associated Env was very low as determined by Western blotting. In many cases, we were unable to detect Env by Western blotting if we normalised cell lysates to 100 ng total protein or PSV and IMC samples according to p24 levels determined by ELISA. In the case of virus production, culture medium containing PSV and IMC was first ultracentrifuged to concentrate virus. To maximise the chance of detecting Env we then loaded the maximum sample volume allowed per well (16  $\mu$ l). After Western blotting Env densitometry was normalised to that of the loading controls (p24 or  $\beta$ -actin).

#### 3.2.2.7.1 Env expression during pseudovirion production

Although expression levels of recombinant Env did not differ across cell types in general, CAP210 E8 and Du151a were expressed and cleaved with differing efficiency as previously shown when Env clones JRFL and YU-2 were compared. This suggested that clones are differentially processed<sup>108</sup>. *In*

*in vivo* expression of Env involves the presence and activity of other viral proteins and we thus determined whether co-transfection with the HIV backbone influenced Env expression and cleavage. For example, it has been shown that the co-expression of Gag influences the conformation and incorporation of Env into viral particles which might be linked to Env trafficking and processing<sup>111</sup>.

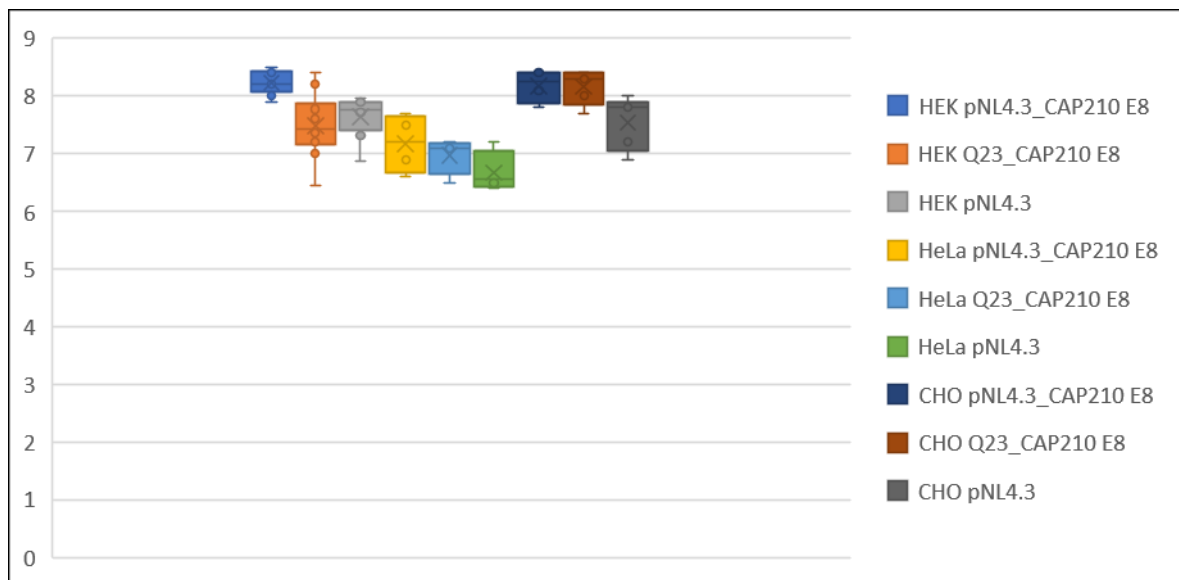
Two different PSV backbones, pSG3 $\Delta$ env and pNL4.3-R-E-luc<sup>+</sup> were included in co-transfections of HEK293T, HeLa and CHO cells with CAP210 E8 gp160. Western blot analysis showed that expression of Env with pSG3 $\Delta$ env and pNL4.3-R-E-luc<sup>+</sup> was higher in HEK293T than in HeLa cells, although this difference did not reach statistical significance. There was no detectable Env in CHO lysates generated in the presence of both backbones. Env expression in the presence of HIV backbones is thus highly dependent on cell type and not affected by the type of backbone (Figure 3.8).



**Figure 3.8 Cell-associated Env after co-transfection of different cell lines with PSV backbones.** A) HEK293T, HeLa and CHO cells were co-transfected with CAP210 E8 and either pSG3 $\Delta$ env or pNL4.3-R-E-luc<sup>+</sup>. Co-transfection with pGL4\_luc determined transfection efficiency. Cells were lysed after 48 hours, luminescence was determined using a luminometer and total protein concentration was determined. The Western blot was loaded with the maximum volume per well per sample and is a representative of two independent experiments. The blots were probed with sheep-anti-gp120 antiserum and  $\beta$ -actin primary antibodies. B) Densitometry analysis was carried out using Image J and normalized to the  $\beta$ -actin loading control and transfection efficiency. The average relative expression of two independent Western blots are indicated with error bars representing Standard deviation. One-way ANOVA was carried out using Prism 5.0 for statistical analysis but yielded no significance.

### 3.2.2.8 Transfection efficiency during production of infectious molecular clones

As in sections 3.2.2.1 and 3.2.2.5, transfection efficiencies did not differ significantly when IMCs were generated using either Q23 or pNL4.3 (Figure 3.9). However, transfection efficiency of HeLa cells tended to be lower than the other cell lines and so we continued to normalise expression data to transfection efficiency.

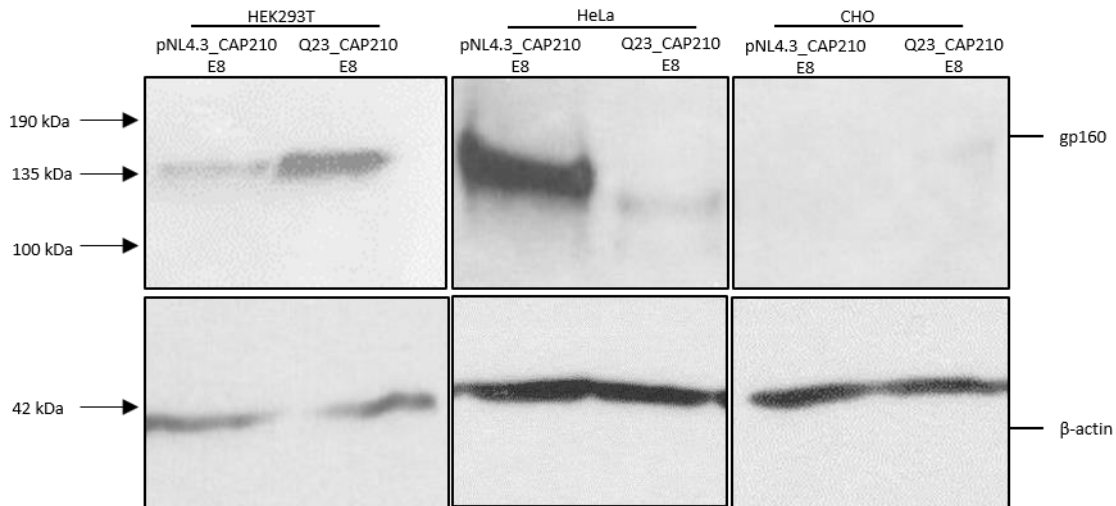


**Figure 3.9: Transfection efficiency in HEK, HeLa and CHO cells during production of IMCs.** Cells were co-transfected with either CAP210 E8 and pNL4.3 or Q23 and pGL4-luc to measure efficiency at which plasmids were taken up and expressed by the different cells. The box and whisker plot depicts the efficiencies for samples transfected into three cell lines. Each box represents the distribution of RLU for 5-10 experiments which each had duplicate measurements. The duplicates were averaged and plotted above. Column statistics analyses was carried out using Prism 5.0 and showed no significant differences between transfection efficiencies between any of the samples.

#### 3.2.2.8.1 Env expression during production of infectious molecular clones

IMCs express *env* from the LTR of the HIV backbone in concert with other viral genes,<sup>12</sup> whereas recombinant Env expression is driven by the CMV promoter. Production of Env in the presence of other viral proteins might influence its expression, cleavage and incorporation into viral particles. We determined whether CAP210 E8 expression, when expressed as IMCs, was influenced by Q23 and pNL4.3 backbones. Compared to Env expression in the absence of other viral proteins, Env was undetectable in CHO cell lysates except for a faint band corresponding to Q23\_CAP210 E8 (Figure 3.10). HEK293T cells expressed higher levels of Env within the context of Q23 than pNL4.3, while HeLa cells expressed pNL4.3\_CAP210 E8 better than Q23\_CAP210 E8. Cleavage of gp160 was not detected for any of the samples. The differences in expression are likely due to host cell-specific factors that preferentially interact with viral proteins expressed by one backbone and not another and/or

preferential binding of transcription factors to the LTR. Overall, this result suggests that when Env is expressed within the context of an IMC backbone, cell type and backbone identity influences experimental outcome.



**Figure 3.10: Cell-associated Env after co-transfection of different cell lines with IMC backbones.** A) HEK293T, HeLa and CHO cells were co-transfected with CAP210 E8 and either Q23 or pNL4.3. Co-transfection with pGL4\_luc was included to determine transfection efficiency. Cells were lysed after 48 hours, luminescence was determined using a luminometer. The Western blot was loaded with the maximum amount of total protein per well per sample and is a representative of two independent experiments.  $\beta$ -actin was included as a loading control. The Western blots were probed with sheep-anti-gp120 antiserum and  $\beta$ -actin primary antibodies. No statistical analysis was performed on this data as the two biological repeats were inconsistent with some bands appearing on one blot but not on the other.

### 3.2.2.8.2 Incorporation

Due to the variation in expression and cleavage of Env that might impact incorporation and infectivity, we next compared incorporation of Env in PSVs and IMCs. Incorporation of high levels of uncleaved gp160 might affect the infectivity of PSVs and IMCs, noting that cleavage is essential for virion infectivity<sup>112</sup>.

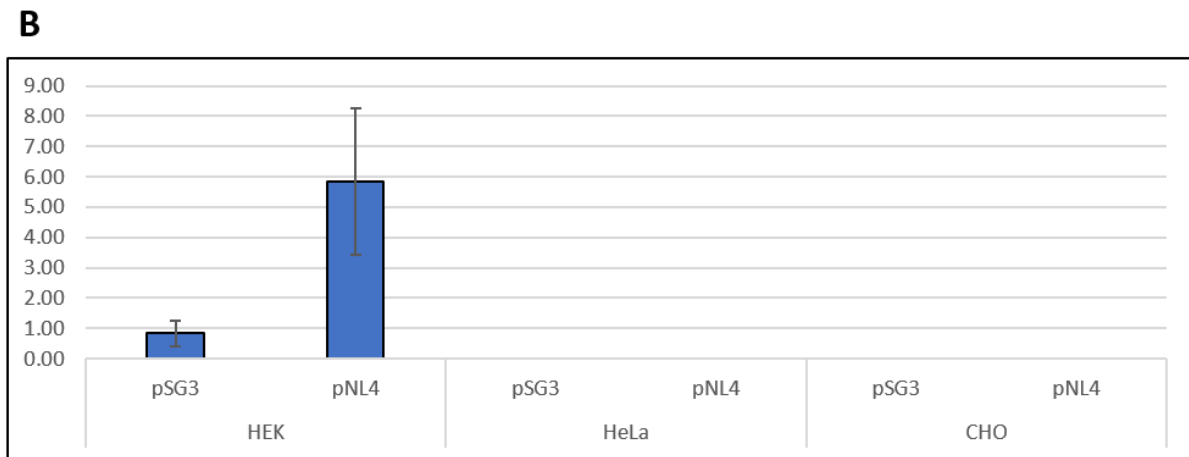
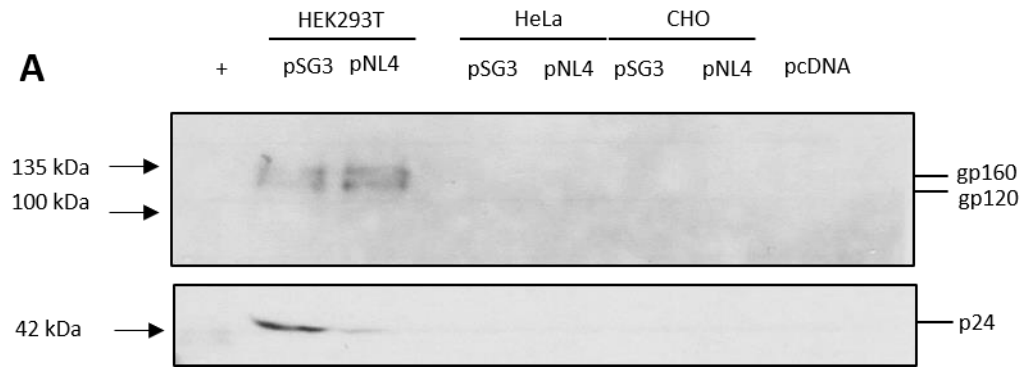
#### 3.2.2.8.2.1 Incorporation of Env into pseudoviruses

HEK293T, HeLa and CHO cells were transfected with pSG3 $\Delta$ env and pNL4.3-R-E-luc<sup>+</sup> as described in chapter 2. Even though the harvested pseudovirus culture medium was ultracentrifuged to concentrate the viral particles, p24 ELISA revealed that CHO and HeLa cells produced very low concentrations of viral particles ( $\sim 5 - 20$  ng/ml for CHO and  $\sim 5 - 30$  ng/ml for HeLa, with each backbone respectively). This was corroborated when no p24 or Env bands were detected by Western blotting. On the contrary, HEK293T cells produced high levels of PSVs ( $\sim 1000$  ng/ml with pSG3 $\Delta$ env and

~200 ng/ml with pNL4.3-R-E-luc<sup>+</sup>) with clear Env bands on the Western blot showing gp160 as well as gp120. The detection of a band corresponding to gp120 indicated that gp160 was cleaved into gp120 and gp41 (Figure 3.11). However, the gp160 band suggested that uncleaved, precursor gp160 was also being incorporated. Moore, et al. <sup>113</sup> reported a heterogeneous mixture of Env oligomers on the virion surface including uncleaved gp160 <sup>113</sup>. Moreover, findings by Dubay, et al. <sup>30</sup> suggested that the more uncleaved gp160 incorporated the less infectious the resulting virus <sup>30</sup>. Taken together, these results indicate that incorporation of uncleaved Env will have an impact on viral infectivity.

Although there was no difference in the levels of cell-associated Env when HEK293T cells were co-transfected with pSG3Δenv and pNL4.3-R-E-luc<sup>+</sup> (Figure 3.8), incorporation of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 was more robust than pSG3Δenv\_CAP210 E8 (Figure 3.11). pNL4.3-R-E-luc<sup>+</sup> differs from pSG3Δenv not only in the presence of the luciferase gene, but also that pNL4.3-R-E-luc<sup>+</sup> does not express functional Nef and Vpr proteins <sup>114</sup>. Nef has multiple functions which include trafficking of proteins and stabilising interactions between gp120 and gp41, affecting the conformation of Env – mechanisms suggested to influence HIV-1 infectivity <sup>115</sup>. It is thus possible that the lack of Nef expression resulted in better trafficking of pSG3Δenv\_CAP210 E8 to the plasma membrane and/or stabilising Env conformations that enhanced incorporation into PSVs compared to that of pNL4.3-R-E-luc<sup>+</sup>.

Even though HeLa cells expressed pSG3Δenv\_CAP210 E8 and pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 (Figure 3.8) there was no detectable virus-associated Env (Figure 3.11). This could be due to the very low levels of expression of Env in conjunction with the poor production of PSV by this cell line. HeLa cells did also have the lowest transfection efficiency (Figure 3.7). pSG3Δenv\_CAP210 E8 and pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 were not detected after transfection of CHO cells and this corresponded to the lack of virus-associated Env. Overall this data suggests that incorporation of functional Env is dependent on the producer cell as well as the PSV backbone.

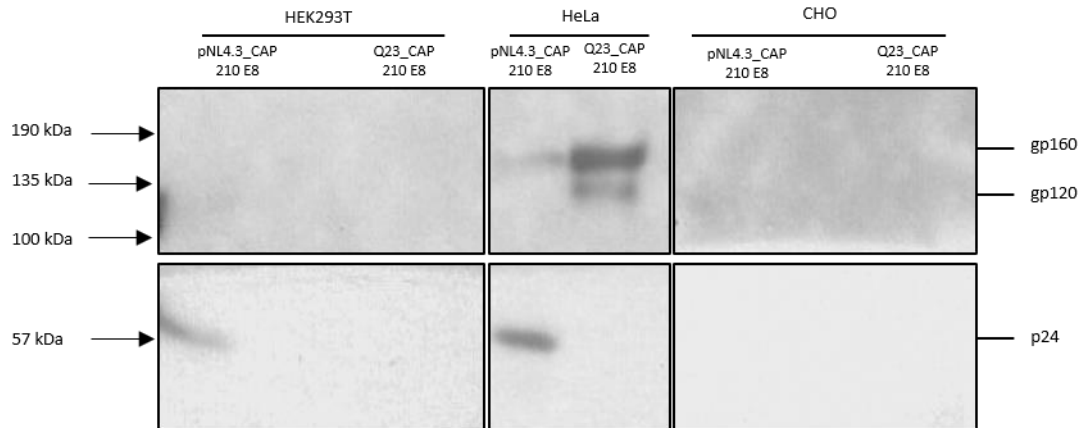


**Figure 3.11: Incorporation of Env into virus particles.** A) HEK293T, HeLa and CHO cells were co-transfected with CAP210 E8 and either pSG3 $\Delta$ env or pNL4.3-R-E-luc<sup>+</sup>. Cells were lysed and the maximum capacity of the well was loaded as we were unable to determine p24 concentrations. The PSV culture medium was harvested and ultracentrifuged for analysis of virion incorporation. The Western blot is a representative of two biological repeats. The blots were probed with sheep-anti-gp120 antiserum and rabbit-anti-p24 primary antibodies. Gp160 and gp120 bands were detected with a MW of 164 and 154 kDa, respectively. B) The cells were lysed for densitometric analysis. The values above represent the relative expression of Env normalized to  $\beta$ -actin and transfection efficiencies.

### 3.2.2.8.2.2 Incorporation of Env into infectious molecular clones

Results obtained for the incorporation of Env into IMCs were largely inconclusive considering that a band corresponding to p24 was only observed for pNL4.3\_CAP210 E8 when IMCs were produced in HeLa and HEK293T cells. This suggested that either cells were not producing sufficient infectious virus for detection, virus was lost during ultracentrifugation and/or experimental error affected Western blotting. It seems likely that the latter was responsible considering that Env was detected in only HeLa-derived IMCs, and in one instance, in the absence of p24. Although p24 was not detected, the

prominent band corresponding to gp120 for HeLa cell-derived Q23\_CAP210 E8 suggested that there was a high level of fully processed, functional Env incorporated into these virions.



**Figure 3.12: Incorporation of IMCs into HEK, HeLa and CHO cells.** Cells were co-transfected with IMC DNA constructs and a helper plasmid. The viruses were harvested and ultracentrifuged to concentrate them for visualisation via Western blot. pNL4.3\_CAP210 E8 is the IMC generated using a pNL4.3 backbone while Q23\_CAP210 E8 is the IMC generated via Q23 backbone. gp160 represents the uncleaved Env proteins and gp120 represents the cleaved portion. p24 was used as the loading control.

### 3.2.2.9 Processing: *N*-glycosylation

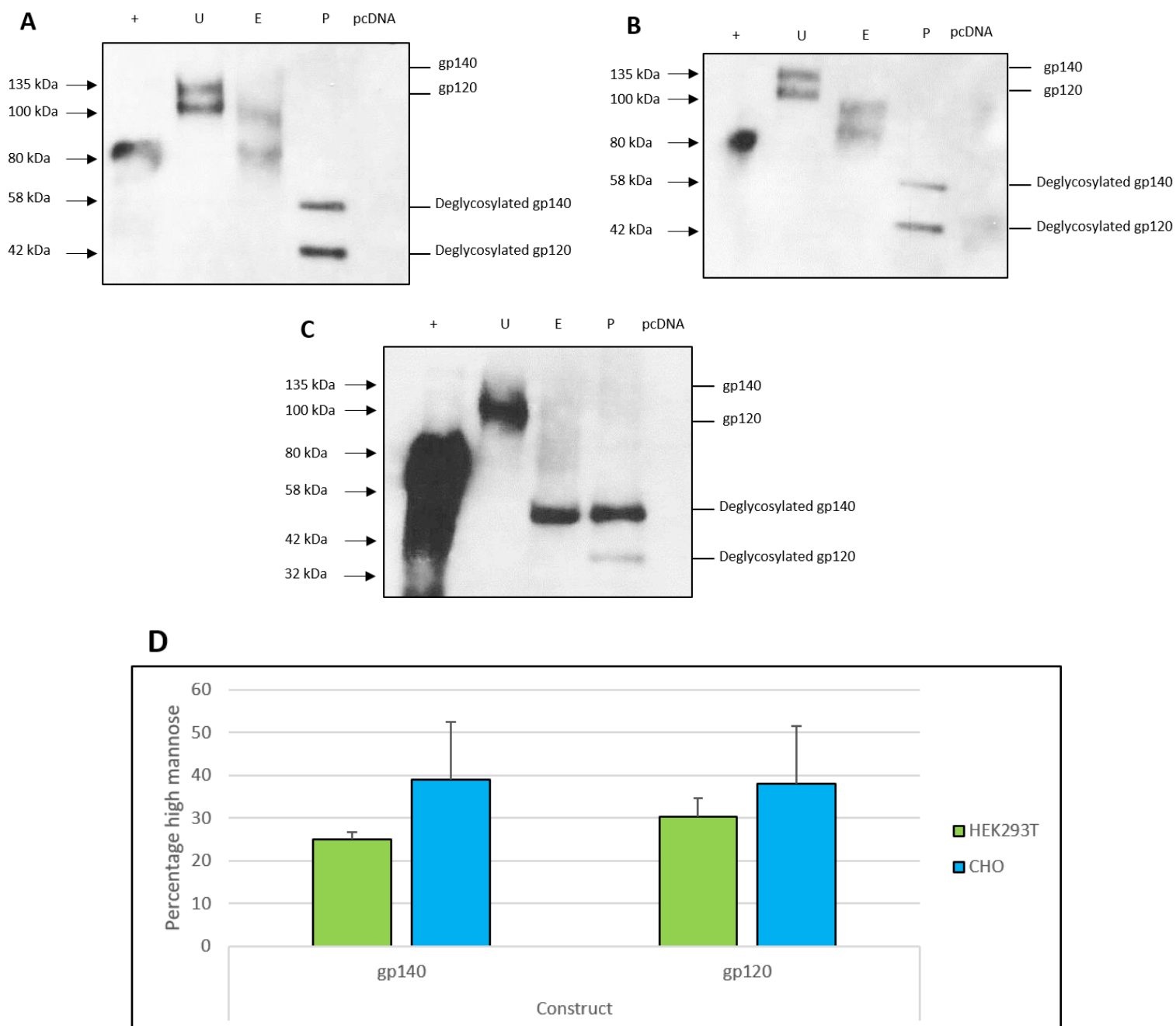
Env expression and processing is highly dependent on the cleavage of the Env signal peptide (Env-SP) in the endoplasmic reticulum (ER). The inherent slow cleavage of the Env signal peptide is essential for the correct folding, and thus, functions of Env. Experiments that included changes in the Env-SP found that increased expression led to changes in Env cleavage, incorporation, viral infectivity and aberrant Env *N*-glycosylation, supporting the link between expression and Env processing<sup>82,116</sup>. Next, we determined whether CAP210 E8 was differentially *N*-glycosylated.

#### 3.2.2.9.1 Differences in *N*-glycosylation between cell types

Given that *N*-glycosylation is dependent on the host cell machinery, we hypothesised that Env would have differential glycosylation patterns if expressed in different cell lines. Furthermore, differences in the MW of recombinant CAP210 E8 and Du151a expressed in CHO, HeLa and HEK293T cells suggested that Env was differentially *N*-glycosylated (Table 3.2). The soluble Env construct, gp140, was used to determine the percentage of high mannose associated with Env by digestion with endoglycosidases. Truncated gp160 lacking the cytoplasmic tail or transmembrane domain such as gp140 is a common method of expressing Env<sup>117,118</sup>.

Env treated with Endo H would be stripped of high mannose *N*-glycans, while PNGase F would lead to deglycosylation. The percentage high mannose was determined by comparing the difference in MW of untreated Env with demannosylated Env relative to the change in MW after deglycosylation. CAP210 E8 gp140 was 25 % and 39 % mannosylated when produced in HEK293T and CHO cells, respectively. Similarly, gp120 was 30% and 38% mannosylated by HEK293T and CHO cells, respectively. Contrary to results reported by Raska, et al. <sup>119</sup>, there was no significant difference in the proportion of high mannose *N*-glycans between HEK293T and CHO cells. Furthermore, the level of mannosylation of gp140 was similar to that identified previously by Go et al, (2011) (20 – 30 %) and less than virion associated gp120 (56 – 79 % oligomannose) <sup>29,55,56,120,121</sup>.

Interestingly, we found that most of the gp140 produced in HeLa cells carried mainly high mannose *N*-glycans as treatment with Endo H deglycosylated the glycoprotein (Figure 3.13 C). A previous study done by Bonomelli, et al. <sup>29</sup> showed that *N*-glycan composition shifted to predominantly high mannose when Env expression was increased <sup>29</sup>. HeLa cells expressed gp140 2-fold higher than HEK293T HeLa cells (Figure 3.5 D), suggesting that higher levels of CAP210 E8 might have led to increased mannosylation. It has been suggested that over expression of Env might overwhelm the cellular machinery so that Env accumulates in the ER and bypasses the normal processing pathway, leading to aberrant *N*-glycosylation <sup>48</sup>. *N*-glycosylation is important for Env function as expression of Env in the presence of tunicamycin, a glycosylation inhibitor rendered the glycoprotein non-functional <sup>88,122 123</sup>. Therefore, CAP210 E8 PSVs and IMCs produced in HeLa cells should not be infectious.



**Figure 3.13: N-glycosylation in HEK293T & CHO cells.** Cells were transfected with gp140, a soluble Env clone. 48 hours post-transfection, the culture medium was collected for binding of gp140 to lectin beads. The bound proteins were then digested with endoglycosidases and run on a Western blot. The graph is a representation of 3 independent experiments. A) Endoglycosidase digests of gp140 produced in HEK293T cells. B) Endoglycosidase digests in CHO cells. C) Endoglycosidase digests in HeLa cells. ‘+’ represents gp120 positive control, ‘U’ is untreated gp140 protein, ‘E’ represents gp140 digested with Endo H, ‘P’ represents gp140 digested with PNGase F and pcDNA was used as a negative control. D) Percentage high mannose of gp140 and gp120 constructs in HEK293T and CHO cells. The shift in molecular weight was calculated using the equation:  $(\text{MW of untreated gp140} - \text{Endo H-treated gp140}) / (\text{MW of untreated gp140} - \text{PNGase F-treated gp140}) \times 100$ . Statistical analysis was performed by one-way ANOVA in Prism 5.0 but yielded no significant differences.

### 3.3 Discussion

A number of studies have compared Env antigenicity and found that neutralisation varied depending on whether Env was cell- or virion- associated. The authors suggested that the conformation of Env differed depending on the expression system, which had important ramifications for immunogen design. This raised a number of questions regarding the most physiologically relevant expression system to use for the design of vaccine candidates <sup>108,124</sup>. It has been argued that the design of an efficacious vaccine depends on understanding HIV transmission and a number of investigations have characterised the Env of transmitted founders. Unfortunately, the studies have produced conflicting results possibly due to variation in expression systems. In this study, we aimed to compare expression systems to determine whether they resulted in differences in Env expression, cleavage, incorporation and *N*-glycosylation.

When CAP210 E8 was expressed in different cell lines, expression levels were very similar. CHO cells, on the other hand, expressed and cleaved higher levels of Du151a gp160 than the other cell lines. These differences suggest that cell-specific host factors interact with some Envs better than others leading to better expression and/or processing. There was no difference in expression of gp140 and gp160 within cell lines, suggesting that truncation of gp41 did not influence expression. However, loss of the cytoplasmic tail changes Env conformation <sup>125</sup> so that gp140 was no longer recognised by the antibody used in Western blotting. However, as we used a polyclonal anti-gp120 serum the effect of antibody affinity was limited.

The MW of recombinant CAP210 E8 gp140 varied according to cell line (ranged from 189 – 151 kDa) but was only marginally differentially mannosylated (HEK293T and CHO cells generated gp140 with 25 % and 39 % mannosylation, respectively and gp120 with 30 % and 38 % mannosylation, respectively). It is possible that the changes in mannosylation might not be the only reason for the variation in MW. Different levels of sialylation and/or fucosylation of complex *N*-glycans can also affect the apparent MW of glycoproteins <sup>126</sup>. Finally, the method we used for detecting mannosylation might not be sensitive enough to detect small changes in *N*-glycosylation and mass spectrometry would have been a better option. However, in our laboratory bulk purification of Env was unsuccessful, preventing analysis by mass spectrometry (unpublished data).

It was suggested that poor Env cleavage might contribute to differences in antigenicity (i.e. Env conformation) between cell-associated recombinant Env and virion-associated Env <sup>108,109</sup>. We did not observe consistent cleavage of CAP210 E8 across all independent experiments and this was likely due to poor expression and cleavage by all cell lines. It has been shown that recombinant Env introduced into mammalian cells by transfection are very poorly expressed and cleaved <sup>106,109</sup>. However, CHO cells

cleaved CAP210 E8 gp160 and Du151a gp160 better than the other two cell lines, potentially due to the expression of a number of proteases that cleave Env<sup>107,110</sup>. We could not reach any conclusions regarding the comparative cleavage efficiency of CAP210 E8 and Du151a due to the limited number of experiments showing consistent cleavage of both clones. However, cleavage of Du151a was consistently observed across all experiments. Provine, et al.<sup>12</sup> showed that cleavage of Env varied according to clone<sup>12</sup>, suggesting that Du151a is more susceptible to cleavage by furin (or other proteases) than CAP210 E8. However, because of low Env expression levels, we might have underestimated cleavage efficiency, especially noting that we did not have multiple biological repeats.

Expression of recombinant Env might not mimic that of *in vivo* expression. When we tested cell-associated Env levels during production of PSVs and IMCs there was no difference in expression of Env in the presence of either PSV backbone. However, HEK293T cells expressed much higher levels of PSV-associated Env than the other cells suggesting that HEK293T cells are better producers of PSVs. The presence of PSV backbones reduced Env expression levels relative to Env expressed on its own. In fact, CHO cells lost the ability to express detectable levels of CAP210 E8 gp160. This would then suggest that cell type and the presence of viral proteins affect Env expression.

When expression was tested in the presence of IMC backbones, CHO cells only expressed a weak band corresponding to Env Q23\_CAP210 E8. HEK293T and HeLa cells on the other hand, expressed Envs to varying levels depending on the IMC backbone. HeLa cells expressed pNL4.3\_CAP210 E8 to much higher levels than matched Q23 IMCs, and HEK293T cells expressed Q23\_CAP210 E8 more efficiently. The difference in expression of Env in the presence of PSV and IMC backbones could be due to interactions with other viral proteins such as Gag-gp41 interactions that influence Env concentration at the cell surface<sup>125</sup>.

Incorporation of Env into viral particles should be proportional to expression<sup>127</sup>. We could not detect Env in PSVs generated in CHO and HeLa cells and this could be due to poor expression. These cell lines produced PSV with very low titres, as measured by p24 ELISA, suggesting that the concentration of PSV was too low to detect Env incorporation. HeLa and CHO cells might produce factors that interfere with virus production<sup>128</sup>. When we tested HEK293T cells, we found that both gp160 and gp120 had been incorporated and this was consistent with the expression of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 and pSG3Δenv\_CAP210 E8. Similar to another study, despite poor cleavage by HEK293T cells, high levels of CAP210 E8 gp120 was incorporated into PSVs. Herrera et al, suggested that this could be due to preferential uptake of gp120<sup>124</sup>. Therefore, despite poor cleavage in HEK293T cells, high levels of CAP210 E8 gp120 might have been incorporated due to preferential uptake of gp120 by PSVs.

There were higher levels of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 incorporated into PSVs than pSG3Δenv\_CAP210 E8. pNL4.3-R-E-luc<sup>+</sup> differs from pSG3Δenv not only in the presence of the luciferase gene, but also that pNL4.3-R-E-luc<sup>+</sup> does not express functional Nef and Vpr<sup>114</sup>. Nef has multiple functions which include trafficking of proteins and stabilising interactions between gp120 and gp41, affecting the conformation of Env – mechanisms suggested to influence HIV-1 infectivity<sup>115</sup>. It is thus possible that the lack of Nef expression resulted in better trafficking of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 to the plasma membrane and/or stabilising Env conformations that enhance incorporation into PSVs.

Incorporation of Env into IMCs was inconclusive, most likely due to very poor viral titres in conjunction with experimental errors and these experiments must be repeated. CHO cells expressed high levels of recombinant Env, but could not produce detectable IMCs and PSVs, consistent with the absence of cell-associated Env. Therefore, CHO cells are apparently not good producers of PSVs or IMCs but produce recombinant Env at high levels, with efficient cleavage. This is supported by analysis of literature where papers do not report on using CHO cells for PSV and IMC production while it is one of the few cell lines licenced to produce recombinant protein vaccines<sup>129</sup>. Furthermore, it has been reported that CHO blocks infection of vaccinia virus and semlika virus, suggesting that it may not support expression of virus in general, including HIV<sup>128,130</sup>.

In conclusion, recombinant Env expression only differed between clones and not cell types, but the introduction of PSV and IMC backbones seemed to reduce expression and the extent of the decrease was determined by cell type. CHO cells expressed high levels of recombinant protein but were very weak producers of IMCs and PSVs, clearly indicating that some cells are not suitable to generate infectious viral particles. HEK293T cells seemed to be the better producer of PSVs than the other two cell lines and this was congruent with good Env incorporation. Overall, there was no clear pattern between expression systems (recombinant Env, PSVs and IMCs) and Env expression, cleavage, incorporation and *N*-glycosylation. However, the overarching outcome of this project is that the current methods used to study and produce Env are highly sensitive to cell type and backbone variation which makes it very difficult to compare the phenotype of different Env clones within and between studies.

## Chapter 4

### Comparing the function of Envelope using pseudovirion single round infection and infectious molecular clone replication assays

#### 4.1 Introduction

As discussed in Chapter 1, studies aimed at characterising TF Env have generated mixed results<sup>10-12,59,83,131</sup>. We hypothesised that the variation might be due to differences in study design, such as comparing PSV Env entry to IMC replication. It was shown previously that the antigenicity of Env varied when PSVs and IMCs were compared, suggesting that the methods used by different studies impact Env conformation and therefore function<sup>10,12</sup>. The objective of this chapter was to determine whether entry efficiency of PSVs and replication of matched chimeric IMCs, generated using the same Env, were similar when produced by different cell lines.

HIV-1 has high genetic diversity with nine subtypes and a number of circulating recombinants (Woodman and Williamson, 2009). It stands to reason that replication competent HIV requires the co-evolution of both structural and accessory genes to maintain functional interactions. If diversification of Env must coincide with concomitant evolution of the entire genome to maintain viral fitness, then the use of primary isolates should be the best method to evaluate viral phenotype and pathogenesis of circulating strains. However, the culture of primary isolates has the disadvantage of poor replication and rapid adaptation to laboratory conditions, influencing studies comparing the fitness of circulating strains<sup>132</sup>.

Studies on Env function usually use purified recombinant protein, PSV and/or IMCs with the latter two approaches using standardised, HIV genomes with high replication capacity. The use of standardised backbones potentially reduces variation when comparing different Env variants. For example, Tat's influence on viral entry will be constant for all PSVs<sup>133</sup>, however, when comparing different Env clones, backbone-derived protein might interact with Env differently due to differences in Env sequence. It was shown that PSVs derived from the same backbone, pNL4.3-R-E-luc<sup>+</sup>- pseudotyped with two different Envs, had different entry efficiency<sup>134</sup>. This could indicate intrinsic variation in function of the two Envs or the impact of backbone-derived proteins on Env processing, incorporation etc. To control for this, we used two different backbones to generate PSVs and IMCs.

The most common isolate modified for PSV and IMC production is the backbone pNL4.3, a subtype B full-length clone derived from the ligation of 5' and 3' fragments of the LAV isolate from a bacteriophage lambda DNA library<sup>104</sup>. For pseudovirus assays, pNL4.3 was modified to include a

luciferase gene within Nef, deletion within Env and inactivation of Nef and Vpr<sup>114</sup>. The subtype B pSG3 backbone was the first full-length genome cloned as a single provirus and became the standardised backbone for PSV neutralisation antibody assays. A stop codon was introduced within Env without disrupting the Vpu open reading frame to form pSG3Δenv<sup>103,135</sup>. This project utilised both pNL4.3-R-E-luc<sup>+</sup> and pSG3Δenv to analyse the entry efficiency of a subtype C Env, raising the question of whether subtype differences might influence the outcome of the experiments. Although a subtype C PSV backbone would have been preferable, at the start of this project, none were available. Furthermore, it has been shown that subtype C viruses replicate poorly and less efficiently than pNL4.3<sup>136,137</sup>.

When we compared chimeric IMC replication we used both pNL4.3 and Q23, a subtype A HIV provirus<sup>5</sup> to investigate the impact of incompatible subtype-subtype viral protein-protein interactions. Both pNL4.3 and Q23 proviruses were adapted for yeast recombination by introducing a URA3 gene within Env which enabled the insertion of CAP210 E8 to generate chimeric IMCs<sup>7,138</sup>. The final backbones: CMV –PBS-LTR-NL4-3Δgp160 URA and pRS315-Q23\_nef-HIS3\_V1-V5-URA3 differed in that Q23 did not express a functional Nef and was controlled by the LTR and not the cytomegalovirus promoter<sup>5</sup>.

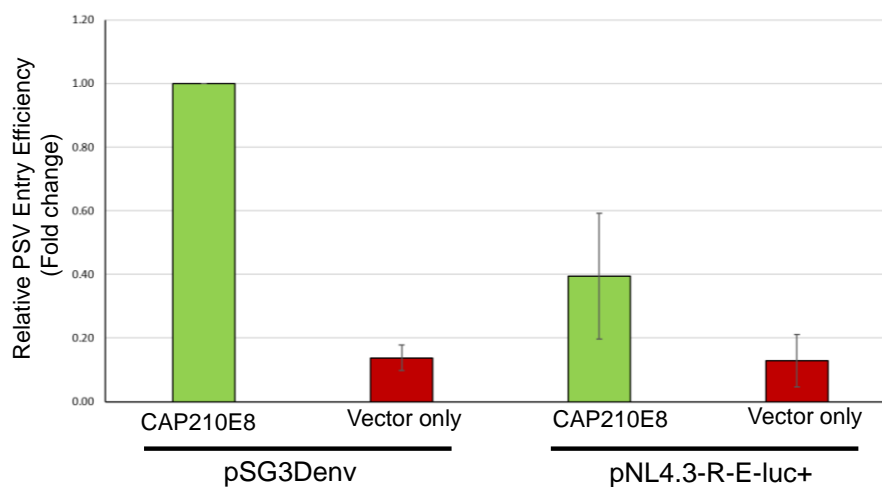
Although, the use of standardised HIV-1 backbones of different subtypes allowed us to determine whether backbones influenced the function of the same Env clone, it did not take into account the cell-specific influence of auxiliary proteins on Env function such as Vpu being essential to virion production in only some cells and not others<sup>139</sup>. This suggests that polymorphisms in backbone-expressed viral proteins might result in cell type specific variation in HIV replication independent of Env. For this reason, we used different cell lines to produce PSVs and IMCs and compared the same Env clone in conjunction with different backbones. Therefore, by comparing multiple factors and keeping confounders constant, we determined whether Env-driven entry of PSV and IMC replication was influenced by cell type (HEK293T, HeLa and CHO) or backbone (pNL4.3-R-E-luc<sup>+</sup> vs pSG3Δenv; pNL4.3 vs Q23).

## 4.2 Results

### 4.2.1 Entry efficiency

#### 4.2.1.1 Pseudovirus entry efficiency of TZM-bl cells

To investigate the effect of producer cell line and PSV backbone, TZM-bl cells were infected with virus pseudotyped with two Env clones. The entry efficiency of PSVs produced in HeLa and CHO cells was equal to the vector only control when generated with both backbones (data not shown), suggesting that these cells were poor producers of PSVs with CHO and HeLa cells producing concentrations of ~ 5 – 20 ng/ml and ~ 5 – 30 ng/ml, respectively even after concentration by ultracentrifugation.



**Figure 4.1: Relative entry efficiency of PSVs produced in different cell types with different PSV backbones.** pNL4.3-R-E-luc<sup>+</sup> and pSG3Denv were pseudotyped with CAP210 E8 or pCDNA3.1 plasmid (vector only control) in HEK293T cells. PSV titre was normalised to 100ng/ml of p24 before infection of TZM-bl cells. Entry of CAP210 E8 PSVs and vector only control was measured via luminescence and normalised to the entry of pSG3Denv\_CAP210 E8 produced in HEK293T cells to reduce error between biological repeats. Entry efficiency assays were done in duplicate and bar graphs represent the average of two independent experiments with error bars indicating standard deviation. One-way ANOVA analysis was carried out using Prism 5.0 but there was no significant difference:  $p > 0.05$

HEK293T cells produced high concentrations of pSG3Denv\_CAP210 E8 PSVs which were able to enter TZM-bl cells with approximately 8-fold higher entry efficiency compared to the vector only control (Figure 4.1). PSVs of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 had approximately 2.5-fold lower entry efficiency than those produced with the pSG3Denv backbone (Figure 4.1). The pSG3Denv backbone is thus better suited for the production of PSVs than pNL4.3-R-E-luc<sup>+</sup>. The lack of statistical significant difference between pSG3Denv\_CAP210 E8 and pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 entry efficiency was likely due to the high variation between biological repeats. However, the apparent difference in entry efficiency of pNL4.3-R-E-luc<sup>+</sup>\_CAP210 E8 and pSG3Denv\_CAP210 E8 suggests that backbone differences impacted the

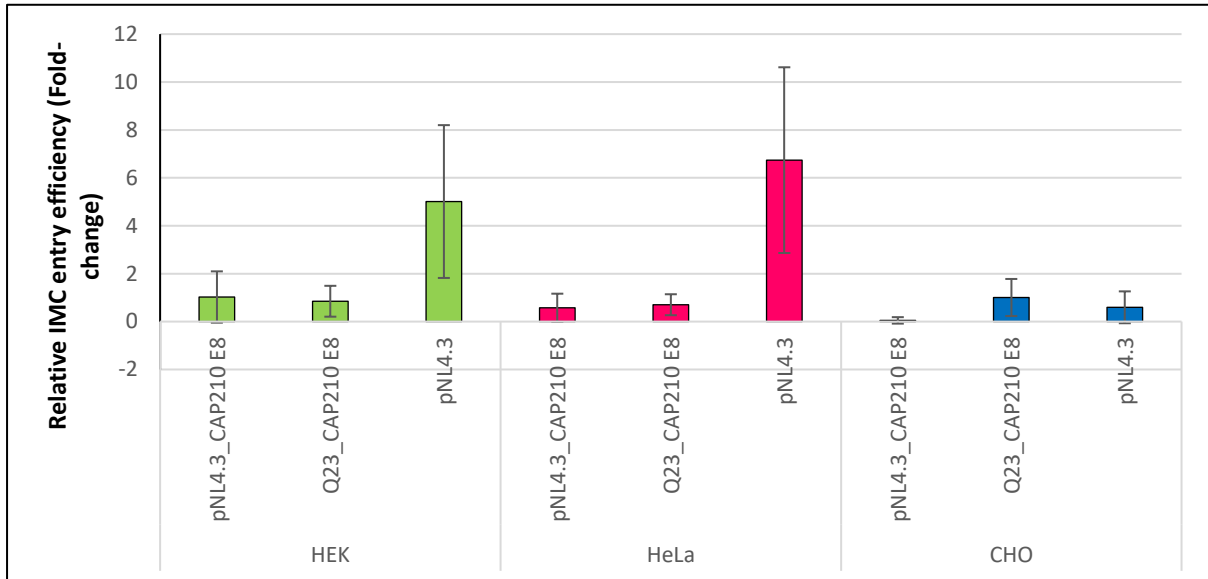
fitness of CAP210 E8. Overall, our results suggest that PSVs generated by different cells using different backbones are not comparable between studies.

#### **4.2.1.2 Chimeric IMC infection of TZM-bl cells**

Q23 and pNL4.3 chimeric IMCs carrying CAP210 E8 were generated by transfection of HEK293T, HeLa and CHO cell lines to compare IMC backbone and producer-cell effects on the ability of Env to mediate entry of TZM-bl cells. After 48 hours, viruses were harvested and quantified by p24 ELISA. However, all samples yielded very low or no p24 values. Thus, all samples were expanded in PBMCs to increase viral titre and subsequently used to infect TZM-bl cells. The deviation from our original protocol limited the extent of our analysis because expansion in PBMCs would likely abrogate all cell-specific effects achieved by transfection of different cell lines. However, to make it easier to distinguish samples we continued to refer to the IMCs based on their producer line.

Surprisingly, not all IMCs were able to enter TZM-bl cells even though they were passaged through PBMCs. This suggested that some cell lines were not good producers of IMCs and that expansion in PBMCs did not make a difference. This was highlighted when CHO cells apparently did not produce infectious IMCs irrespective of backbone and HEK293T and HeLa cells did not produce detectable pNL4.3\_CAP210 E8 and Q23\_CAP210 E8 IMCs (Figure 4.2). It is possible that all cell lines were unable to produce infectious IMCs carrying CAP210 E8 or the viral titre was too low for detection by luminescence.

When HEK293T and HeLa cells were transfected with the pNL4.3 provirus, the expanded IMCs infected TZM-bl cells similarly: approximately 5- and 7-fold, respectively, better than the cells only control (Figure 4.2). The similar infectivity of IMCs produced by the two cell types is likely because we are effectively comparing the same virus after expansion in PBMCs. As far as we know, the only difference between pNL4.3 provirus and pNL4.3\_CAP210 E8 is the presence of different Envs. Detectable replication of pNL4.3 provirus and not pNL4.3\_CAP210 E8 is likely due to Env-specific differences. On the other hand, IMC replication was also affected by backbone seeing that Q23\_CAP210 E8 IMCs infection of TZM-bl cells was not detected probably because the viral titre was too low. Therefore, at this point, we can only suggest that overall, the CHO cell line is not suitable for IMC production, HEK293T and HeLa cells produce detectable infectious IMCs for only certain Envs and pNL4.3 might be a good candidate backbone to use for IMC replication in PBMCs.



**Figure 4.2: Infection of IMCs produced in different cell types with different IMC backbones.** HEK293T (green), HeLa (pink) and CHO (blue) cells were transfected with the DNA of pNL4.3\_CAP210 E8, Q23\_CAP210 E8 and pNL4.3 provirus (AIDS Reagent Programme, NIH). TZM-bl cells were infected with IMCs after expansion in PBMCs and infection was measured by luminescence after 72 hours using BrightGlo (Promega). Infection was normalised to cells only control. Bars represent the average of two biological repeats each done in duplicate with error bars indicating standard deviation. Labelling is as follows: pNL4.3\_CAP210 E8 is the IMC generated using the pCMV-PBS-LTR-NL4-3Δgp160/URA backbone, Q23\_CAP210 E8 is the IMC generated with the Q23 backbone and pNL4.3 is the HIV-1 provirus which served as a positive control. No significant differences were found between any of the samples using the one-way ANOVA test in Prism 5.0.

## 4.2.2 Replication kinetics of infectious molecular clones

### 4.2.2.1 Identification of donor PBMCs permissive to IMC replication

Blood from the Western Province Blood Transfusion Service was obtained from 15 donors and used to isolate PBMCs for IMC expansion and replication assays. As PBMCs from different donors do not always support HIV replication, we tested the donors used in the replication assay for the ability to support replication of pNL4.3 provirus generated in HEK293T cells. Viruses were first expanded in a mixed pool of donor PBMCs before replication in specific donor PBMCs for +/- 14 days after which p24 concentrations were determined using an ELISA. IMCs of pNL4.3 provirus equivalent to 100 ng/ml p24 were able to replicate in two PBMC donors (Table 4.1).

**Table 4.1: Replication of pNL4.3 provirus in donor PBMCs after 14 days.**

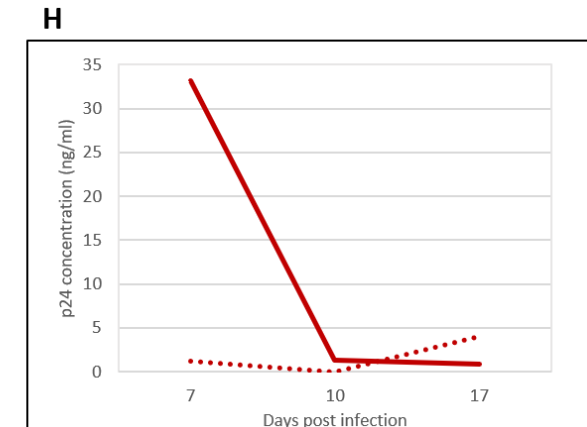
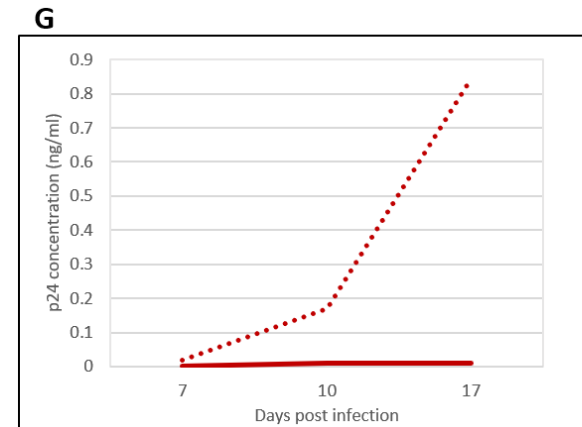
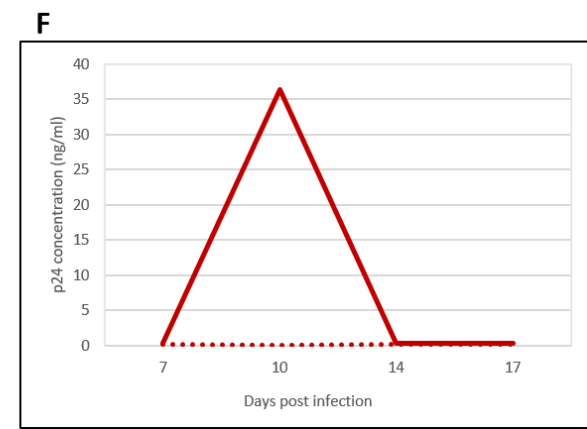
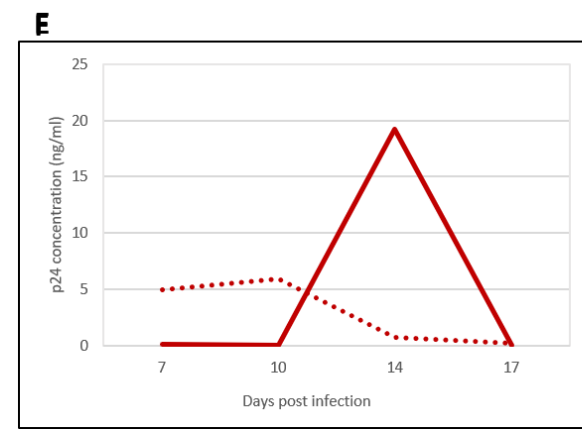
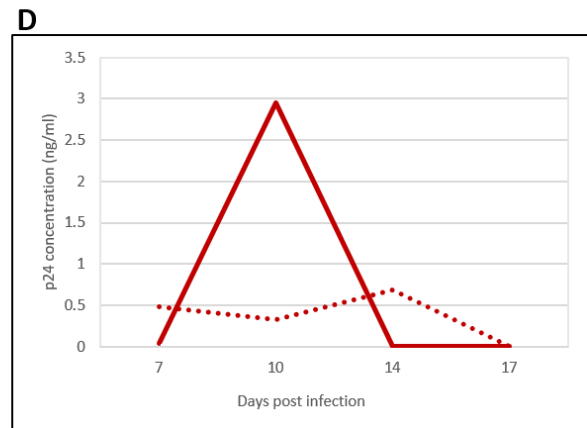
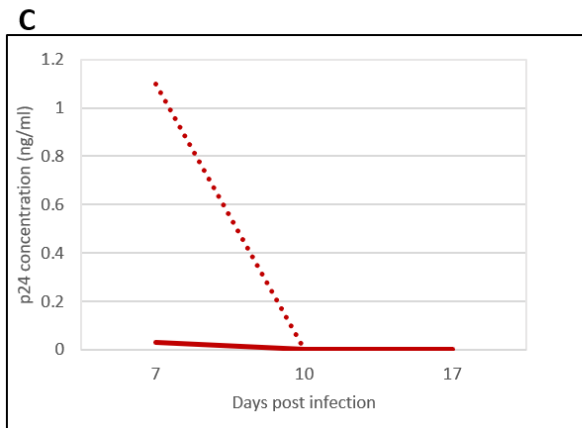
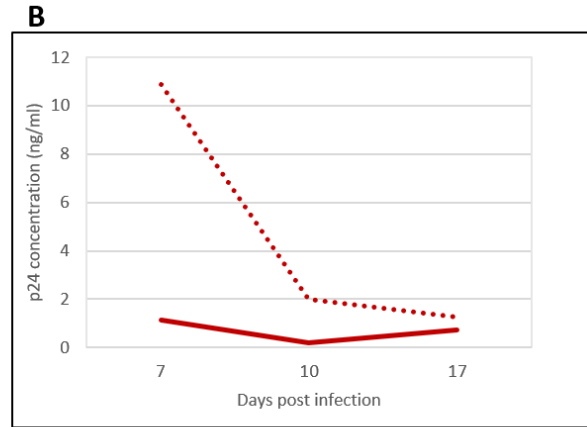
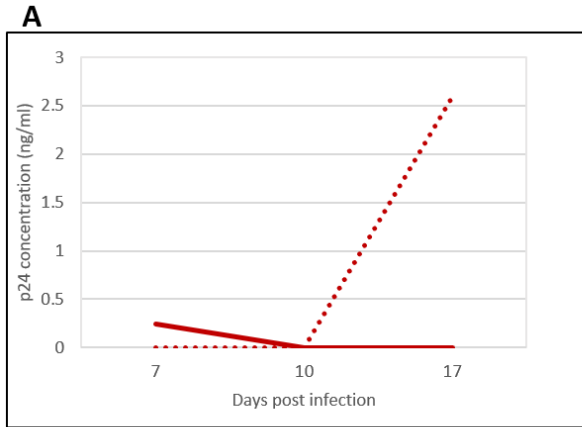
Donor	Donor 11		Donor 12	
	Day 4	Day 14	Day 4	Day 14
p24 concentration (ng/ml)	62.7	75.7	56.6	75.1

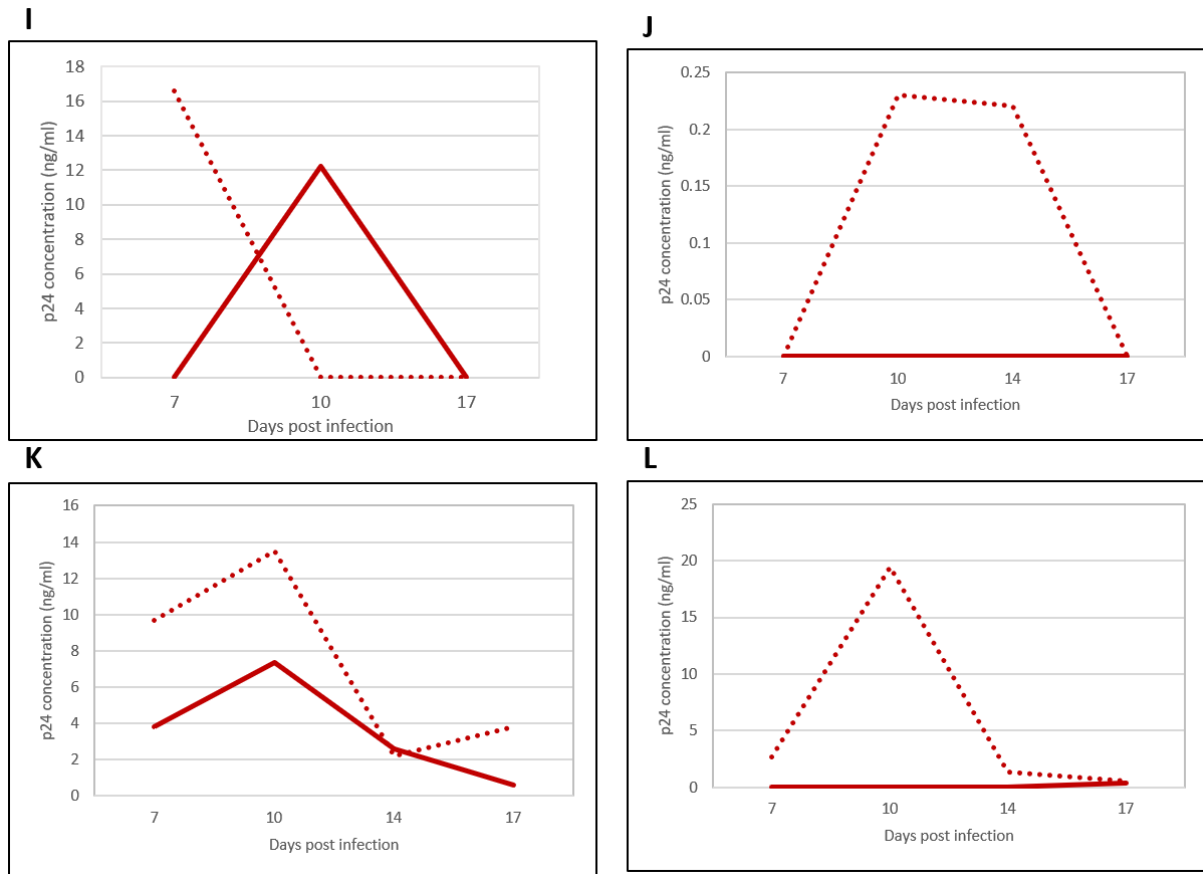
#### **4.2.2.2 Determining whether Env clones influence the replication of pNL4.3\_CAP210 E8 and pNL4.3 provirus**

As infection of TZM-bl cells was not consistently detected when inoculated with low virus concentration, samples were ultracentrifuged to concentrate the virus before replication in PBMCs. Despite expansion and ultracentrifugation, the concentration of virus was too low to determine TCID50 or p24 ELISA. PBMCs were thus infected with IMCs normalised to 15 x background luminescence obtained by infection of TZM-bl cells. As the titre of CHO-produced IMCs was lower than 15 x background total volume of virus was used for infection of PBMCs.

IMCs replicated for 7, 10, 14 and 17 days after which p24 concentrations were measured by ELISA. We first compared the impact of Env clones (pNL4.3 and CAP210 E8) on the replication of pNL4.3 IMCs. Figure 4.3 shows that the replication of the IMCs was highly variable. However, overall, there seemed to be consensus as the majority of experiments (8/12) indicated that pNL4.3\_CAP210 E8 replicated to higher titres than pNL4.3 provirus (Figure 4.3). As the IMCs were expanded in PBMCs, one would expect that all pNL4.3 provirus would replicate to the same level. However, 50 % of the experiments (6/12) indicated that pNL4.3 provirus was unable to replicate in PBMCs irrespective of donor and biological repeat. This unexpected finding could be due to the sensitivity of these assays to experimental error especially noting the variation between biological repeats using the same donor.

The results from PBMC replication experiments are contrary to the TZM-bl infection data where most pNL4.3 provirus were able to infect the reporter cell line (Figure 4.2). It is possible that the differences between these assays is because of variation in TZM-bl and PBMC host factors such as transcription factors that recognise the LTR enhancer sites. When we compared producer cell lines; in 3/4 experiments HEK293T cells produced 'non-infectious' pNL4.3 provirus compared to CHO cells that produced IMCs unable to replicate in PBMCs in only one experiment. This could indicate that producer cells might have an effect on subsequent IMC replication in PBMCs. This is unexpected as all viruses were expanded in PBMCs and should no longer carry any signatures derived from their producer cell line. It is interesting to speculate however that epigenetic changes to the LTR in the producer cells lines influence replication in PBMCs <sup>140</sup>.





**Figure 4.3: Comparison of the replication of pNL4.3\_CAP210 E8 and pNL4.3 provirus produced in different cell lines.** IMCs were generated in A, D, G, J) HEK293T, B, E, H, K) HeLa and C, F, I, L) CHO cells with pNL4.3 carrying either pNL4.3 Env (solid red line) or CAP210 E8 Env (dotted red line). PBMCs from donors 11 (A-F) and 12 (G-L) were infected with the maximum possible pNL4.3 provirus and pNL4.3\_CAP210 E8 produced in all three cell lines for biological repeats 1 and 2. All viruses were normalised to 15X background based on infection of TZM-bl cells as p24 concentrations were not detected. IMCs produced in CHO cells were not normalised to background and total virus was used for infections. Culture medium was collected on days 7, 10, 14 and 17 post-infection and viral titre quantified by p24 ELISA (ng/ml). pNL4.3\_CAP210 E8 is a chimeric IMC made using the pCMV-PBS-LTR-NL4-3Δgp160/URA backbone. Graphs indicate data of biological repeat 1 using donor 11 (A, B, C) and donor 12 (G, H, I) and biological repeat 2 using donor 11 (D, E, F) and donor 12 (J, K, L). Biological repeat 1, day 14 values were not included due to experimental error.

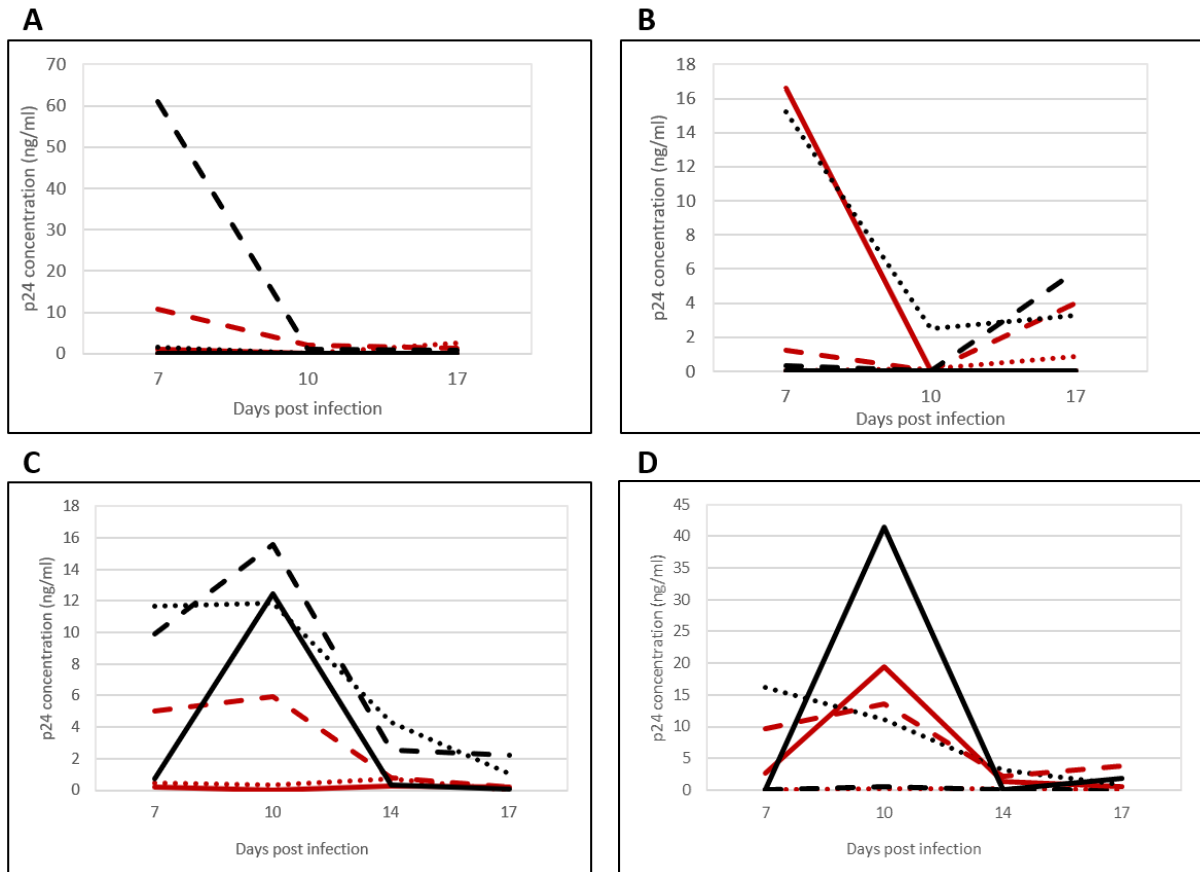
It is possible that we can no longer directly compare the replication pNL4.3 backbone constructs because manipulation during plasmid construction of pCMV-PBS-LTR-NL4-3Δgp160/URA backbone for homologous recombination might have altered the pNL4.3 provirus. In addition, the low concentrations of pNL4.3\_CAP210 E8 and pNL4.3 provirus IMC inoculum used in this project might also have influenced the outcome of the study. Overall, the results suggested that CAP210 E8-driven IMC replication might have higher replicative ability compared to IMCs carrying the pNL4.3 Env.

#### 4.2.2.3 Determining whether backbone differences influence the replication of pNL4.3\_CAP210 E8 and Q23\_CAP210 E8

After finding that Envs seemed to impact replication of IMCs, we sought to compare the effect of backbones on IMC replication. We transfected HEK293T, HeLa and CHO cells with pNL4.3\_CAP210 E8 and Q23\_CAP210 E8, harvested the virus, ultracentrifuged and expanded in PBMCs. After 17 days of expansion, the viruses were ultracentrifuged again and then used to infect PBMC donors 11 and 12 in two independent experiments. There seemed to be a trend where 7/12 experiments indicated that Q23\_CAP210 E8 were able to replicate to higher titres than pNL4.3\_CAP210 E8 (Figure 4.4) (Table 4.1). However, there was a high level of variation which might have influenced the findings. High variation in donor PBMCs has been reported <sup>141</sup>. To make definitive conclusions regarding the effect of backbone on replicative fitness, these experiments need to be repeated.

**Table 4.2 Rank order of Q23 and pNL4.1 replicative fitness according to cell line used to produce IMCs**

	Biological Repeat 1		Biological Repeat 2	
	Donor 11	Donor 12	Donor 11	Donor 12
HEK293T	Q23_CAP210 < pNL4.3_CAP210	Q23_CAP210 < pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210
HeLa	Q23_CAP210 > pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210	Q23_CAP210 < pNL4.3_CAP210
CHO	Q23_CAP210 < pNL4.3_CAP210	Q23_CAP210 < pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210	Q23_CAP210 > pNL4.3_CAP210



**Figure 4.4: Comparison of the replication of pNL4.3\_CAP210 E8 and Q23\_CAP210 E8 produced in different cell lines.** pNL4.3\_CAP210 E8 (red lines) and Q23\_CAP210 E8 (black lines) IMCs were generated in HEK293T (dotted line), HeLa (dashed line) and CHO (solid line) cells. A) Donor 11 and B) donor 12 PBMCs, were infected with either IMC equivalent to 15X background luminescence or the total volume of virus obtained after ultracentrifugation as we did not obtain p24 concentrations. Virus supernatants were collected on days 7, 10, 14 and 17 post-infection and quantified by p24 ELISA (ng/ml). pNL4.3\_CAP210 E8 is a chimeric IMC made by inserting subtype C CAP210 E8 into the pCMV-PBS-LTR-NL4-3Δgp160/URA<sup>5</sup> backbone and Q23-CAP210 E8 was generated by inserting subtype C CAP210 E8 into pRS315-Q23\_nef-HIS3\_V1-V5-URA3<sup>7</sup>. Data represents biological repeat 1 (A and B) and 2 (C and D).

#### 4.4 Discussion

Considering that many HIV studies employ the PSV and IMC systems for determining Env function, it is reasonable to question whether using different backbones and cell types influence study outcomes. The results obtained in this study suggest that expression systems and backbone identity do influence the ability of the virus to enter cells.

Firstly, we must acknowledge the variation we observed between biological repeats using the same constructs and donor PBMCs. Here we see that even using the same donor, the assay is highly susceptible to variation. We plan to do more biological repeats using the same donor PBMCs and taking into account the titre of the infecting IMCs as this was likely the biggest confounding factor of

this study. Secondly, the intention of these experiments was to compare IMCs that came directly from HEK293T, HeLa and CHO cells. However, due to the low titres obtained after the initial transfection of these cell lines, the viruses were expanded in PBMCs prior to investigating their replication. This means that the effect of cell type on IMC production may have been lost after multiple rounds of passage through PBMCs. Therefore, to make proper comparisons of cell type effect on IMCs, we will have to repeat these experiments without expansion in PMBCs.

Despite this consideration, we compared 1) the entry efficiency of PSVs generated by the different cell lines and 2) the ability of IMCs generated by different backbones and carrying different Envs to infect TZM-bl cells and replicate in PBMCs.

When PSVs were generated with pSG3Δenv and pNL4.3-R-E-luc<sup>+</sup>, only HEK293T cells were able to produce virions that infected TZM-bl cells, whereas PSVs produced in HeLa and CHO cells were not detected using the reporter cell line. During production of PSVs Env was expressed best in HEK293T cells, weakly in HeLa cells and apparently, not at all by CHO cells. As HeLa cells expressed Env we were surprised that PSVs could not infect TZM-bl cells. HeLa cells and CHO cells have been shown to produce tetherin which prevents the release of viral particles at the cell membrane <sup>142,143</sup>. On the other hand, HEK293T cells do not produce tetherin <sup>144</sup>. It is thus possible that HeLa and CHO cells are unable to release high levels of PSV due the expression of tetherin. However, pSG3Δenv and pNL4.3E-R-luc<sup>+</sup> express Vpu, which counters the effect of tetherin.

It is the expression of Vpu that enables PBMCs to support HIV infection. <sup>145</sup>. In order for tetherin to prevent the release of HIV, it must be trafficked to the plasma membrane. At the cell surface, it binds virus and inhibits release. Vpu reduces tetherin levels at the cell surface by sequestering tetherin in the trans golgi network <sup>87</sup>. A functional Vpu was expressed by all PSV and IMC backbones used in this project. Therefore, IMCs of pNL4.3 provirus, pNL4.3\_CAP210 E8 and Q23\_CAP210 E8 were able to replicate in PBMCs due to the presence of a functional Vpu, albeit inconsistently with pNL4.3\_CAP210 E8 unable to replicate to detectable levels in some experiments and then outgrowing pNL4.3\_CAP210 E8 in others.

The lack of consistency between experiments could be due to a number of factors. When IMCs were produced in HeLa and CHO cells, tetherin might have reduced the amount of virus released so that less virus was added to the TZM-bl cells and PBMCs. The titre of IMCs was probably too low to induce a detectable luciferase signal after 72 hours infection of TZM-bl cells but that repeated cycles of replication in PBMCs increased virus concentration sufficient for detection by p24 ELISA. However, the low concentration of the inoculum would have affected how rapidly the virus was able to replicate in PBMCs to a detectable level. This would explain why IMCs produced by CHO cells were able to 1)

replicate in PBMCs 2) but were unable to enter TZM-bl cells and 3) did not produce detectable levels of PSVs.

Another confounding factor that might have influenced direct comparison between pNL4.3 Env and CAP210 E8 and the ability of IMCs to mediate entry of TZM-bl cells and PBMCs is that CAP210 E8 is R5- and pNL4.3 is X4-tropic. However, TZM-bl cells, derived from HeLa cells also express endogenous CXCR4 co-receptor, similar to PBMCs so it is highly unlikely that tropism affected the study outcome<sup>146</sup>. However, comparing Envs with the same tropism expressed by the same backbone would have helped limit confounding factors. There might also have been other factors that played a role in the variation in our findings, noting the low expression of Env by some cell lines discussed in chapter 3. Finally, the variation between experiments was also affected by experimental error worsened the poor virus production.

Results from the study conducted by Ndung'u et al. (2001) suggested that a subtype C IMC replicated significantly less efficiently than a subtype B IMC, pSVIII/89.6. When considering the results obtained for the replication assay in our study, it is possible that the subtype used to generate IMCs might have played a role in the production of the virus. Q23\_CAP210 E8, the subtype A backbone, apparently did not produce IMCs able to infect TZM-bls, similar to pNL4.3\_CAP210 E8, the subtype B backbone. However, comparisons between Q23\_CAP210 E8 and pNL4.3\_CAP210 E8 IMC replication in PBMCs suggested that Q23 was more compatible with CAP210 E8 than pNL4.3 as it replicated more often to higher titres. However, the high variation between backbones and donors as well as between biological repeats suggests that more than one factor influenced the ability of CAP210 E8 to mediate infection of the IMCs.

Overall, this data suggests that the selection of cell lines to produce PSVs and IMCs are crucial to the outcome of experiments looking at entry of a reporter cell line such as TZM-bl cells and that comparison of Env phenotype should be done using a compatible backbone. Therefore, comparison of Env function is most likely best achieved through replication of chimeric IMCs, generated using the same backbone, in PBMCs isolated from a number of donors to reduce the effect of donor variability.

# Chapter 5

## Conclusion

### 5.1 Introduction

Previous studies conducted by Louder, et al. <sup>10</sup> and Provine, et al. <sup>12</sup> presented conflicting results regarding the characteristics of PSVs and IMCs. Their findings were important as a number of studies utilised either PSVs or IMCs to characterise HIV transmission. We therefore compared the effect of producer cell type, HIV backbone, and Env clone on the entry and replication of PSVs and IMCs and whether any variation could be explained by differences in Env expression, cleavage, incorporation and *N*-glycosylation. In this chapter we will provide an overview of our data to highlight the importance of standardising methods across laboratories, especially when identifying characteristics of TF for vaccine development. The data shown in the tables below were derived from graphs in chapters 3 and 4 and in order to compare the data, positive controls or background controls were used as references to generate relative values depicted as either + (weak), ++ (moderate) or +++ (strong).

### 5.2 Comparison of Env expression and incorporation, with PSV entry and IMC replication

**Table 5.1: Summary of characteristics of matched CAP210E8 pseudovirus and chimeric infectious molecular clone generated with the pNL4.3 backbone**

Cell type	PSV Env expression	IMC Env expression	PSV Env incorporation	IMC Env incorporation	PSV entry	*IMC entry	#*IMC replication
HEK293T	+++	++	+	-	+++	-	+++
HeLa	+	+++	-	++	-	-	++
CHO	-	-	-	-	-	-	+++

\* This was based on IMC expanded in PBMCs.

# Average relative to pNL4.3 provirus replication shown in Figure 4.3. Rough analysis: CAP210 E8 = pNL4.3: ++; CAP210 E8 > pNL4.3: +++; CAP210 E8 < pNL4.3: +

In Table 5.1 PSV entry appeared to be host cell-specific as the HEK293T cell line were the only cells that produced PSVs able to infect TZM-bl cells. This coincided with expression and incorporation of Env into PSVs; HeLa and CHO cells did not generate PSVs with detectable levels of Env. CAP210 E8 gp140 was highly mannosylated by HeLa cells and we hypothesised that PSVs produced by these cells would be non-infectious. It has also been shown that incorporation of Env is cell type-dependent because of host cell factors interacting with the cytoplasmic tail of gp41 <sup>127</sup>. Our experiments corroborate these findings and suggest that HEK293T cells might express factors that enhance Env incorporation into PSVs resulting in higher entry of TZM-bl cells compared to other cell lines. As

discussed before, HeLa and CHO cells might also produce tetherin which might have influenced release of HIV, affecting viral titre.

Contrary to PSV entry data, HEK293T cells did not produce IMCs able to infect TZM-bl cells. This was an unexpected result as HEK293T cells are routinely used for IMC production and it is likely that experimental error led to very low levels of virus production rather than non-infectious IMCs. After transfection of all cell lines, p24 ELISA did not detect CAP210 E8 IMCs and we thus expanded the IMCs in PBMCs which were then normalised based on luciferase readings in TZM-bl cells. We isolated too little virus to calculate p24 levels or TCID50 and it is possible that we miscalculated the titre which ultimately affected the TZM-bl entry and IMC replication assays. As the IMCs were passaged through PBMCs we could not directly compare the effect of producer cells on IMC entry with that of PSV entry.

To measure IMC replication, expanded virus was ultracentrifuged, normalised to TZM-bl luciferase readings and used to infect PBMCs. Contrary to the TZM-bl entry data, we detected replication with IMCs produced in all cell lines with those originally generated in HEK293T and CHO cells being better producers of infectious virus than HeLa cells. However, this difference is also likely due to experimental error as any cell-specific effect of producer cells on Env function and/or processing and IMC replication would have been lost during expansion in PBMCs. Louder, et al. <sup>10</sup> found that passaging IMCs through HEK293T cells altered neutralisation sensitivity<sup>10</sup>, supporting that expression in PBMCs resulted in Env with altered conformation. Therefore, irrespective of what cell produced the IMC, after passage in PBMCs, they were ostensibly identical viruses. PSVs on the other hand might still be influenced by host cell-specific processing.

### 5.3 Comparison of expression, incorporation and entry of PSVs produced by two backbones

**Table 5.2: Summary of the effect of pSG3DEnv and pNL4.3R-E-luc<sup>+</sup> backbones on pseudovirus produced in HEK293T, HeLa and CHO cells.**

Cell type	HEK293T			HeLa			CHO		
	Expression	Incorporation	Entry	Expression	Incorporation	Entry	Expression	Incorporation	Entry
pSG3 *	++	+	+++	+	-	-	-	-	-
pNL4.3 R.E-luc <sup>+</sup> *	++	+	++	+	-	-	-	-	-

\* PSVs were produced with CAP210E8

When comparing the overall results for PSVs, we see a cell type-dependent effect. HEK293T cells expressed detectable Env in the presence of both backbones which corresponded to entry into TZM-bl cells. Although HeLa cells were able to express low levels of Env in the presence of both backbones, the expression did not seem to result in incorporation and entry of PSVs. PSVs produced in CHO cells were unable to enter TZM-bl cells and this corresponded to lack of Env expression and incorporation.

Backbones, pSG3Δenv and pNL4.3R-E-luc<sup>+</sup> did not have an effect on PSV production suggesting that viral protein-Env interactions were not important in this assay.

#### 5.4 Comparison of Env expression, incorporation and replication of infectious molecular clones generated by two backbones

Unlike in the case of PSVs, when we compared the effect of Q23 and pNL4.3 on Env expression and incorporation and IMC replication, the results were not consistent between backbones (Table 5.3).

**Table 5.3: Summary of the effect of pNL4.3 and Q23 backbones on the production of CAP210 E8 chimeric infectious molecular clones passaged in PBMCs**

Cell type	HEK293T			HeLa			CHO		
	Expression	Incorporation	*Replication	Expression	Incorporation	*Replication	Expression	Incorporation	*Replication
Q23	+++	-	+++	+	+	+	-	-	+++
pNL4.3	+	-	-	+++	+++	+	-	-	++

\*Replication of IMCs relative to one another

Although, producer cell should not affect IMC replication after passage in PBMCs, there seemed to be some association. The lack of Env incorporation and replication of IMCs produced by HEK293T cells was likely due to experimental error as HEK293T cells are common producers of infectious virus in the literature. CHO cells, however, seemed to consistently demonstrate poor Env expression and incorporation which suggests that these cells do not produce high titres of HIV. It has been shown that CHO cells appear to be resistant to virus infection and this might have influenced our ability to detect Env.

The experimental error and possible resistance of CHO cells to produce virus to high titres influenced our ability to confirm whether pNL4.3 or Q23 were better at generating replication competent IMCs. In most experiments, CHO cell-derived IMCs were not normalised to 15 x background due to poor titre. However, this did not seem to affect the replication of these IMCs as many out-replicated the other variants. Overall, Q23\_CAP210 E8 performed better in most replication experiments suggesting that there could be backbone-dependent effects on IMCs. We saw similar results when we compared PSV backbones, with pSG3Δenv PSVs better able to infect TZM-bl cells. Therefore, comparison between matched PSV and chimeric IMC phenotype should be done in the same backbone to limit variation between experiments.

In the study conducted by Provine, et al.<sup>12</sup>, significant differences were found between matched pairs of PSVs and IMCs. The authors suggest that the differences found between these two systems cannot

be attributed to differential cleavage nor incorporation alone. Within the context of the earlier study, our findings suggest that each of the factors investigated herein had some impact on the resulting viruses, whether PSV- or IMC-derived. Experiments of IMC replication and incorporation must be repeated to obtain higher viral titre to circumvent expansion of virus in PBMCs. Overall, we can conclude that both PSV and IMC assays are very sensitive to variation and numerous biological repeats need to be performed for reproducible results. Furthermore, before direct comparison between studies that have used IMCs and PSVs, we must take into account the variation between the assays and that even repetition with the same cells and matched backbones might not yield the same results when comparing different Envs. The research community must standardize methods of characterising Envs in order to best understand its function and pathogenesis of HIV.

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# Appendix

10 20 30 40 50 60 70 80 90 100  
CAP210\_E8 ATGAGAGTGATGGGGATACAGAGGAATTGGCAGCAATGGGGGATATGGGGCTCTTAGGCTTTTGGCTGTTAATGATCTGTAGTGGGATGGGAACTTGT  
Q23CAP210E8

110 120 130 140 150 160 170 180 190 200  
CAP210\_E8 GGGTCACAGTATATATGGGGTACCTGTGTGGAAAAGAGCCAAAACCCTTATTTTGTGCATCAGACGCTAAAGGATATGACACAGAAAGTGCATAATGT  
Q23CAP210E8

210 220 230 240 250 260 270 280 290 300  
CAP210\_E8 CTGGGCTACACATGCCGTGTATCCCAACAGACCCCAACCCCAAGAAATAGTTTGGAAAATGTAAACAGAAAACCTTAAACATGTGGAAAATGACATGGTG  
Q23CAP210E8

310 320 330 340 350 360 370 380 390 400  
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Q23CAP210E8

410 420 430 440 450 460 470 480 490 500  
CAP210\_E8 CATATAAATAATGGTACGAATAGTACTGATCTATGAAAATTTGTTCTTCAATGCAACCACAGAACTAAGAGATAAGAAAAGAGGAGTATGCACTTTT  
Q23CAP210E8

510 520 530 540 550 560 570 580 590 600  
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Q23CAP210E8

610 620 630 640 650 660 670 680 690 700  
CAP210\_E8 TGTCCAAAGGCTCTTTTGACCCCAATCCTATACATTATGTGCTCCAGCGGGTTATCGGATTCCTAAAGTGTAAATAAAGACATTCATGGAACAGGAC  
Q23CAP210E8

710 720 730 740 750 760 770 780 790 800  
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Q23CAP210E8

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Q23CAP210E8

910 920 930 940 950 960 970 980 990 1000  
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Q23CAP210E8

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100  
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Q23CAP210E8

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200  
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Q23CAP210E8

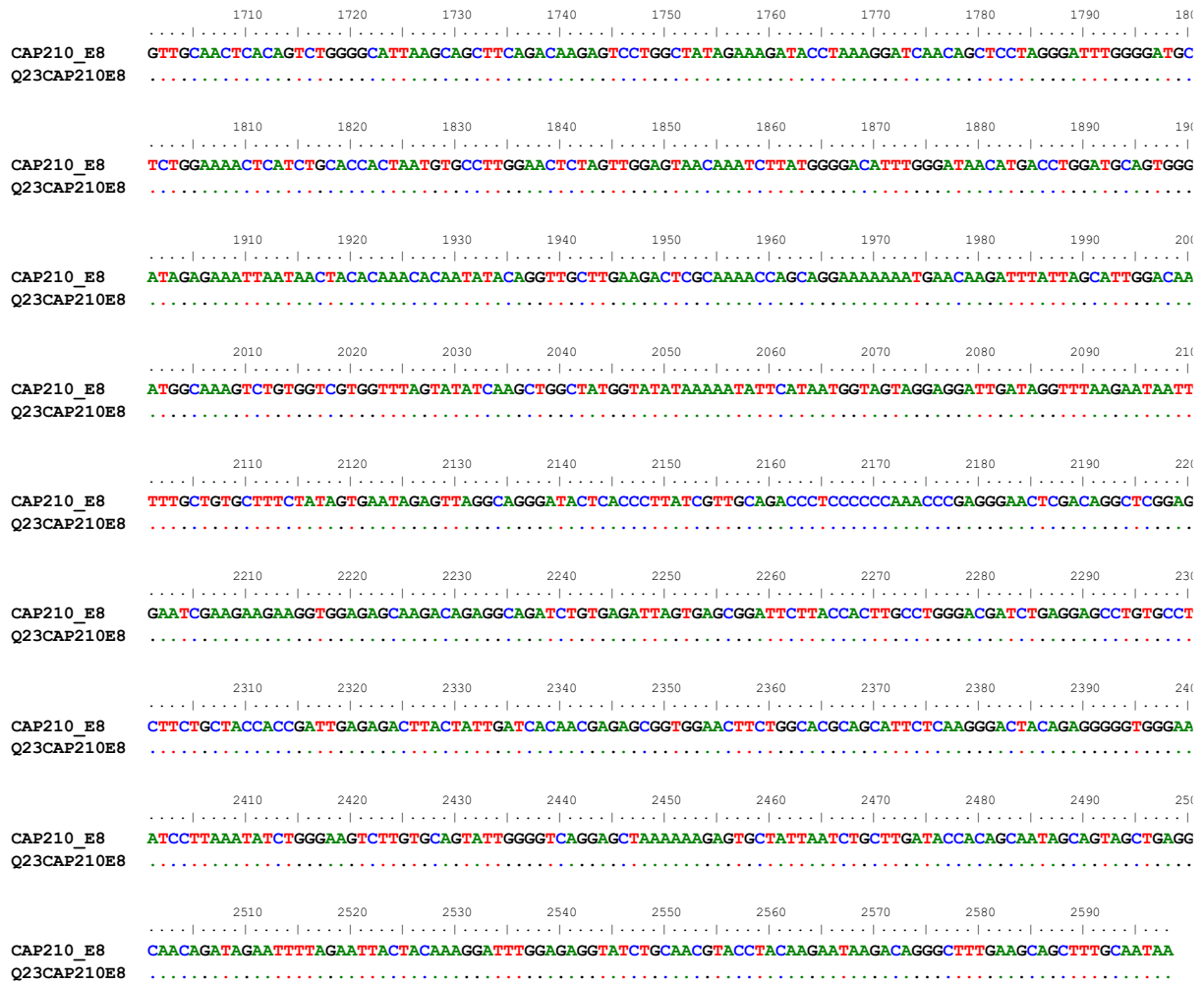
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300  
CAP210\_E8 AGTACAGCAGAAAACAGGAAATTCACAAATACAAAACATCAGCCTCCCATGCAAGATAAGGCAAAATTAACCATGTGGCAGGAGGTAGGACGAGCAATGT  
Q23CAP210E8

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400  
CAP210\_E8 ATGCTCCTCCAGTAAAGGGAAACATAACATGTATATCAAAACATCAGGACTATTACTGACCGCTGATGGAGGAGAAAACAAAACAGAGAAATAATGACAC  
Q23CAP210E8

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500  
CAP210\_E8 AGAGATATTCAGGCCTGGAGGAGGAGATGAAAGCAATTTGGAGAAGTGAATTTATAAATAAAGTAGTAGAAATTAACCATTAGGAGTAGCACCC  
Q23CAP210E8

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600  
CAP210\_E8 ACTAGAGCAAAAAGGAGATGGTGGAGAGAGAAAAGAGCAGTGGGAATAGGAGCTGTGTTCTTGGTTCCTTGGGAGCAGCAGGAAGCCTATGGGGC  
Q23CAP210E8

CAP210\_E8 CAGCATCCATTAACGCTGACGGTACAAGCCAGCAAAATTAATTTCTGGTATAGTGCAGAGCAAAAGTAAATTTCTGAGAGCTATAGAGCCCAACAACATAT  
Q23CAP210E8



**Figure A1: Full sequence alignment of chimeric IMC, Q23\_CAP210 E8 to CAP210 E8.** *E. coli* was transformed with chimeric IMC DNA. Positive colonies were selected and sent for sequencing. The alignment above shows that the CAP210 E8 Env was successfully inserted into the Q23 vector.