Phenotypic characterisation of Human Immunodeficiency Virus type 1 Envelope entry efficiency of transmitted/founder variants circulating in Mbeya, Tanzania

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Abstract

Background: Altered fitness of transmitted founder (T/F) HIV-1 variants has been linked to slowed disease progression, yet almost all of these studies have focussed on the role of attenuating immune escape mutations in T/F Gag or entry efficiency of Envelope (Env) in long-term non-progressors (LTNP) and elite suppressors (ES) during chronic stages of infection. As Env could also play an important role in viral fitness during early infection, we investigated if T/F Env entry efficiency, prior to immune selection, affected viral loads of progressors *in vivo*.

Findings: Functional Env clones from eight progressors and one LTNP, representing the T/F virus responsible for clinical infection, were constructed. Subsequently Env pseudoviruses (PSVs) were generated and confirmed to be all R5 but not macrophage-tropic. PSVs had a ~80 fold range in entry efficiencies using TZM-bl cells with the LTNP being the poorest enterer. Enhanced entry was associated with increased viral load at 3 months although this association was reduced by 12 months. Finally, we show that entry efficiency was influenced by fusion capacity, CCR5/CD4 dependency, and/or incorporation of gp120 into pseudovirions.

Conclusion: The variation in TZM-bl entry efficiency of T/F viruses was due to one or more advantageous Env attributes. Viruses with high entry efficiency may have a replication advantage subsequent to transmission, leading to increased viral loads during early infection of progressors.

Keywords: HIV, transmission, entry efficiency, Mbeya, Tanzania

1.0 **Introduction**

HIV transmission is associated with a severe genetic bottleneck where usually only one virus survives to establish clinical infection (Abrahams *et al*., 2009; Keele *et al*., 2008; Haaland *et al*., 2009; Nofemela *et al*., 2011). Low viral fitness of these transmitted founder (T/F) viruses has been linked to slower disease progression (Martinez-Picado *et al*., 2006; Goepfert *et al*., 2008; Chopera *et al*., 2009; Song *et al*., 2012; Prince *et al*., 2012; Yue *et al*. 2015) which is of significance to vaccine design as an attenuated T/F might provide long-term viral control. However, most of these studies have focussed on Gag Cytotoxic T cell (CTL) escape mutations that attenuate viral replication, and no studies have investigated whether entry efficiency of T/Fs could also influence disease progression. However, Env also plays a major role in the competitive and adaptive ability of the virus (Rangel *et al*., 2003; Travers *et al*., 2005) and entry efficiency has been shown to be a determinant of overall viral fitness (Ball *et al*., 2003; Marozsan *et al*., 2005) and disease progression (Lassen *et al*., 2009; Casado *et al*., 2013). The influence of entry efficiency on disease progression has been limited to the study of long-term non-progressors (LTNP) and elite suppressors (ES) which showed that these individuals carried viruses in the chronic stage of infection with lower En*v* entry efficiency compared to progressors (Lassen *et al*., 2009; Casado *et al*., 2013). Furthermore, while there have been several studies characterizing the properties of transmitted viruses (Ochsenbauer *et al*., 2012; Ping *et al*., 2013; Salazar-Gonzalez *et al*., 2009), there is limited information on the association between Env phenotypic properties of T/F variants and disease progression. Therefore, this study aimed to determine whether T/F entry efficiency as a function of viral fitness could influence viral loads of progressors, and to understand better the underlying role of other phenotypic properties of T/F viruses, such as tropism, CCR5 and CD4 dependency, fusion capacity and Env incorporation.

2.0 **Methods**

2.1 **Cohort description**

The HIV Superinfection Study (HISIS) high-risk cohort from Mbeya, Tanzania recruited drug-naive participants within three months of their previous HIV-negative sample (Herbinger *et al*., 2006; Riedner *et al*., 2006). Analysis of 212 Env sequences from 22 participants (n = 10 sequences per participant) generated using single genome amplification (SGA) indicated that sixteen participants were infected with a single infectious unit at transmission (Nofemela *et al*., 2011). Neighbour-joining trees and HIGHLIGHTER plots (http://www.hiv.lanl.gov) of the SGA sequences were used to identify the sequence of the T/F virus, and the amplicons identical to the consensus sequence were cloned from nine acute stage participants as part of the Vaccine Immune Monitoring Consortium, Collaboration for AIDS Vaccine Discovery (VIMC – CAVD) (http://www. cavd.org). Analysis of longitudinal viral loads indicated that eight of the participants were progressors, and one was an LTNP based on classification by Casado *et al*., (2010).

2.2 **PCR amplification and cloning of the *env* gene.**

The PCR amplification of the HIV-1 *env* gene was done using the SGA approach previously described (Abrahams *et al*., 2009; Keele *et al*., 2008). The second round PCR reaction was repeated using the high fidelity Phusion Hot Start DNA Polymerase (Finnzymes), together with, 0.2 mM dNTPs (Roche), 4 μM of Env 1A-Rx (5' CAC CGG CTT AGG CAT CTC CTA TAG CAG GAA GAA 3') and EnvN (5’ CTG CCA ATC AGG GAA AGT AGC CTT GT 3') in a final volume 50 μl. The cycling conditions for this reaction were as follows: denaturing at 94 ºC for 5 minutes, 45 cycles of 94 ºC for 30 seconds; 55 ºC for 30 seconds; 72 ºC for 4 minutes, and a final extension step at 72 ºC for 10 minutes. The amplicons were gel purified and cloned into the mammalian

expression vectorspcDNA3.1D/V5-His-TOPO (Invitr ogen) or pTarget (Promega, US) according to the manufacturer's instructions. Functional Env clones were selected using a 96-well plate format pseudovirion entry efficiency assay based on relative light units (RLU) that are 2.5 times above background.

2.3 **Pseudovirion entry efficiency assays**

Pseudoviruses were generated by co-transfection of HEK 293T cells with 2.5 μg *env* and 5 μg of either the subtype B HIV-1 backbone pSG3.1Δ*env* (a gift from L. Morris, NICD) or the subtype C backbone pBR264F-MluΔ*env* (a gift from B. Hahn, UAB) using PolyFect Transfection Reagent (QIAGEN). After 48 hours, the viral supernatant (2 ml) was harvested and clarified through a 0.45 μm filter and stored at -80°C. Pseudovirus titer was normalized using p24 ELISA (Vironostika HIV-1 Antigen microelisa system, Biomerieux, FRA) according to the manufacturer's instructions. TZM-bl cells (1 X 10^4 cells) were infected in triplicate with 50 ng p24 of pseudovirus for 48 hours before the medium was removed, and cells were lysed with Bright-Glo buffer (Promega, US). Luciferase activity (RLU) was measured using a Glomax 96-well microplate luminometer (Promega, US). The functional subtype C clone, Du151a, an R5 tropic T/F variant with high similarity to subtype C consensus sequence was used as a positive control (Williamson *et al*., 2003; Burgers *et al*., 2006) and included in all experiments so that clonal Env entry efficiency could be measured as a percentage relative to DU151a to control for inter-experimental variation. Pseudovirion stock generated by the transfection of the pSG3.1Δ*env* backbone was used as a negative control, and the RLU reading of cells only was considered as background signal. A RLU reading of 2.5 times above background was considered a positive infection measurement based on the TCID50 methodology of Reed and Meunch (Fouda *et al*., 2013).

2.4 **Coreceptor phenotype**

Pseudoviruses were generated by co-transfecting Env clones with the luciferase-encoding pNL4-lucR^-E^- backbone (NIH Aids Reagent Programme). U87 cell lines that express CD4, and either CCR5 or CXCR4 coreceptor were infected with 50 ng p24 of virus and luciferase activity was measured after 48 hours. Viruses that were able to infect the U87 - CD4 - CCR5 cells were classified as R5 tropic, and viruses that infected U87 – CD4 - CXCR4 were classified as X4-tropic. The Env clones QHO (Li *et al*., 2005), RPI (Cilliers *et al*., 2005), and Du179 (van Harmelen *et al*., 2001) were obtained from P. Moore (NICD), and were used as positive controls for R5-, X4-, and dual-tropic R5X4 variants, respectively. A pseudovirion stock generated with the transfection of the pNL4-lucR^-E^- backbone was used as a negative control

2.5 **Pseudovirus entry inhibition assay**

TZM-bl cells (1 X 10^4 cells per well) were infected in triplicate with pseudovirus (50 ng p24) in the presence of increasing concentration of TAK779 (0.04 - 400 nM) and T-20 (0.3 – 25 μg/ml) (NIH Aids Reagent Programme), and luciferase activity was measured after 48 hours, and the IC50 was determined using GraphPad Prism software 5 (CA, USA). Soluble CD4 IC50 was measured as part of the VIMC-CAVD (http://www.cavd.org) study and included in this analysis.

2.6 **Cell-cell fusion assay**

HEK 293T cells (4 x 10^5) were seeded in 6-well plates overnight, and co-transfected with 3.75 μg of each individual *env* plasmid and 3.75 μg of pSVtat72 (NIH AIDS Reagent Programme) for 48 hours using the PolyFect Transfection Reagent (QIAGEN, US) according to the manufacturer's instructions. The cell medium was removed, and the cells were lifted using 0.04% EDTA. The 293T cells (1 X 10^4 cells per well) were added to TZM-bl cells (1 X 10^4 cells per well) and allowed to incubate at 37^{o}C for 24 hours. The media (100 μl) was removed from the cells, and 100 μl of Bright-Glo buffer (Promega, US) was added. The plate

was incubated for 2 minutes at room temperature with gentle shaking. After cell lysis, 150 µl was transferred to a Co-Star black plate, and the plate was read immediately using a Glomax 96 microplate luminometer (Promega, US). The percentage fusion capacity was measured relative to the reference clone, Du151a.

2.7 **Pseudovirion infection of Affinofile cells.**

Pseudoviruses were generated by co-transfecting HEK 293T cells with 5 µg of *env* clone and 10 µg of the luciferase-encoding pNL4-lucR$^-$E$^-$ backbone (NIH Aids Reagent Programme). Pseudovirus-containing cell culture medium was collected after 48 hours, and filtered (0.45 µM) before p24 levels were measured (Vironostika HIV-1 Antigen microelisa system, Biomerieux, FRA). The Affinofile cell lines, a generous donation from B. Lee (UCLA), were induced and infected as previously described by Lassen *et al*., (2009) and colleagues. Briefly, the 293 Affinofile cells (1 X 10^4 cells per well) were seeded in 24-well plates, and induced with a concentration range of minocyline (0 – 5 ng/ml) and ponasterone A (0 - 4 µM) to induce CD4 and CCR5 expression, respectively. The cells were collected after 24 hours and washed with PBS before staining. The cells were stained with PE anti-human CD4 and PE anti-human CD195 (CCR5) (BioLegend, US) for 30 minutes at room temperature. The absolute number of CD4 and CCR5 was quantified using the Quanibrite beads (BD Biosciences, US) according to the manufacturer's instructions. The data acquisition and flow cytometry analysis was done using Cellquest and Flowjo software, respectively.

Affinofile cells (1 X 10^4 cells per well) were seeded in a 96-well plate for 48 hours, and were induced with minocyline (2.5 – 5 ng/ml) and ponasterone A (2 - 4 µM) for 24 hours. The cells were then infected with 100 ng p24 pseudovirus and infection was determined after 48 hrs using luminescence. Entry efficiency in 293Affinofile cells was determined based on RLU readings. CCR5 dependence (percentage infection relative to high CD4/high CCR5) was determined using the following equation: RLU (high CD4/low CCR5)/RLU (high CD4/high CCR5) X 100 (Ping *et al*., 2013).

2.8 **Pseudovirus Env Western Blotting**

Pseudoviral stocks were generated by co-transfecting HEK 293T cells (2 X 10^6 cells) with 5 µg of cloned *env* and 10 µg of the subtype B HIV-1 backbone pSG3.1Δ*env*. After 48 hours, the clarified culture medium was centrifuged at 26 000 rpm for 2 hours through a 20% glycerol cushion at 4°C in an RW55 rotor (Beckman Coulter, GER) to harvest pseudovirus. The viral pellet was resuspended in PBS for p24 determination and viral quantitation using the Vironostika HIV-1 Antigen microelisa system (Biomerieux, FRA) before viral lysis in SDS Laemelli loading buffer [40 mM Tri-HCL (pH 6.8), 10% glycerol, 10% β-mercaptethanol, and 1% SDS]. The equivalent amount (1.5 ug) of total p24 pseudoviral lysate was resolved using 10 % SDS-PAGE before transfer to nitrocellulose membrane. The primary antibodies (sheep anti-gp120 and rabbit ARP 432) raised against gp120 and p24, respectively were obtained from the NIH AIDS Reagent Programme. Primary antibodies for gp120 and p24 were detected with horseradish peroxidase-conjugated goat-anti-sheep IgG (Sigma, US) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, US) secondary antibodies respectively. The gp120 Western blot was detected using the LumiGlo chemiluminescent substrate (KPL) and exposed to X-ray film, and the p24 Western Blot was visualised with NCIP/BCIP tablets (Roche, GER).

2.9 **Statistical analysis**

The association of entry efficiency with viral load, IC50 measurements and fusion capacity was calculated using the Spearman correlation test. These figures were constructed using GraphPad Prism 5 software (CA, USA).

Table 1. Description of transmitted/founder Env clones from a high-risk opulation of female bar workers from Mbeya, Tanzania

Clone ID	Time post-infection (Days)	Sub type	Fiebig Staging	Entry efficiency (% infection relative to Du151a)	Viral Load at 3 months Post-infection (copies/ml)	Viral Load at 12 months Post-infection (copies/ml)	Cell Tropism	Soluble CD4 IC50 (nM)	TAK779 IC50 (nM)	T-20 IC50 (nM)
390T/FTZ	45	C	V	159.6	>750 000	>750 000	R5	2.50	21.28	414
89T/FTZ	14	CD	I/II	121.4	>750 000	322 000	R5	48	9.84	258
216T/FTZ	14	C	VI	80.0	>750 000	914	R5	>1087	125.50	537
605T/FTZ	41	AC	V/VI	63.5	434 000	>750 000	R5	161	20.32	166
401T/FTZ	45	CD	ND	52.4	51 500	56 600	R5	217	60.77	300
234T/FTZ	45	C	V/VI	17.7	509 000	71 400	R5	117	101.33	318
569T/FTZ	45	C	V/VI	15.9	23 800	6060	R5	313	42.94	735
346T/FTZ	41	C	VI	4.8	26 700	22 700	R5	34	33.63	1215
398T/FTZ	45	C	ND	2.0	2 378	1 070	R5	356	8.18	>5564

The T/F Env clones characterized in this study were generated from samples at Fiebig stages I/II to VI. The plasma viral loads at the time of sampling ranged from 2 378 to >750 000 copies/ml

>750 000 was the maximum detection limit of the assay

>1087: Resistant to 1087 nM sCD4; >5564 nM: Resistant to 5564 nM T-20.

TZM-bl cells (1 X 10^4 cells per well) were infected in triplicate with 50 ng p24 of pseudovirus (PSV) in the presence of a serial dilution of inhibitor and it's IC50 was determined using GraphPad Prism software 5 (CA, USA).

* Clone identification: participant number_transmitted/founder_country of origin Env clones with high entry efficiency are highlighted in bold.

Clone 398 is classified as a long-term non-progressor (LTNP) due to plasma HIV RNA levels that are equal to or below 2000 copies/ml for ~ 4 years without antiretroviral therapy.

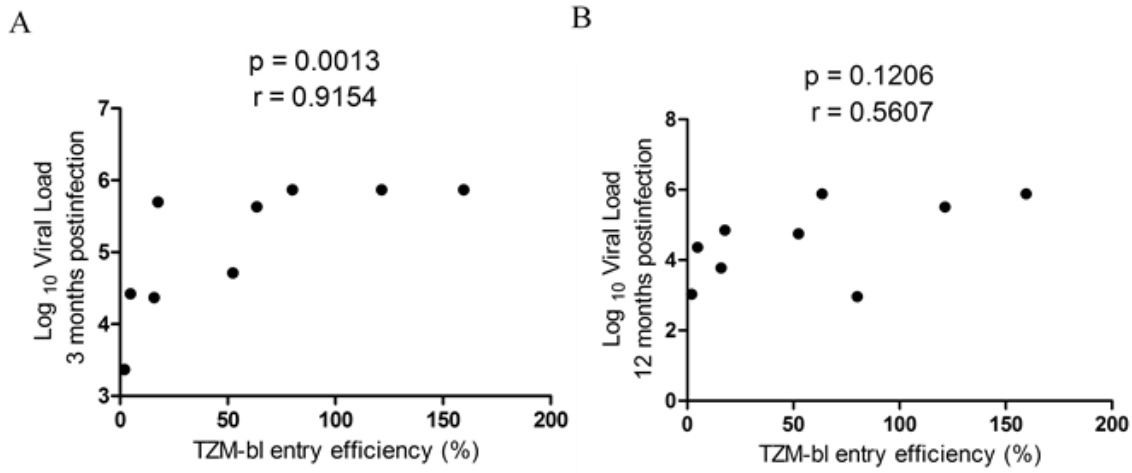


Figure 1. **Association between Env entry efficiency of T/F variants and viral load**

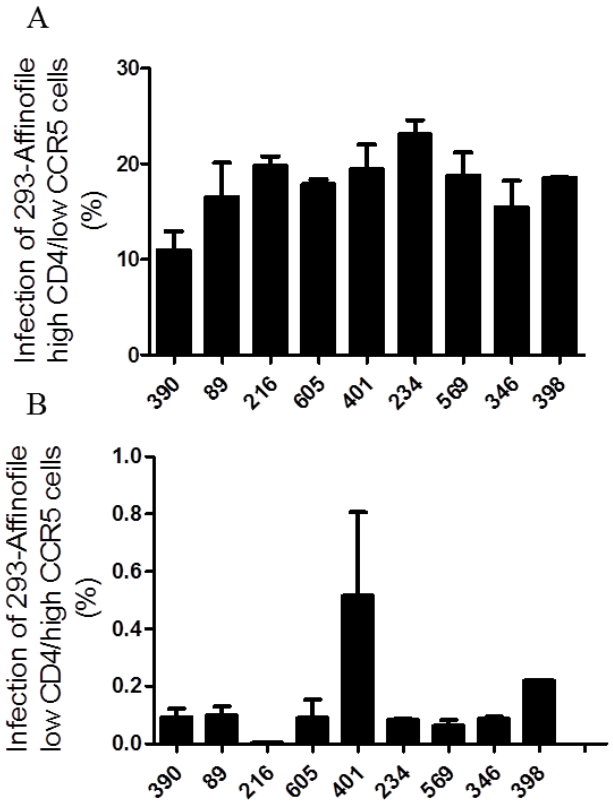
Pseudovirions were generated using the Subtype B backbone pSG3.1Δ*env* and entry efficiency was measured relative to Du151a (%). The association between relative infectivity in TZM-bl cells and viral load at 3 (A) and 12 (B) months post-infection was analyzed using a Spearman correlation test.

3.0 **Results**

Functional Env clones were constructed from nine participants infected with either HIV-1 subtype C (n = 6) or recombinant CD (n = 2) or AD (n = 1) who were previously found to be infected with a single variant (Nofemela *et al*., 2011). All clones were identical to the consensus of the SGA-derived *env* amplicons, and assumed to represent the T/F virus responsible for clinical infection (Keele *et al*., 2008). Pseudovirions (PSVs) were generated to determine the effect of entry efficiency on viral load and to identify phenotypic properties that could influence this relationship. The only LTNP of this study had the lowest entry efficiency supporting the two previous studies that showed that viruses from HIV LTNP and ES had lower entry efficiency compared to chronic progressors (Lassen *et al*., 2009; Casado *et al*., 2013). We thus wanted to determine the impact of entry efficiency of T/F PSVs on viral loads from progressors subsequent to transmission. We firstly wanted to determine the relationship between viral loads and entry efficiency of progressors only and thus excluded the LTNP from the analysis.

We found that PSV entry efficiency of TZM-bl cells was significantly associated with viral load at 3 months post-infection (mpi) (p = 0.0072; r = 0.8783) but not at 12 mpi (p = 0.1966; r = 0.5150). Our results were similar when we included the LTNP in the analysis (at 3mpi p = 0.0013; r = 0.9154 and at 12 mpi p = 0.1206; r = 0.5607) (Figure 1). The same trend was observed with PSVs generated using the subtype C backbone at both 3 mpi (p = 0.0760; r = 0.6272) and 12 mpi (p = 0.0857; r = 0.6193) (data not shown). Overall, there was either a significant or a trend for an association between entry efficiency and viral loads, irrespective if the LTNP was included in the analysis or not.

Next we set out to identify whether co-receptor usage, CCR5/CD4 levels and fusion capacity contributed to the wide range of entry efficiencies observed for the PSVs. All the PSV clones only infected U87-CD4+CCR5+ cells and not U87-CD4+CXCR4+ cells, confirming R5 tropism



(Table 1). To assess the dependency of PSV entry on fusion, and CCR5 and CD4 levels, we first measured

Figure 2. Pseudovirion entry efficiency in dual-inducible HEK293-Affinofile cells at A) High CD4 and Low CCR5 levels and B) Low CD4 and High CCR5 levels. Entry efficiency is calculated as % infection relative to entry of cells with $CD4^{high}/CCR5^{high}$ levels. This figure is a result of two independent experiments using two pseudovirus preparations, and the error bars represent the standard deviation between the two assays.

their sensitivity to entry inhibitors. A large range of inhibition could be observed for TAK779 (IC50 = 8.18 – 126.50 nM), T-20 (IC50 = 166 - 5564 nM), and sCD4 (IC50 = 34 – 1087 nM). One PSV (clone 398) was resistant to T-20, and another (clone 216) was resistant to sCD4. However, there was no significant association between IC50 values and PSV entry efficiency in TZM-bl cells for any of these inhibitors. As a more direct measure to determine the impact of receptor levels on entry efficiency we also used 293-Affinofile cells expressing either 1) high CD4 (146 397 molecules/cell) and low CCR5 (2 566 molecules/cell); or 2) low CD4 (2 397 molecules/cell) and high CCR5 (428 032 molecules/cell). All of the PSVs were able to infect when CCR5 levels were lowered to resemble that of T lymphocytes (% infection relative to $CD4^{high}/CCR5^{high}$; median = 18.6%; range = 10.9% - 25%) (Figure 2a). However, when CD4 receptor levels were lowered, and CCR5 levels remained high, resembling macrophages, 7/9 PSV clones did not enter, with the remaining two clones (398 and 401) entering at much lower levels (% infection relative to $CD4^{high}/CCR5^{high}$ of 0.21% and 0.51%, respectively) (Figure 2b). Consistent with the lack of correlation with TAK779 sensitivity, the analysis revealed no association between entry in 293-Affinofile cells with high CD4/low CCR5 and entry in TZM-bl cells ($p = 0.5517$). In addition to measuring T-20 sensitivity, we also performed a cell-cell fusion assay to assess fusion capacity of the PSVs. Again no statistically significant association could be observed between fusion and entry efficiency ($p = 0.2298$) (Figure 3). Finally, we measured Env incorporation into our PSV particles as this could play a role in entry efficiency (Bachrach *et al*., 2005). The relative position of gp120 of the PSV clones was determined relative to the gp120 positive control although bands representing gp160 and gp120 of clones 89 and 605 were incompletely resolved and thus not included in this analysis (Figure 4). The level of gp120 incorporation varied between the clones and for some of the PSVs there seemed to be an association between TZM-bl entry efficiency and the level of gp120 incorporation. PSV clones 234, 398 and 569 with relatively low TZM-bl entry efficiency (Table 1) had low gp120 incorporation and PSV clones with high entry efficiency (clones 390 and 216) had elevated relative levels of gp120 incorporation, suggesting that gp120 incorporation could be influencing TZM-bl entry efficiency (Figure 4). This apparent association was lost when

considering PSV clones 346 and 401, suggesting that alternative factors could be playing a role in the entry of these clones.

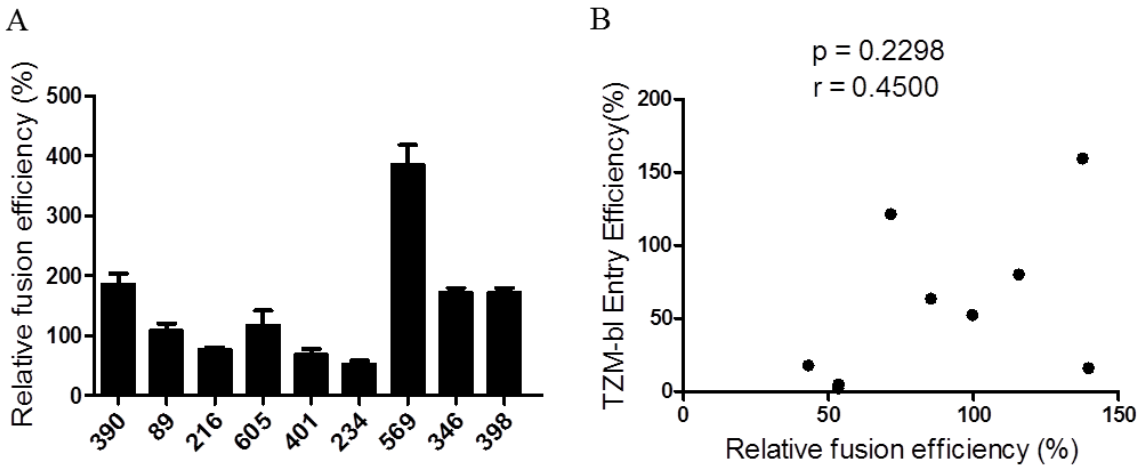


Figure 3. Cell-cell fusion capacity A) Env fusion capacity of the T/F variants was determined using a cell-cell fusion assay and indicated as percentage fusion capacity relative to Du151a. The cell-cell fusion assay utilised HEK 293T cells (4 x 10^5) co-transfected with 3.75 µg of each individual *env* plasmid and 3.75 µg of pSVtat72 for 48 hours before the cells were lifted and added to TZM-bl cells in a 1:1 ratio and relative light units (RLU) were measured. B) The association between relative fusion capacity and infectivity in TZM-bl cells was analysed using a Spearman correlation test.

4.0 **Discussion**

A few studies have shown a link between Env function and HIV replicative fitness (Kong *et al*., 2008; Rangel *et al*., 2003) and replicative fitness and disease progression (Quinones-Mateu *et al*., 200; Miura *et al*., 2010). In this study we report on high-risk women from Tanzania, recruited soon after infection, and show that the entry efficiency of T/F Envelope PSVs was positively correlated with viral loads at 3 months, suggesting that entry efficiency of transmitted viruses could influence disease outcome during early infection. However, the relationship between viral loads and entry efficiency lost significance at 12 mpi, suggesting that other factors, such as immune responses, could be influencing the association during later stages of infection.

Overall, our results support the findings of Lassen *et al.*, (2009) and Casado *et al*., (2013) who found that entry efficiency of viruses from seven ES and three LTNP, respectively were lower than those from chronic progressors, suggesting that viruses with poor entry could lead to viral control. In support, one of our PSV clones, 398, a LTNP with longitudinal viral loads below 2000 copies/ml (Casado *et al*., 2010) had the lowest entry efficiency. More importantly, we show that the effect of Env entry efficiency on disease progression is not restricted to ES and LTNP. Due to the overall relationship between Env function and viral loads, we wanted to identify the underlying biological factor(s) driving Env entry efficiency and thus potentially, replication fitness. As HIV replication fitness may be influenced by the level of CD4 and CCR5 at the surface of T cells (Poncelet *et al*., 1991), we investigated the effect of limiting host receptor levels on entry efficiency using Affinofile cells induced to express different levels of CD4 and CCR5. High levels of CD4 were shown to be

important for entry of these pseudoviruses as low CD4 levels resulted in a marked reduction in entry compared to when CCR5 coreceptor numbers were lowered. The inability to mediate entry at low levels of CD4 by most (7/9) pseudoviruses suggests that the T/F viruses are unlikely to be macrophage-tropic, which supports findings in recent studies on subtype B and C viruses (Salazar-Gonzalez *et al*., 2009; Alexander *et al*., 2010; Ochsenbauer *et al*., 2012; Ping *et al*., 2013). Preliminary studies in our laboratory using IMCs also show that transmitted variants replicate well in PBMCs, but very poorly in macrophages (data not shown). In terms of cell-cell fusion, there was no correlation between entry efficiency and cell-cell fusion, despite some viruses with high entry efficiency having high cell-cell fusion capacity. This discrepancy could be due to the two assays measuring different parameters, with the pseudovirus assay being dependent on Env incorporation into viral particles, and the cell-cell fusion assay reliant on Env expression on the surface of cells.

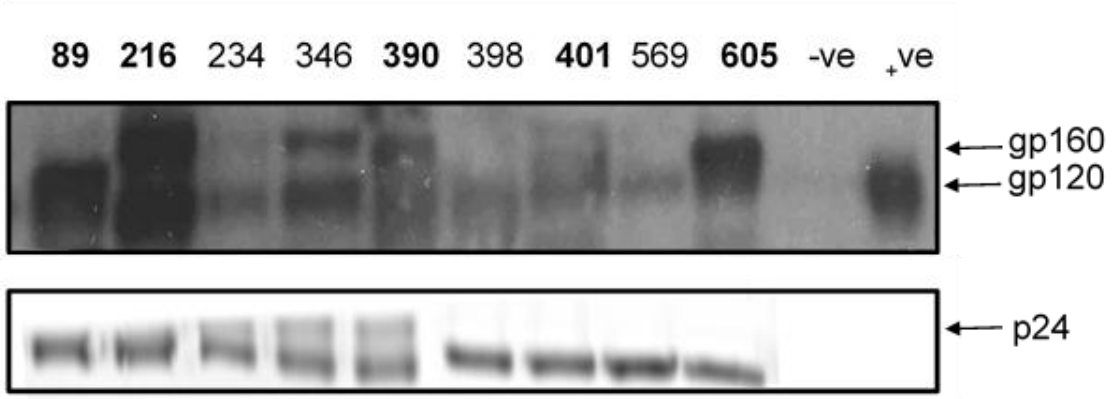


Figure 4. Pseudovirion incorporation of Env clones Pseudovirions were generated in HEK 293T cells, and culture supernatants were layered over 20% glycerol and ultracentrifuged at 26 000 rpm for 2 hours. The pelleted viruses were lysed, and 1.5 ug of p24 was loaded per lane. Negative control (-ve) comprises viral particles without envelope and the positive control is HIV-1 gp120 CM cat #2968 (NIH AIDS Research and Reference Reagent Programme). The bottom panel indicates the amount of p24 loaded per lane. Clones with high entry efficiency are indicated in bold. The blot was probed with sheep anti-gp120 and rabbit ARP 432 raised against gp120 and p24, respectively (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH).

As previous studies have shown that high levels of surface Env expression result in increased infectivity (Bachrah *et al.*, 2005; Parker *et al*., 2012; Provine *et al*., 2009), we investigated whether the entry efficiency of the T/F Env clones was due to enhanced Env incorporation. This study found that incorporation of Env into pseudovirions may impact entry efficiency as some T/F viruses with the highest entry efficiency had better gp120 incorporation compared to other viruses. A recent study by Parrish *et al*., (2013) found that T/F viruses are more infectious than chronic viruses, and this was partly due to the presence of higher number of Envelope particles on the surface. However, the relationship between Env incorporation and entry efficiency was not consistent for all the clones in this study, suggesting that other factors may be playing a role in the entry efficiency of the T/F viruses.

5.0 **Conclusion**

In conclusion, high PSV entry efficiency was

linked to higher viral loads during early infection, suggesting a possible role of T/F Env entry efficiency in disease progression. Furthermore, as T/F Env fusion capacity, CCR5 and CD4 dependency, and Env incorporation influenced PSV entry efficiency, viruses with any one or more of these advantageous Env characteristics could replicate to higher titres that could in turn impact disease progression during early infection.

6.0 **Competing interests**

The authors declare that they have no competing interests.

7.0 **Author's contributions**

AN, CW, and ZW were involved in the conception and design of the study, and also the reviewing of the manuscript. PS was involved in data analysis and preparing the manuscript. GB, RT, JM performed some of the experiments. LM, MH co-ordinate the study cohort in Mbeya Tanzania, and are also involved in the acquisition of samples.

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