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Viral Determinants of Subtype C HIV-1 Transmission in Zambian Heterosexual Couples

By

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B.A., University of Maryland, 2006

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Abstract

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By Zachary Ende

Heterosexual transmission of subtype C HIV-1 is the predominant route of infection worldwide. A dramatic loss in HIV-1 genetic diversity occurs during HIV-1 transmission by sexual exposure, where approximately 80% of new infections are established by single viral variants. These breakthrough transmitted/founder viruses may have unique properties that confer a higher capacity to transmit, or may in contrast be selected at random from the quasispecies of the HIV infected source partner. Appreciating the balance of random and natural selection pressures operating during transmission, by determining transmitted virus properties, could help inform the rational design of vaccines and enhance our understanding of the molecular details of mucosal transmission.

Characteristics of genome-length authentic primary viruses from an infected individual's quasispecies and across transmission in acutely infected partners was lacking prior to the investigations undertaken here. We applied novel molecular virological and immunological techniques to study viruses from a cohabiting heterosexual couples cohort in Zambia to investigate the genetic and phenotypic properties of viruses from the plasma of infected donors, along with viruses that transmitted to their epidemiologically linked acutely infected partners.

The results presented here demonstrate transmitting viruses harbor more consensus residues across the entire genome, and are slightly more sensitive to concurrent donor plasma antibody neutralization, indicative of selection pressures acting on virus fitness at the transmission bottleneck. However, we observed that newly infected recipients were permissive to infection with viruses that have a range of phenotypic properties. Transmitted viruses had variable replicative capacities, particle infectivities, interferon resistance profiles, HLA-I downregulation signatures, and susceptibility to NK cells. These data provide the first characterization of authentic genome-length virus clones from transmission pairs, and will serve as useful tools in the study of HIV infection.

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Chapter I: Introduction

Why study HIV transmission?

The human immunodeficiency virus (HIV) was identified as the likely cause of the deadly acquired immunodeficiency syndrome (AIDS) in 1983, after being cultured from a lymph node biopsy[1], leading to a 2008 Nobel Prize. The study of HIV and AIDS has led to efficacious drug therapies that have saved millions of lives[2], and treatment at any stage of infection has led to reduced transmissions, causing a slowdown in the spread of new infections[2-5]. However, therapy alone cannot end the pandemic, since even in an environment such as the United States of America, 20% of infected people are unaware of their HIV status, and only 20% of HIV infected individuals are fully virally suppressed—numbers that are far worse in the heart of the pandemic in parts of Sub-Saharan Africa[6]. There is still no cure for those on therapy, and no vaccine to prevent new infections. Thus, understanding viral properties and viral transmission for the sake of its ultimate potential to help millions of people is still being pursued vigorously.

In addition to the aforementioned worthy reasons to study HIV transmission, the study of the unknown often has unforeseeable consequences, and the study of retroviruses is a prime example of such a phenomenon. HIV is a retrovirus, and its official classification is the following: a Group VI single stranded RNA reverse transcriptase encoding virus (ssRNA-RT), in an unassigned order, in the *Retroviridae* family, in the *Orthoretrovirinae* subfamily, in the *Lentivirus* genus (https://talk.ictvonline.org/taxonomy/). Identifying HIV would not have been possible without the 1970 discovery of reverse transcriptase in what are now relatively obscure

RNA tumor viruses[7-9] (1975 Nobel Prize). Reverse transcriptase is an enzyme that transcribes RNA into DNA (an RNA-dependent DNA polymerase), and its discovery has had far reaching impacts on modern biology, aside from its significance to HIV and AIDS. Oncogenes (i.e. Ras, Src, EGFr; potential cancer causing genes) were discovered by reverse transcribing, and ultimately sequencing, acutely transforming retroviruses[10, 11], leading to a revolution in cancer biology (Nobel Prize 1989). Second, reverse transcription of cellular RNA into complementary DNA by reverse transcriptase is a ubiquitous and fundamental tool for molecular biology. Lastly, and perhaps most surprisingly, reverse transcriptases were found encoded in human "junk DNA", within class I transposable elements, or retrotransposons, which include endogenous retroviruses, that in total make up ~40% of the human genome[12-14]. Some retrotransposons are estimated to be as old as eukaryotic cells themselves, stretching back 600 million years. Differences can also be observed on more recent time scales; for instance, humans and chimpanzees have ~10,000 differentially present retrotransposons[12, 13, 15, 16]. Others have speculated that the ancient transition between an RNA to a DNA world >3.5 billion years ago necessitated reverse transcriptase, especially since reverse transcription is found in some prokaryotes as well[17]. Therefore, the study of retroviruses has illuminated our past and present, and has had implications beyond viruses themselves, illustrating how the study of HIV transmission may have practical applications to human health, or may lead in unexpected directions.

The primary aim of this dissertation work was to study HIV transmission from the perspective of the virus, in order to determine if there are viral correlates of transmission,

or viral properties associated with transmitted viruses, which might elucidate the molecular details of transmission. Finding transmission associated viral properties potentially impacts upon strategies aimed at interrupting viral spread. To understand the goal of this project, and how we set out to add to the current understanding of HIV transmission, more background is provided in the sections below.

Origins of HIV

Since the discovery of HIV, much has been learned about its origins and closely related *Lentiviruses*. Although the first patients suffering from AIDS were reported around 1981[18-21], historical descriptions of patients with AIDS-symptoms date back to 1959[22]. More definitive proof of earlier HIV infections came from divergent West African HIV sequences amplified from a 1959 banked plasma sample[23], and a paraffin-embedded lymph node biopsy from 1960[24]. Along with other studies of available HIV sequences[25], the consensus is that HIV has been spreading for approximately 100 years in humans as two species, HIV-1 and HIV-2.

HIV-1 is the major species and is made up of multiple groups (M, N, O and P), of which group M, which entails multiple subtypes (A-K, and circulating recombinant forms) accounts for >95% of the worldwide pandemic, while subtype C within group M is >50% of infections. Such diversity is partially the consequence of multiple cross-species transmission events of simian immunodeficiency virus (SIV) from chimpanzees (HIV-1 group M and N), gorillas (HIV-1 likely group O and P), and sooty mangabeys (HIV-2)[26-30]. The pandemic group M strain is derived from chimpanzees, specifically the subspecies *Pan troglodytes troglodytes*[31], whose range is around Cameroon, Gabon

and the Democratic Republic of the Congo—the same area from where the earliest HIV sequences were derived and hunting chimpanzee for bush meat has been reported[26-28, 31]. Studies involving primates isolated thousands of years ago on Bioko island off the coast of Cameroon have estimated SIV to be 80,000 years old[32], suggesting humans have been exposed to HIV-related retroviruses for thousands of years. Pandemic HIV-1 group M has been spreading for approximately 100 years, though multiple cross-species transmission events have occurred at different times, meaning that cross-species transmissions may have occurred much earlier or re-emerge in the future.

Routes of HIV transmission

HIV-1, the causative agent of the AIDS pandemic, continues to contribute to over 1 million deaths every year in sub-Saharan Africa alone[23]. The spread of HIV through the human population occurs, in the case of hetero- and homosexual transmission, primarily from sexual exposure of genital mucosa to HIV-containing genital fluids, and in the case of mother-to-child transmission, predominantly through perinatal and milk-borne exposure of mucosal surfaces. Heterosexual transmission of HIV remains the predominant mode of transmission, particularly in sub-Saharan Africa, accounting for nearly 70% of new transmissions worldwide. In developed countries of the Western Hemisphere, transmission through men having sex with men (MSM) represents nearly 50% of those newly acquiring HIV. Transmission via direct blood-to-blood contact through contaminated blood transfusions or intravenous drug use (IDU) represents a small percentage (<10%) of infections in the U.S., but transmission among IDUs is a significant contributor in parts of Southeast Asia and Eastern Europe[2]. HIV risk of

infection is highly dependent on the route of transmission; exposure in MSM via anal intercourse has an infection probability of 1 in 20 to 1 in 300, whereas infection by exposure of the male genital tract has a risk of approximately 1 in 300 to 1 in 7000. Infection via exposure of the female genital tract carries a risk of 1 in 200 to 1 in 2000. The aforementioned frequencies of transmission vary substantially based on a number of factors including the behavioral environment of the specific epidemic (high risk vs. low risk behaviors), the stage of infection of the transmitting partner (early or acute vs. chronic infection), and perhaps the circulating HIV subtype in a given area, pointing to a role for viral characteristics[33-36].

HIV infection of the female genital tract and systemic spread

The SIV-macaque intra-vaginal challenge model has been especially informative in shaping concepts of the steps involved in successful transmission and dissemination. Studies by Haase, Miller, and colleagues[37-43], using high doses of SIVmac239 and SIVmac251, led to the model shown in Figure 1A, whereby SIV interaction with the cervico-vaginal mucosa induces an innate response that signals activated T cells to migrate to the site of infection. This increased availability of $CD4^+$ target cells allows the initially infected cell, a partially activated mucosal $CD4^+$ T cell, to amplify the infection over the first few days to a level where virus or virus- infected cells could exit the mucosal tissue and travel to distal sites. These include the gut- associated lymphoid tissue (GALT), where the bulk of early viral replication and T cell depletion occurs. An inhibition of infection by vaginal application of glycerol monolaurate, an inhibitor of inflammation, prior to infection, supported this model of a localized inflammatory

response playing a role in transmission[42]. A more detailed analysis of these early events[39], has suggested that vaginally inoculated SIV, rapidly induces chemokine expression in the cervical epithelium (CCL3, CCL20, and CXCL8), which is associated with early recruitment of macrophages and plasmacytoid dendritic cells. Additional chemokine (CCL3 and CXCL8) production by these cells generates a chemokine gradient that is spatially correlated with the recruitment of $CD4^+$ T cells.

In a recent large study where 44 animals were infected intravaginally with a high dose of 5×10^4 $TCID_{50}$ SIVmac251 and serially necropsied on days 0, 1, 3, 7, and 10, virus was occasionally detectable in distal tissues, such as the gastrointestinal tract, in addition to the site of inoculation, on day 1[44]. On day 3, 89% (8 of 9) animals had detectable levels of viral RNA in at least one distal tissue, suggesting that at least some viral dissemination out of the vaginal mucosa can occur rapidly. Nevertheless, the role of these distal infections in the establishment of systemic infection remains unclear since in a majority of animals, plasma viremia was not detected until day 10. It is also important to recognize that these high dose infections, resulting in an average of five transmitted viral variants per animal, may not be directly comparable to human sexual transmission where one variant typically initiates infection (discussed further below in the section on the transmission-linked genetic bottleneck).

Using a high titer single-round non-replicating SIV construct that expressed luciferase and m-Cherry, Stieh *et al.*[45] were able to demonstrate preferential infection of vaginal and cervical tissue, with signal also being detected within the ovaries and occasionally in the draining lymph node, consistent with some early dissemination of virus. Recent studies, using a similar approach, suggest that Th17-lineage $CCR6^+$ $CD4^+$ T

cells are the predominant targets of SIV during vaginal transmission[46]. This cell type plays a key role in maintaining the integrity of the gut mucosa and is rapidly depleted following both SIV and HIV infection[47-53].

Factors influencing the efficiency of transmission

Both viral and host factors influence the efficiency of transmission, and it is the interplay between these that defines the frequency with which transmission occurs. Viral load (VL) in the transmitting partner has been shown to be a determining feature, with higher VL facilitating transmission[35, 54-60]. Studies in HIV-1 discordant couples have shown that partners with VL less than 1,000 copies/ml almost never transmit to their partners, while those with VL greater than 100,000 copies/ml transmit much more frequently, with an average 2.5-fold increase in risk with each $\log_{10}$ increase in VL[54, 55]. In Zambian discordant couples, this increased risk was most evident in female-to-male transmissions[55]. The increased risk for high VL may reflect higher VL in the genital tract of the transmitting partner[57, 61] and a greater chance of virus reaching the genital mucosa. This is also consistent with non-human primate studies where the number of transmitted variants increases with the dose of the inoculum[62, 63].

Inflammatory responses to both the existent microbiome as well as to sexually transmitted infections clearly increase the frequency of HIV-1 transmissions[64, 65] (Fig 1B). Recent studies of young women in a South African cohort have shown that a cervicovaginal microbiome enriched in diverse populations dominated by *Gardnerella* and *Prevotella*, but not *Lactobacillus*, correlates with increased genital pro-inflammatory cytokine concentrations[66]. This ecologically diverse, *Lactobacillus*-deficient, microbiome, was also associated with a higher risk of HIV-1 acquisition[67]. Moreover,

sexually transmitted infections (STI), and herpes simplex type 2, which induce inflammation and ulcers in an uninfected partner, as well as similar infections in the transmitting partner, are known to increase the risk of transmission[35, 54, 55, 64, 68, 69]. Genital inflammation in the uninfected partner has also been associated with a greater number of transmitted variants[70].

Finally, the stage of infection (early vs. asymptomatic vs. late) can also play a key role in defining the efficiency of transmission, with the risk of infection from patients with acute infection (within the first month) and early infection (within the first 4 months) being higher than that in established infection[71-73]. This likely reflects high VL observed in acute infection[61, 74, 75] and lack of neutralizing antibody[76], which may inactivate a majority of virions in established infection. Indeed, in the macaque model, SIV in the plasma from animals in the acute stage of infection has an infectivity almost 100 times greater than that of virus in the plasma from chronically infected animals[77].

The Transmission-linked Genetic Bottleneck

The concept that transmission of HIV involved a genetic bottleneck, in which one or a limited number of variants from the diverse population present in the transmitting partner establish productive infection in the previously uninfected partner, was first established in studies over two decades ago. These landmark studies published in 1991-1993, analyzed viral sequences from early time points for the first time in primary HIV infection, as well as, in some cases, viral sequences from the donors of a small number of linked heterosexual, homosexual, hemophilia and mother-to-child transmissions[78-85].

Wolinsky *et al*.[78] analyzed sequences of the V3-V5 region of the HIV envelope glycoprotein (Env) from three separate mother-to-child transmission events and determined that sequences within the infant after transmission were less diverse than those found in their mothers. Studies by Wolfs *et al.*[79], Pang *et al.*[84], Zhu *et al.*[80] and Zhang *et al.*[81] presented similar findings in early primary infections of heterosexual, homosexual, and parenteral transmission events where almost all the Env sequences in recipients were genetically homogeneous. Kleim *et al.*[82] and Simmonds *et al.*[83] discovered similar results in hemophilia patients. Zhu *et al.*[80] also observed that in the two heterosexual transmissions described, the transmitted virus appeared to be a minor variant in the blood of the transmitters.

The difficulty of obtaining samples at early time points after infection, and from linked donors of HIV transmission, hindered the successful investigation of transmitted viral characteristics for over a decade. A more in depth analysis was performed using samples from heterosexual transmissions in a previously established cohort of serodiscordant couples in Lusaka, Zambia[86]. Derdeyn *et al*.[87] sequenced almost 300 cloned HIV-1 Subtype C *env* gene amplicons derived from 8 heterosexually linked transmission pairs shortly after transmission, and performed genetic and phenotypic studies. A strong genetic bottleneck was observed in each transmission pair, in that the *env* derived from each linked recipient emanated from a single branch on their respective donor phylogenetic *env* tree (Fig 2A), indicative of a single transmitted or distinct founder virus establishing infection in each case.

More definitive analyses of the viral populations present early after infection have allowed an accurate and precise description of these transmitted/founder (TF) viruses and

sequences evolving from them during the critical period when productive clinical infection is established[88-91]. These studies have relied on the use of end-point dilution PCR, termed single genome amplification (SGA), to amplify sequences from multiple single genomes present in plasma very early after primary infection, followed by direct sequencing of the DNA amplicon[92]. This approach, in contrast to bulk PCR followed by cloning, avoids sequencing errors introduced by the Taq polymerase, *in vitro* recombination induced by template switching during the PCR reaction, and non-proportional representation of sequences as a result of template resampling. Furthermore, by applying to these sequences a mathematical model of early virus evolution[93], it has been possible to define the nucleotide sequence and number of TF variants, assuming the absence of immune selection during primary infection, wherein replicating genomes accumulate random mutations at a constant rate defined in large part by the error rate of the reverse transcriptase (Fig 2B). Using this SGA approach, Keele and colleagues analyzed more than 3,000 complete *env* sequences derived from 102 subjects with acute subtype B HIV-1 infection, and showed that 78 of the subjects had evidence of productive infection by a single virus, while the remaining 24 had been infected by a minimum of two to five viruses[89]. Applying this same method to 20 subtype A and C transmission pairs for whom multiple sequences from both partners were derived, Haaland *et al.*[70] determined that a single virus established infection in 90% of cases, while an analysis of 69 newly infected subtype C individuals from South Africa by Abrahams *et al.*[94] showed that 78% involved single variant transmission. In the latter studies and that reported by Keele *et al.*[89], it is clear that, in those individuals where multiple viruses initiate infection, the number of infecting variants does not follow a

Poisson distribution, which is inconsistent with each variant being transmitted independently with low probability. Most likely, in these cases, factors such as sexually transmitted infections, and potentially for young women the use of hormonal contraceptives, lower the barrier to transmission[70, 95]. This is consistent with the model portrayed in Figure 3, which shows that virus transmission can fail at multiple steps following inoculation onto a mucosal surface. Thus STI could abrogate the barrier imposed by an intact mucosa by inducing breaks in the epithelial lining thereby allowing more virus variants to initiate infection in the mucosal tissue; in addition, inflammation inducing genital infections could increase the availability of activated CD4 cells required to establish a spreading infection, in this way allowing infections that would have otherwise failed due to lack of target cells to expand (Fig 1B). It is still not defined, under conditions of low multiplicity infections, where the probability of infection is less than 1% per coital act, how many viruses initiate an abortive infection in the mucosa.

Transmission of multiple variants occurs most often in the case of infections of men having sex with men, and intravenous drug users, where on average 75% and 60% of transmissions are caused by a single viral variant, respectively, although there is a wide range of estimates from different studies[96-99]. This is consistent with the higher incidence of transmission observed during intra-rectal intercourse[68] and likely reflects the reduced barriers to infection imposed by these routes. A somewhat higher frequency of multiple variant infections is also seen from recent sequencing studies in mother-to-child transmissions, with an average of 35% resulting from multiple variants, although there is also significant variation from one study population to the next (18%-53%)[100-104].

A powerful tool for experimentally investigating the transmission process is the non-human primate model of HIV infection, where it has been possible to reproduce the transmission-linked genetic bottleneck. In this system, rhesus macaque monkeys are challenged repeatedly with low doses of simian immunodeficiency virus (SIV) intra-vaginally or intra-rectally. Macaques challenged multiple times via the rectal route with a quasispecies of SIVmac251 or E660 were found, using the same SGA approach as in infected people, to be infected with a limited number of genetic variants—a majority with a single variant[63]. The kinetics of virus replication in these animals resembles that observed in acutely infected people[75, 105]. A similar genetic bottleneck, with predominantly single variants establishing infection, was observed when macaques were challenged intra-vaginally[106] or through the penile route[107], although both were significantly less efficient. Increasing the challenge dose via the intra-rectal route above 10^7 viral RNA copies resulted in infection being established by multiple (>10) variants, suggesting that in this model system the genetic bottleneck could be overcome by increasing the size of the input inoculum[62].

Evidence for Selection during Transmission

There has been considerable debate since the discovery of the HIV genetic bottleneck was discovered in the early 1990's, over whether the transmission of HIV-1 is simply a stochastic process, where the low chance of a transmission event usually results in the transmission of a single genetic variant, or whether certain viral phenotypes, which confer transmissibility on that variant, are selected for during the transmission process.

It is very clear that some aspects of the transmission process do involve chance: the genetic variant must be present in the genital fluid of the transmitting partner at the time of intercourse; must interact with the genital or rectal mucosa; must cross the epithelial barrier and infect a susceptible $CD4^+$ T-cell; and must have a sufficient number of secondary target cells for infection to spread and establish a localized then systemic infection (Fig 1A, Fig 3). Despite these stochastic aspects of transmission, there is strong evidence that selection pressure is applied and that viruses with specific traits are selected for during the transmission process.

The first evidence for selection came from early studies on isolates from early stages of infection which showed that they generally grew more slowly and did not induce syncytia between infected cells[80, 108]. After the discovery of HIV obligate coreceptor usage in the mid 1990s, and the observation that HIV can use different coreceptors, it became clear that previously defined phenotypes were dependent on coreceptor tropism. Viral use of CCR5 and CXCR4, the main coreceptors for HIV, define what at the time were called macrophage-tropic/non-syncytium-inducing and T-cell tropic/syncytium-inducing phenotypes, respectively[109-116]. This, and the finding that newly transmitted viruses utilized CCR5, provided the first evidence that CXCR4 tropic viruses were selected against, while CCR5 tropic viruses were selected for, during transmission. The discovery that protection from HIV acquisition by the mucosal route is conferred by a deletion of 32 amino acids in the CCR5 gene, provided further evidence that only CCR5-tropic viruses could infect by this route and that CXCR4 viruses were selected against during HIV transmission[117-121]. The propensity for CCR5 tropism

remains unexplained[122] but could be due to target cell availability at portals of entry[123]

Evidence for selection pressures acting on HIV during transmission has been supported by phylogenetic analyses of HIV sequences from heterosexual transmission pairs. Sagar *et al*.[124] provided the initial evidence that newly infecting (recipient) viruses were evolutionarily closer to the most-recent common ancestor (MRCA) than transmitting partner (donor) viruses by calculating distances of recipient and donor *env* sequences to their MRCA from 10 subtype D and 3 subtype A linked transmission pairs. This work was supported by two separate studies of cohorts containing subtypes A, B, and D, where intra-host diversity was found to be greater than inter-host diversity[125, 126]. A longitudinal analysis of viruses from donors also found that the virus that transmitted more closely resembled earlier viruses in the donor than the viruses circulating at the time of transmission[126]. These observations have been supported and extended by additional studies that have included genes outside of *env*. In a study of 137 epidemiologically linked heterosexual transmission pairs, a selection bias for consensus amino acid residues and against non-consensus polymorphisms was found in *gag*, *pol* and *nef*, suggesting an *in vivo* fitness and transmission advantage for variants with a greater fraction of consensus residues[127]. The selection pressure for consensus residues was influenced by gender, with female-to-male transmission imposing a greater selection bias on the virus than male-to-female transmission. Interestingly, selection bias was reduced in men with genital ulcers or inflammation (GUI), and where the donor partner exhibited a high viral load[127]. Both factors are known to increase the risk of infection and number of genetic variants transmitted (see above factors influencing the efficiency of

transmission), demonstrating the interplay between general susceptibility and the genetic bottleneck.

Some of the first observations of the HIV genetic bottleneck suggested that transmitted viruses were minor variants of the donor's plasma[80, 128]. A follow up of the earliest studies that compared viral genetic diversity within and between partners in transmission pairs by analyzing virus from plasma, seminal fluid and seminal cells, suggested that the transmitted virus could be identified in both cell-free and cell-associated forms in the donor genital tract, and that it was generally a minor variant of the genital tract[129]. A more recent analysis utilizing SGA of the V1-V4 region of Env from genital tract and plasma samples of eight subtype C infected heterosexual transmission pairs reported very similar results[130], indicating that, despite significant compartmentalization of viral genotypes with discrete populations within the genital compartment, the virus in the donor that most resembled the TF was a minor variant, either of the genital tract or the plasma[130]. Both studies therefore provide additional evidence for HIV-1 selection during transmission.

Properties of the Transmitted/Founder Virus

Evidence of a genetic bottleneck during sexual transmission of HIV-1 has stimulated efforts to define biological characteristics of TF viruses that could favor their transmission over a majority of the viruses that are circulating in the quasispecies of the transmitting partner. Other than CCR5 tropism, no single trait has been consistently identified across different studies and cohorts reported to-date. In some respects this might be expected given the differences in the cohorts under study (e.g., men having sex

with men vs. heterosexual), as well as the stringency of the barriers to infection (e.g., +/- inflammation) the virus must face. Nevertheless, a number of properties have been linked to transmissibility and will be discussed below (labeled 1-5).

1) *Tropism*: The observation that CCR5-tropic viruses are preferentially transmitted has been demonstrated in most studies, including those where discrete TF virus envelopes and full-length viruses were examined[89, 91, 131-133]; however, this is not absolute since infrequent CXCR4-tropic transmitted strains have been observed[108, 134-136]. Moreover, mutations (CCR5Δ32) that lead to the absence of CCR5 expression on the surface of $CD4^+$ T cells are not fully protective from HIV infection[118, 136]. Nevertheless, there is genetic and phenotypic evidence, other than overrepresentation of CCR5-tropic variants in newly infected individuals that point to the importance of CCR5 during transmission. A statistically significant genetic signature around the CCR5 binding site in gp120 V4, along with a complex signal surrounding functionally important amino acids in and around this site, were identified from an analysis of over 6,000 subtype B sequences derived from more than 200 acutely and chronically infected individuals[137]. In a humanized mouse model, infection with the CXCR4-tropic strain, HIV_{NL-E}, resulted in a low level persistent infection, that was suppressed in animals dually infected with the CCR5-tropic $HIV\text{-}1_{NLAD8\text{-}D}$, suggesting that even if CXCR4-tropic viruses cross the mucosal barrier, their restricted replication in $CCR5^+$ $CD4^+$ T cells can result in them being outcompeted by CCR5-tropic variants[138]. Thus, while the exact mechanism for CCR5 selection remains unclear and may be multifactorial[122], the simplest explanation may be that it is driven by target cell availability. This would be

consistent with observations in monkey challenge studies, where the number of $CCR5^+$ target cells in the mucosa correlated with susceptibility[139, 140]. It is also consistent with the observation that $CCR5^-CXCR4^+$ $CD4^+$ T cells are a minor population (<25%) in both the epidermis and dermis of inner and outer foreskin compared to $CCR5^+CXCR4^-$ and $CCR5^+CXCR4^+$ $CD4^+$ T cells[123]. Furthermore, CCR5 expression is high on human vaginal epithelial CD4+ T cells[141]. It should be noted, that transmitted founder virus Envs mediate inefficient infection of macrophages and show a requirement for high levels of both CD4 and CCR5, with no evidence for preferential use of an alternate coreceptor[88, 89, 124, 133, 142]. These studies argue that neither infection of macrophages nor alternate coreceptor usage is advantageous for HIV-1 transmission.

2) *Variable loops, glycosylation and neutralization sensitivity*: Cogent evidence that transmission might select for single variants with traits other than co-receptor usage came from a comparison of viral *env* sequences from both partners of eight subtype C HIV-1 transmission pairs. It was found that within each pair, whether male-to-female or female-to-male, the newly transmitted viruses encoded shorter, less glycosylated V1-V4 regions than their chronic counterparts[87], raising the possibility that more compact envelope glycoproteins better interacted with critical target cells in the genital mucosa. The observation was confirmed using SGA in an additional 10 subtype C transmission pairs[70]. Similar results were obtained in a study comparing Envs from subtype A HIV-1 acutely infected sex workers to a database of matched chronic virus sequences, as well as in a study of thirteen subtype D and A transmission pairs from the Rakai district of Uganda[124, 143]. In contrast, such differences have not been seen in most studies of

recently transmitted subtype B HIV-1, whether the risk factor was associated with male homosexual or heterosexual transmission, suggesting that subtype differences in the virus or maturity of the epidemic may define a role for such a phenotype[143-145]. However, in a study comparing SGA-derived subtype B *env* sequences from 135 acutely infected and 140 chronically infected individuals, statistically fewer N-linked glycosylation (PNLG) sites were found in the gp120s from early infection, with a trend towards fewer PNLG in the V1V2 loops and reduced V4 lengths[137].

Previous studies in the Zambian subtype C transmission cohort indicated that HIV-1 gp120 variable loop length was negatively correlated with neutralization susceptibility[146, 147], and the addition of N-linked glycosylation sites is often associated with escape from neutralization by antibodies[148]. Longer loop length and increased N-linked glycosylation could be residual effects from antibody specific escape[149], suggesting that transmitted variants would be expected to be more sensitive to neutralization in the donor since they have less N-linked glycosylation and shorter variable loops on average[87]. Indeed, subtype C heterosexually transmitted viruses have modestly increased sensitivity to donor plasma taken near the time of transmission[87], though not to broadly neutralizing antibodies[150] or pooled plasma[87]—a finding largely unexplained to-date. One hypothesis suggests bound antibodies could enhance infection, similar to antibody-dependent enhancement of dengue infection, through Fc receptor capture by target cells of interest in the mucosa of the new host, as has been reported for infected volunteers in the VAX004 vaccine trial[151], but this has not been demonstrated in non-vaccinated populations. Nevertheless, subtype B variants have shown differing results, with one study showing increased sensitivity to the broadly

neutralizing antibodies b12 and VRC01[145], while others have shown little or no difference with broadly neutralizing antibodies[89].

3) *Interactions with the integrin α4β7*: The HIV gp120 V2 region binds to α4β7 with various affinities depending on the Env variant under study—a discovery originating from the enigmatic binding of HIV to NK cells[152]. Since $\alpha4\beta7^{+}$ $CD4^{+}$ T cells are highly susceptible to HIV infection[153], and home to mucosal sites including the genital tract[154], there have been several studies aimed at defining the role of this interaction during transmission. A comparison of transmitted/founder and early Envs to later isolates from the same individual, showed high affinity α4β7 binding was lost over time. This appeared in part to be due to absence of glycosylation at sites in V1 and V2 of the transmitted/founder gp120, since mutation of these sites in the chronic Envs increased α4β7 binding[155]. In addition, an analysis of viruses from the CAPRISA acute infection cohort from South Africa showed that dependence on α4β7 for *in vitro* replication was high for transmitted/founder *env* chimeras, particularly ones encoding a P/SDI/V tripeptide binding motif in the V2 region of gp120. This dependence on α4β7 was lost during the first 2 months of infection, but regained at 39 months post infection for three individuals followed longitudinally[156]. In this subtype C cohort individuals with bacterial vaginosis and an associated IL-7/IL-8/IL-1a cytokine signature were infected with viruses with an enhanced α4β7 dependence[156]. Earlier studies of subtype C infectious molecular clones of transmitted/founder and chronic control viruses did not observe differential inhibition of infection by a blocking antibody to α4β7, and frequently observed enhanced replication[150]. However, dissecting inhibition from

ligand binding activation of cells complicates the interpretation of this data. Despite these differences *in vitro*, administering a blocking antibody to α4β7 prior to SIV challenge in 24 rhesus macaques decreased the number of animals infected and increased the number of challenges for infection to occur[157]. Moreover, treated but infected animals showed a significant reduction in CD4 loss in the GALT and evidence for limited viral dissemination out of the genital mucosa[157]. Treatment with anti-α4β7 antibody during acute infection[158], or during withdrawal of suppressive antiretroviral therapy [159], also lead to lower viral load set-points. Thus enhanced α4β7 affinity may indirectly increase transmission by increasing the efficiency by which virus infected cells are trafficked to the GALT.

4) *Infectivity and replicative capacity*: One of the most compelling hypotheses suggests TF variants replicate faster than other variants. Such an edge in replicative capacity would grant a competitive advantage during the initial events of viral growth and dissemination[36]. This would also be compatible with the selection bias for consensus amino acid residues, which are predicted to increase structural stability and functionality of the Gag and Pol proteins[127].

With respect to infectivity, in general, studies have compared both infectivity in single round infection assays, and replication in multi-round infection assays. Evidence that transmitted variants have enhanced infectivity varies between different cohorts and studies[91, 160, 161]. In a study of 27 subtype B and C TF variants compared to 14 chronic controls, TF variants were 1.7-fold more infectious as a group[91]. Enhanced infectivity for early variants was also indicated by genetic evidence from subtype B

sequences, where mutation away from a histidine in the signal peptide of chronically infected individuals suggested potentially higher Env expression for early variants[137]. A subsequent study of pseudoviruses showed histidine and other basic amino acids at the aforementioned signature position in the signal peptide are associated with higher infectious titers, Env expression, and Env incorporation[162]. Corroborating the genetic signature to a degree, a study of subtype B and C transmitted/founder viruses showed 1.9-fold more Env per particle for transmitted variants[91], pointing to the mechanism of enhanced infectivity coming from increased number of expressed trimers. Genital ulceration and inflammation is known to increase general susceptibility[33, 35] and the number of transmitting variants[70] as well as a decrease in viral sequence selection[127], indicating that an intact mucosal bottleneck perhaps necessitates higher viral infectivity. Indeed, an analysis of 27 virus isolates and Env pseudoviruses generated from those isolates from the CAPRISA 004 tenofovir gel trial, showed that variants transmitted to women with genital inflammation were less infectious[160]. Nevertheless, a study of transmission pairs utilizing *env* expression vectors did not observe a selection for more infectious transmitted/founder viruses [133].

Most studies have not demonstrated a replicative advantage for transmitted variants measured in multiple round infections *in vitro*, instead showing transmission of viral variants with a range of replicative capacities. For instance, Gag chimeric viruses amplified from acute and early time points from subtype B[163] and subtype C[164-166] strains exhibited a broad range of replicative capacities, suggesting both relatively low and high replicative capacity variants have the potential to transmit. The replicative capacities of these chimeric viruses containing patient *gag* genes correlate well with those

of infectious molecular clones[166]. Subtype C transmitted variants chimeric for patient derived *gag* genes that were generated from 149 individuals a median of 45 days post estimated date of infection ranged over 3 $\log_{10}$ by log transforming the slope of virus replication *in vitro*[165], though other studies have not observed as wide of a range[163, 164, 167]. *In vitro* replicative capacity correlated with measurements of immune activation and viral load demonstrating *in vivo* relevance[166]. A range of replicative capacities has also been observed for full-length TF viruses[88, 90, 91, 168]. Subtype A transmitted/founder isolates from two heterosexually acquired infections showed a 10-fold difference by 6 days of replication *in vitro*, matching their divergent outcomes *in vivo*[168]. Similarly, 27 Subtype B and C TF variants showed a 100-fold difference in replication at peak *in vitro*. No differences in average replicative capacity between transmitted variants and those from chronic infections were found[91].

Studies of chimeric variants have also shown little difference in replicative capacity when comparing acute and early strains to chronic controls, or viruses from donor partners that were not transmitted. In a study of 869 subtype B infected individuals, of which 66 were in acute/early infection, Gag-Protease chimeric NL4-3 viruses replicated similarly when comparing the mean of both acute and chronic virus groups[163]. Similarly, subtype C Gag-Protease chimeric NL4-3 viruses from 60 acute/early infections[169] and 406 chronic infections[164] replicated similarly. Similarly, studies on NL4-3 based Env chimeric variants from subtype D transmission pairs or subtype B IDUs showed no increased replicative capacity for the Envs derived from acutely infected individuals[170, 171]. Overall, the current *in vitro* data is relatively unclear as to whether replicative capacity is selected during transmission.

5) *Interferon resistance*: The innate immune response, in particular the production of type I interferons (IFN), is very important in a number of viral infections including lentiviruses[172-176], and treatment of macaques with IFNα2 or IFNβ, increases the number of challenges required to establish systemic SIVmac251 and SHIV infection[177, 178]. While IFNs and their downstream genes are upregulated in the early stages of infection[178]—though often lagging behind detection of viremia in SIV infected macaques[43, 62]—a recent large study of acute infection found that during the first few days following infection, SIVmac239 down-regulated the IFN response and it was not until day 10 post-infection, when plasma viremia was evident, that significant expression of interferon stimulated genes were detected[44]. Nevertheless, a number of recent papers have presented evidence that TF variants are relatively resistant to interferon[91, 179, 180]. The first evidence of interferon resistance in transmitted variants came from subtype B viruses, wherein variants derived from chronic infection showed a marked sensitivity to interferon-α *in vitro* when compared to subtype B TF variants; however, in the same study, subtype C TF and chronic variants exhibited similar sensitivity to interferon[91]. Moreover, in a comparison of subtype B and subtype C TF variants to 6-month consensus viruses derived from the same individuals, TF were more resistant to IFN-α[179]. A specific restriction factor associated with co-receptor usage, IFN-induced transmembrane protein 1 (IFITM1), determined the resistance phenotype of the TF and 6-month virus pairs[181], though VPU-tetherin interactions have also been implicated as major determinants of resistance for some of these variants[182]. Both interferon induced restriction factors IFITM1 and tetherin act at the plasma membrane

and interact with the viral envelope, and Env has been associated with interferon resistance in an investigation of SHIVs passaged in macaque cells *in vitro*[183]. Moreover, HIV-1 binding to either DC-SIGN or Langerin determines restriction in dendritic cells, highlighting how receptor binding can alter viral restriction[184].

However, not all studies have reported results consistent with these most recent observations[170, 185, 186], though these studies were not published at the initiation of this thesis. Observations in the same study that showed differences in subtype B interferon resistance, showed subtype C TF variants were not different from chronic controls[91]. Furthermore, acute Env chimeras derived from 7 acute subtype B IDU infections did not display greater interferon resistance when compared to chronic controls[170]. Additionally, 12 subtype B and C acute and chronic viruses, assessed for activity of accessory proteins that often counteract interferon induced restriction factors, showed similar activity *in vitro*[186].

The Role of Accessory Proteins in Viral Transmission

Accessory proteins of HIV are encoded by genes that are not essential for replication *in vitro*. For HIV-1 and SIV precursors (SIV from chimpanzees and gorillas), these proteins include Nef, Vpu, Vpr and Vif, while for HIV-2 and its SIV precursors (SIV from sooty mangabeys and rhesus macaques), it also includes Vpx, a paralog of Vpr. The accessory proteins enhance *in vivo* fitness, primarily through evasion of innate and adaptive immunity, though multiple roles have been discovered for each protein, especially for Nef (~200 amino acids long) and Vpu (~90 amino acids long), which is the focus of Chapter 3 of this thesis[175, 187]. Nef and Vpu have parallel functions that help

HIV-1 evade innate and adaptive immunity by antagonizing retroviral restriction factors like tetherin and serine incorporator 5 [175, 187-189], manipulating NK and CD8 T cell ligands though HLA-I molecules[190-198], increasing viral infectivity through downregulation of CD4[188, 199-209], altering surface proteins important for cell trafficking like CXCR4, CCR5 and CD62L[210-213] and enhancing induced replication from resting cells[214-216]. Nef and Vpu therefore have a part to play in evasion from vaccine induced immunity dealing with viral transmission and dissemination, as well as evasion from cure strategies that impact upon viral persistence.

Nef and Vpu downregulate arguably the most important cell-surface proteins of the immune system, the human leukocyte antigen class I (HLA-I) molecules. HLA-I downregulation aids evasion of adaptive immunity from CD8 T cells that recognize infected cells through T cell receptor peptide:HLA contacts. Similarly, innate immunity is impacted through HLA-I binding to NK cells via NK killer immunoglobulin-like receptors. HLA-A, HLA-B, and HLA-C are downregulated from the surface of infected cells[192, 193, 195, 197, 198, 217, 218], though initial studies focusing on Nef suggested HLA-C, typically an NK inhibitory ligand, was not downregulated, as a mechanism of evading NK cell mediated killing of infected cells[193, 219-221]. Few studies contradicted this narrative[197] before the 2016 discovery of Vpu-mediated HLA-C downregulation by HIV-1 primary strains—a finding previously missed likely because commonly used lab-adapted strains like NL4.3 lacked this ability[192], highlighting the importance of investigating primary viruses in studies of HIV attributes, despite the fact that primary viruses are often more difficult to work with *in vitro*.

Nef and Vpu have overlapping functions, aside from their interactions with HLA-I molecules, which are related to viral infectivity and infected cell trafficking. Both of these molecules contribute to a much reduced cell surface expression of CD4 and the homing receptor L-selectin (CD62L)[207, 211, 222-226]. CD62L is a major determinant of T cell movement between peripheral lymphoid tissue and blood, and helps T cells bind high endothelial venules (HEV)[227-229]. Antigen stimulation leads to shedding of CD62L, allowing T cell migration out of secondary lymphoid tissues[230, 231]. Disrupting CD62L trafficking to the plasma membrane with a mechanism independent of CD62L shedding[211, 222, 232] could alter trafficking and viral dissemination[233], an important step in establishing systemic infection following transmission, although little is known about the impact of natural sequence variation in Nef or Vpu on CD62L downregulation. CD4 downregulation prevents its interaction with Env during intracellular transport and budding, in order to enhance viral infectivity, prevent the cytotoxic effects of superinfection[234-238], and reduce susceptibility of infected cells to antibody-dependent cell-mediated cytotoxicity (ADCC)[239].

Nef also plays a role in the emergence from quiescent cells, which was discovered using cell culture systems where resting $CD4^+$ T cells rather than pre-activated cells were infected[214, 215]. These quiescent cells were then stimulated days later in order to induce virus production. The presence of Nef significantly boosted the amount of virus that emerged from primary cells, though less so from cell lines[214, 215]. This Nef associated trait has a bearing on transmission. A majority of SIV infected $CD4^+$ T cells in the vaginal mucosa 12 days after inoculation are resting, and approximately half of infected cells from human lymph nodes and tonsils from acute and early infection are

resting CD4+ T cells (HLA-DR-Ki67-)[38]. Thus, being able to infect and replicate in minimally activated cells may serve a benefit to transmitting variants that per chance encounter cells at various stages of activation. The functional diversity of Nef in this respect has not been characterized for primary viruses with intact Nef reading frames, and the specific viral traits that impact this phenotype are not completely understood[188, 189].

What is known about Nef and Vpu with respect to transmission is limited compared Env for instance. In a study of subtype C transmission pairs, a strong selection bias for consensus residues in functional domains of Nef was observed, in areas related to CD4 and HLA downregulation[127]. However, a study of CD4 surface expression on transfected SupT1 lymphoblasts with patient derived Nef proteins, showed preservation of CD4 downregulation capacity in six male to male subtype B transmission events[240]. No differences were observed in CD4 or HLA-A2 downregulation by *nef* clones isolated from acutely infected individuals versus those isolated one year later [241]. A study that included three subtype C transmitted/founder and three subtype C chronic virus *nef* and *vpu* genes found no significant differences in Nef or Vpu mediated CD4 or HLA-I downregulation[186]. Most of these studies include the use of cell lines, expression vectors, and chimeric viruses [191, 240-246]. The extent to which there is functional diversity of Nef and Vpu associated functions in a quasispecies has rarely been addressed[198, 240, 243, 247], indicating that some characterization of primary viruses in primary cells is needed before apt comparisons with transmitted/founder viruses can be made.

Impact of the TF Phenotype on Disease Progression

It is very clear that traits of the transmitted virus influence the course of disease. VL set-point in the donor correlates with that in the infected partner [248-252], despite significant variability in the recipient's immune response to the infection, high viral diversity, and a restrictive genetic bottleneck during transmission. The heritability of VL is consistent with the concept that viral phenotypes are transmitted from person to person, and VL itself is a significant predictor of disease progression. A number of studies have successfully demonstrated a link between transmitted viral traits and disease progression[166, 168, 169, 250, 253-255]. A study of 149 linked transmission pairs showed that replicative capacity conferred by transmitted subtype C *gag* sequences correlated with VL set-point, and that low replicative capacity was protective against CD4 decline for three years after transmission[165]. A follow up study showed that replicative capacity of transmitted variants predicted CD4 decline independently of other risk factors 5 years after transmission, and was associated with very early (~45 days) increases in inflammatory cytokines, T-cell activation/dysfunction, and central memory $CD4^+$ T cell viral burden[166]. These findings confirmed earlier studies with gag-chimeric viruses derived from acutely infected women in South Africa showing evidence for more rapid CD4 decline associated with higher RC viruses[169]. Similar conclusions came from a study of two subtype A transmission pairs where recipients shared three HLA-I alleles but had divergent disease outcomes, in that the non-progressor was infected by a low replicative capacity TF variant and the rapid progressor by a higher RC variant[168]. These studies indicate that low RC variants are able to be sexually transmitted and, when they are, induce a slower CD4 decline and progression to AIDS.

Thus, the viral phenotype of the transmitting virus can have profound impacts on disease progression through the heritability of replicative capacity.

Other traits of transmitted viruses impact VL and subsequent disease progression along with replicative capacity—one of these is viral adaptation to the cellular immune response. HIV-1 adapts to a host through mutations that facilitate immune escape, and many of these can be passed on during transmission[127]. In a study of 114 linked Zambian transmission pairs, transmitted escape mutations in Gag near $CD8^+$ T cell epitopes led to lower viral loads in subtype C infected recipients[253], a finding mirrored in other studies[254]. The importance of viral adaptation to T-cell responses and disease progression was highlighted in a multicohort study of 4,522 individuals in chronic infection and 471 in acute and early infection from both subtype B and subtype C cohorts. It demonstrated that HLA alleles that are typically associated with lower VL and slower disease progression become much less effective when incoming viruses are already well adapted to those alleles[250], and that an infection with a pre-adapted viral sequence to the recipient's HLA alleles was associated with higher viral loads and lower CD4 counts[250]. A concurrent study from Monaco *et al.*[255] also demonstrated the importance of pre-adaptation in a study of 169 subtype C-infected Zambian heterosexual transmission pairs. It found that on average, in the transmitted virus, almost one-third of possible HLA-linked target sites for the recipient's cellular immune response to Gag were already adapted, and that transmitted pre-adaptation significantly reduced early immune recognition of epitopes[255], as was also reported in subtype B infected individuals[250]. Transmitted pre-adapted polymorphisms and polymorphisms that were not linked to the recipient's HLA alleles (non-associated polymorphisms) showed

opposing effects on set-point VL and the balance between the two was significantly associated with higher set-point VLs in a multivariable model including other risk factors. Transmitted pre-adaptation was also significantly associated with faster CD4 decline (<350 cells/μl) and this association was stronger after accounting for non-associated polymorphisms, which were linked with slower CD4 decline[250, 255]. Thus, even before an immune response is mounted in the new host, the balance of adaptation and viral fitness can significantly influence the outcome of HIV-1 infection. Likely related to pre-adaptation is the observation that transmission of multiple variants is associated with faster disease progression. A study of 156 female sex workers from Kenya showed that, in women infected with multiple variants, $CD4^+$ T cell counts declined to less than 350 cells per milliliter in 3 years, compared to 5 years in women infected with a single variant[256]. Another study of 69 heterosexual men and women in southern Africa found viral loads were nearly twice as high at 12 months post-infection for those infected by multiple variants[94]. Furthermore, analysis of 163 subtype B and subtype recombinant CRF01_AE infections from STEP and RV144 vaccine trial participants showed that those infected with multiple variants, as measured either by single genome amplification or *env* diversity thresholds, had higher viral loads (~0.4 $\log_{10}$)[257]. In the group infected by multiple variants, recombination between infecting variants could allow generation of virus most adapted and least recognized by the immune system.

Summary to introduction

Evidence for genetic and phenotypic selection at the mucosal surface for transmitted/founder viruses with distinct characteristics has been mounting since the HIV-1 genetic bottleneck was discovered in the early 1990's. And yet, the nature and extent of the viral-specific bias that occurs during transmission is still under dispute. The impact of transmitted viral properties reaches beyond the initial infection events, since transmitted viral traits influence subsequent disease progression. In order to halt transmission and blunt disease progression in the unfortunate circumstance of HIV infection, a better characterization of viral properties is imperative.

At the outset of this thesis, it was unknown how primary genome-length viruses derived from transmission pairs behaved. In general, full-length primary viruses were relatively unavailable for characterization due to technical constraints. Since closely related viruses from a donor quasispecies with a complement of co-evolved native proteins present had yet to be examined, it was unknown if the transmitted/founder virus and the quasispecies near the time of transmission would show a preference for consensus residues over the whole genome, be uniquely resistant to interferon, have higher replicative capacities or infectivities. These areas and more are the subject of Chapter 2. Characterization of HLA-I downregulation and the subsequent impact on NK cells is the subject of Chapter 3, while additional patient characteristics, viral traits, and technical findings are presented in the Appendix.

Figures

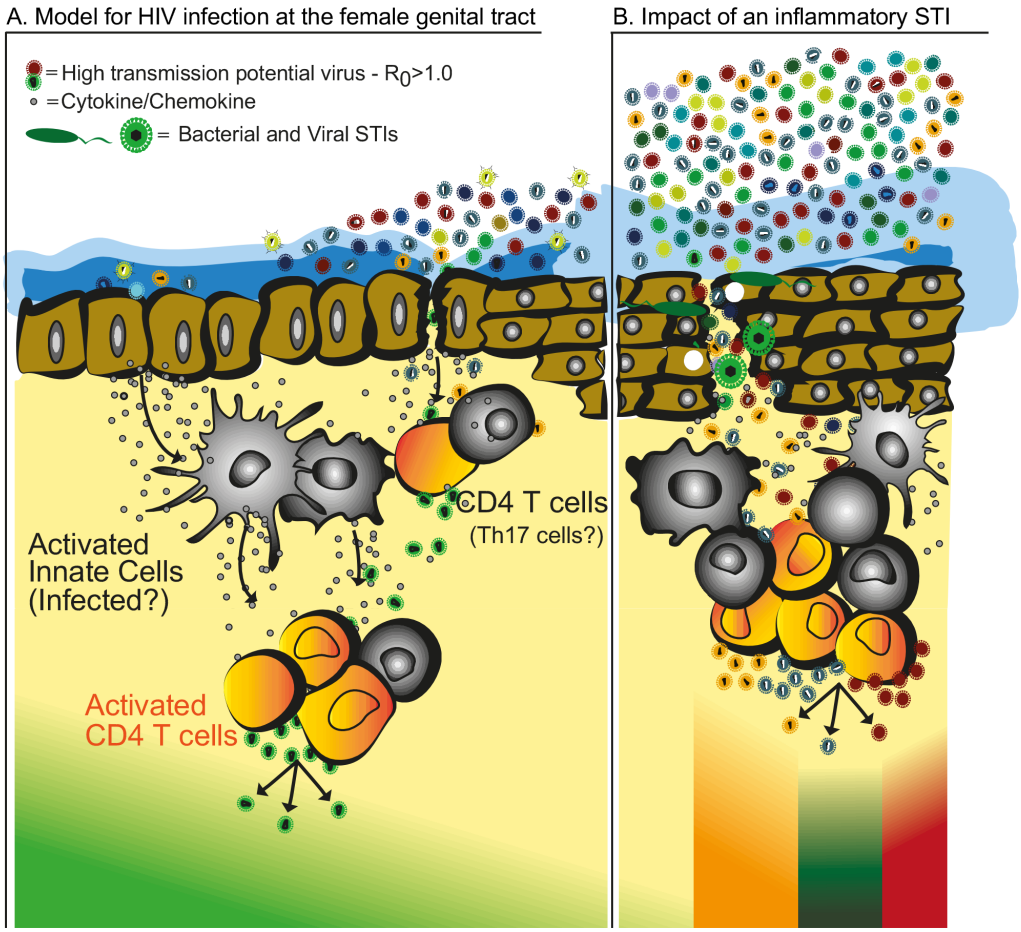


Fig 1. Model of HIV infection from mucosal exposure

A. The HIV quasispecies is genetically, phenotypically and numerically diverse and interact with the exposed genital mucosa (i.e., rectal, vaginal, cervical, penile etc.) of the uninfected individual. Recent studies in the SIV model suggest that virus binding to the genital epithelium signals production of cytokines, which bring in macrophages and

dendritic cells. These in turn generate a cytokine gradient that attracts activated $CD4^+$ T cells to the site of initial infection. Initial target cells are likely susceptible $CD4^+$ $CCR5^+$ T cells, a majority of which may be Th17 $CD4^+$ cells, but infection of macrophages and dendritic cells have been reported. These cells can replicate virus locally or traffic to nearby secondary lymphoid structures—virus could also diffuse there directly. Once virus reaches local lymph nodes and disseminates throughout the body, specifically to the gut mucosa, viral load increases exponentially in the blood. Approximately 70-80% of mucosal infections are established by single variants. **B.** High viral loads in the inoculum or inflammatory conditions from GUI (i.e. HSV, or bacterial infection) mitigate normal barriers to infection. Under these conditions, more activated $CD4^+$ $CCR5^+$ target cells are present in the area due to inflammatory cytokines and chemokines produced locally. As a result, more than one variant often establishes infection in the new host, and may have fewer phenotypic or genetic traits that favor its transmission since selection pressures are absent or lessened.

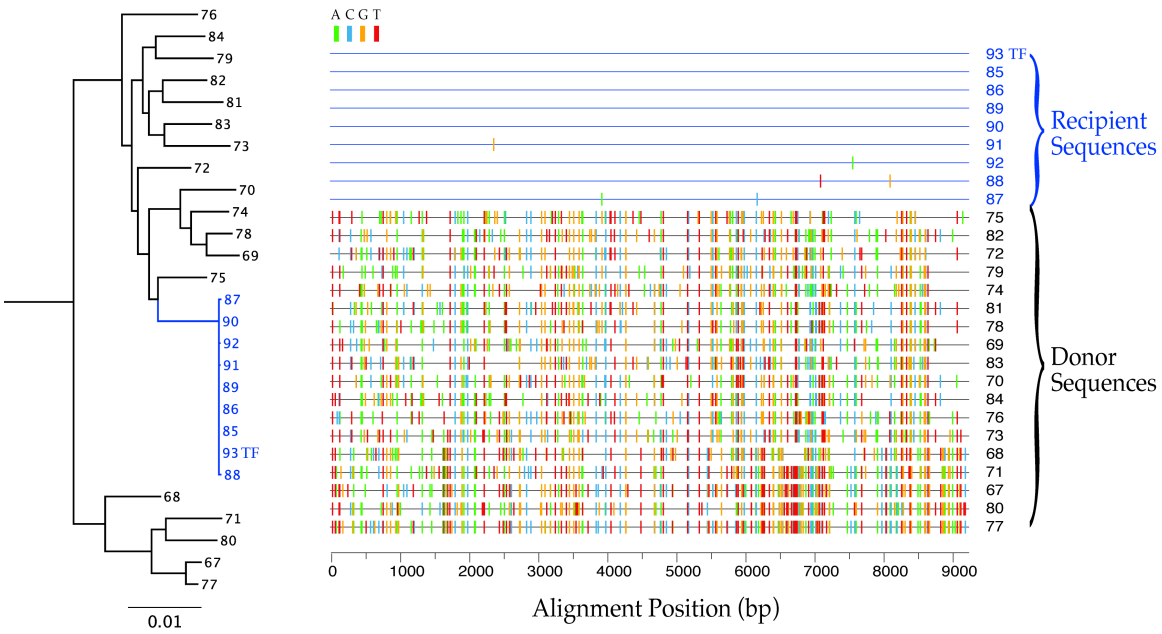
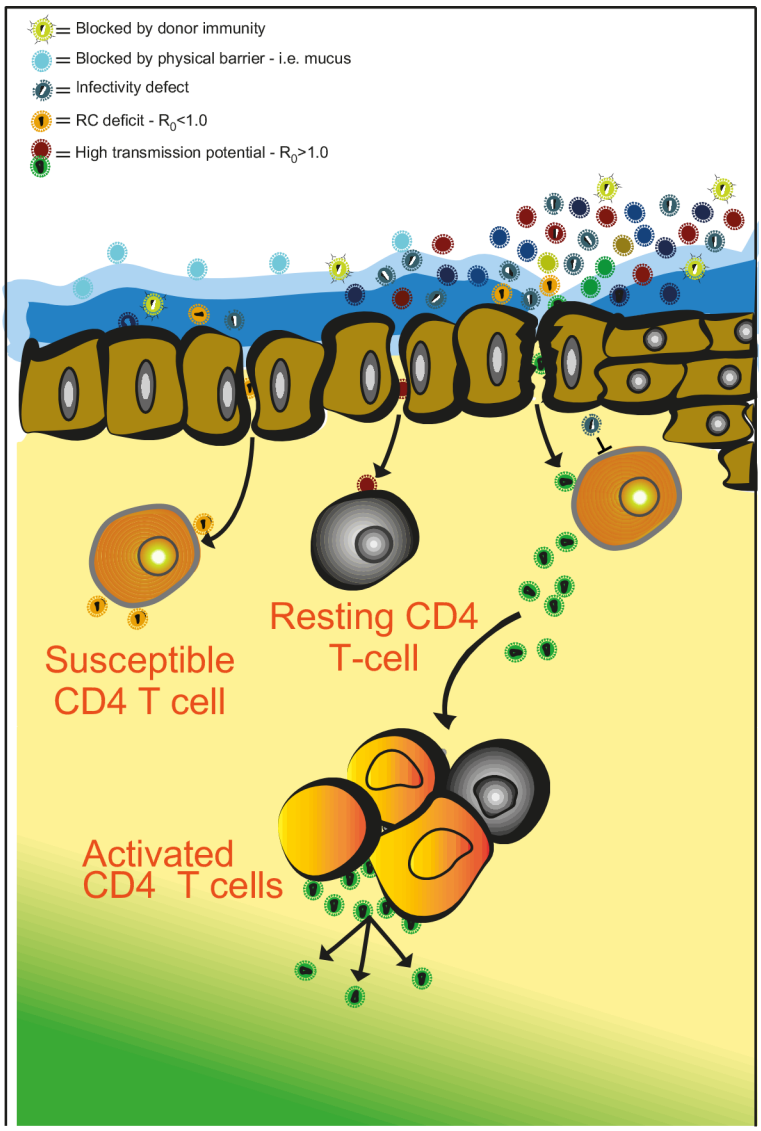


Fig 2. Analysis of sequences from a transmission pair

A. Phylogenetic tree of viral sequences from a Zambian transmission pair. Eighteen near-full length genomic amplicon sequences from the transmitting partner (Donor, Black) and nine from the newly infected partner (Recipient, blue) are displayed in a maximum-likelihood phylogenetic tree. The donor sequences show sequence diversity (horizontal distances between branch tips) with some genomes differing by 0.34% to 6.5%, while the recipient sequences exhibit very limited diversity, differing by a maximum of 0.09%. **B.** Highlighter analysis of transmission pair sequences. All sequences are compared to the recipient consensus, TF sequence represented by amplicon 93. A majority of the recipient amplicons differ from this sequence by only one or two nucleotide changes, which represent random errors by the reverse transcriptase in this very early acute infection sample. In this case the donor sequences exhibit multiple changes from the TF virus and from each other.



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17 **Fig 3. Viral traits that may be advantageous during transmission**

18 During transmission the virus encounters a number of barriers to infection including mucous and

19 epithelial layers that can block access to target cells. However, viruses can penetrate host

20 defenses perhaps most easily through yet unrepaired breaks in the epithelium. The entering

viruses must interact with susceptible $CD4^+$ $CCR5^+$ T cells in order to propagate, a viral trait that may entail selection for more infectious strains. Entry into non-permissive resting $CD4^+$ T cells will result in non-productive infection, or limited viral reproduction. Similarly, if the replicative capacity of the virus is unable to sustain a spreading infection (R_0<1.0), even infection of susceptible cells will not result in dissemination. It is likely that viruses with a replicative advantage will out compete those that replicate less efficiently. As described in Figure 1A, continued amplification of infection may require an influx of activated $CD4^+$ T cells.

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Chapter II: Heterosexual transmission of subtype C HIV-1 selects consensus-like variants without increased replicative capacity or interferon-α resistance

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Abstract

Heterosexual transmission of HIV-1 is characterized by a genetic bottleneck that selects a single viral variant, the transmitted/founder (TF), during most transmission events. To assess viral characteristics influencing HIV-1 transmission, we sequenced 167 near full-length viral genomes and generated 40 infectious molecular clones (IMC) including TF variants and multiple non-transmitted (NT) HIV-1 subtype C variants from six linked heterosexual transmission pairs near the time of transmission. Consensus-like genomes sensitive to donor antibodies were selected for during transmission in these six transmission pairs. However, TF variants did not demonstrate increased viral fitness in terms of particle infectivity or viral replicative capacity in activated peripheral blood mononuclear cells (PBMC) and monocyte-derived dendritic cells (MDDC). In addition, resistance of the TF variant to the antiviral effects of interferon-α (IFN-α) was not significantly different from that of non-transmitted variants from the same transmission pair. Thus neither *in vitro* viral replicative capacity nor IFN-α resistance discriminated the transmission potential of viruses in the quasispecies of these chronically infected individuals. However, our findings support the hypothesis that within-host evolution of HIV-1 in response to adaptive immune responses reduces viral transmission potential.

Author Summary

Despite the available HIV-1 diversity present in a chronically infected individual, single viral variants are transmitted in 80-90% of heterosexual transmission events. These breakthrough viruses may have unique properties that confer a higher capacity to transmit. Determining these properties could help inform the rational design of vaccines and enhance our understanding of viral transmission. We isolated the transmitted variant and a set of related non-transmitted variants from the transmitting partner near the estimated date of transmission from six epidemiologically linked transmission pairs to investigate viral correlates of transmission. The simplest explanation that transmitted variants are inherently more infectious or faster replicators *in vitro* did not hold true. In addition, transmitted variants did not replicate more efficiently than their non-transmitted counterparts in dendritic cells or in the presence of interferon-alpha *in vitro,* suggesting that they are not uniquely adapted to these components of the innate immune system. More ancestral genomes that were relatively sensitive to antibody neutralization tended to transmit, supporting previous reports that mutational escape away from the adaptive immune response likely reduces the ability to transmit. Our investigation into the traits of transmitted HIV-1 variants adds to the understanding of viral determinants of transmission.

Introduction

HIV-1 transmission is characterized by an extreme genetic bottleneck, the basis of which is unclear. Studies of both the highly diverse envelope glycoprotein [1-3] and full HIV-1 genomes [4] demonstrated that 80-90% of heterosexual transmissions are initiated by a single virus variant selected from the diverse viral quasispecies present in the chronically infected transmitting partner. These variants, which are different in each transmission event, have been named transmitted/founder (TF) viruses. Studying TF viruses could enhance our understanding of viral transmission and inform HIV prevention strategies.

The TF is rarely the dominant variant in the plasma or genital tract of the transmitting partner [5,6], which suggests that transmission is not entirely stochastic and may involve selection. A number of prior studies have identified distinctive properties of TF variants [4,7-19], particularly in analyses of the TF viral envelope (Env) glycoprotein. Reported characteristics of TF virus Envs include a selection for CCR5-tropism [2,20], a predominance of shorter and less glycosylated Env proteins [1,11,15,18,19], a preference for binding α4β7 [10,21] and a selection for more ancestral variants [8,22]. Although these studies observed selection of viral traits, others found that acute and chronic variants had similar characteristics. By generating infectious molecular clones (IMC) with the *env* genes from linked recipients and transmitting partners in a common viral backbone, acute and chronic donor viruses displayed similar CD4 and CCR5 requirements for cell entry, low macrophage tropism, and no preferential usage of alternative coreceptors [23,24]. Furthermore, studies of *env* only clones from acute

infection compared with chronic control viruses have shown similar CD4 T cell subset tropism, low macrophage tropism, and a lack of effect of blocking α4β7 on infection [25].

Selection of viral traits outside of the *env* gene has also been observed during heterosexual transmission. We recently described a selection bias during transmission for more consensus-like HIV-1 variants, in *gag*, *pol* and *nef* genes, from a cohort of 137 subtype C infected epidemiologically-linked transmission pairs [7]. This study suggested that *in vivo* fitness of consensus-like HIV-1 variants increased their likelihood of transmission [7]. Studies of full-length infectious molecular clones of TF viruses, in comparison to control viruses derived from chronic infection, have also demonstrated increased particle infectivity, as well as an enhanced resistance to interferon-α (IFN-α) in TF viruses [13,17].

While informative, conclusions of these previous studies are limited in that only individual genes were examined, or corresponding non-transmitted (NT) variants from the transmitting partner were unavailable as controls. HIV-1 IMC with the full complement of HIV-1 proteins have not been generated from both partners of transmission pairs nor evaluated for genetic and phenotypic signatures during transmission. Characterizing TF variants in comparison to NT variants from epidemiologically-linked partners could provide further insight into the viral requirements of HIV-1 transmission, potentially leading to new targets for intervention.

Here, we describe genetic and phenotypic comparisons of full-length genome TF and NT variants from six subtype C epidemiologically-linked heterosexual transmission pairs. We amplified and sequenced near full-length HIV-1 genomes by single genome amplification (SGA) to assess genetic selection during transmission. In addition, we

cloned the complete TF genome along with a representative panel of NT variants. These clones were used to assess the relative *in vitro* fitness of TF variants as measured by particle infectivity, neutralizing antibody resistance, replicative capacity in PBMC and dendritic cells, as well as IFN-α resistance. We found a strong selection bias toward consensus sites across the entire genome, at both the amino acid and nucleotide level, in all six pairs. The TF variants were also more sensitive to neutralization by donor antibodies than NT variants. However, no evidence was found for TF variants exhibiting increased particle infectivity, replicative capacity, or IFN-α resistance when compared to the transmitting partner's NT variants. Thus, in these six subtype C transmission pairs the transmission potential of TF variants is not discriminated by inherent *in vitro* replicative capacity or interferon resistance, and may be determined by alternate phenotypes difficult to dissect in these *in vitro* systems.

Results

Transmission pairs and amplification of near full-length genomes

Full-length genome HIV-1 variants derived from linked transmission pairs have yet to be evaluated for characteristics associated with transmission. To define whether TF variants exhibit distinct properties, we compared them to their NT counterparts in six heterosexual epidemiologically-linked transmission pairs. We selected five female-to-male and one male-to-female therapy-naïve subtype C epidemiologically-linked transmission pairs from the Zambia-Emory HIV Research Project (ZEHRP) based on the availability of plasma samples at the nearest time points following transmission (average 28 days post estimated date of infection) (Table 1). We PCR amplified, using a high-fidelity polymerase, and sequenced a total of 167 HIV-1 near full-length single genome amplicons as described previously [26]. All six linked recipients were in Fiebig Stage II of infection, and were infected with a single variant from the donor quasispecies, as demonstrated by star-like phylogeny in a median of 8 near full-length genome amplicons per sample [2,4]. This allowed us to infer an unambiguous consensus TF sequence from the genetically homogeneous population of sequences in each linked recipient.

For phylogenetic analyses, we aligned full-length nucleotide sequences as well as concatenated full proteome amino acid sequences of 115 HIV-1 single genomes (each TF virus represented by a single consensus sequence), with the HIV-1 consensus/ancestral alignment from the Los Alamos National Laboratory (LANL) HIV database. We generated maximum likelihood trees of the full-length genome and proteome alignments for all six transmission pairs, and confirmed that all pairs were epidemiologically linked, since each TF variant fell clearly within the branches of the linked donor virus variants.

Each transmission pair clustered independently on the phylogenetic tree with bootstrap values of 100 (Fig. 1). All six linked donor partners were chronically infected and demonstrated viral diversity in their plasma near the time of transmission (Fig. 1).

Consensus-like nature of TF and NT viruses

We previously demonstrated a consistent transmission bias for variants with consensus-like amino acid residues across the Gag, Pol and Nef proteins by population sequencing in a cohort of 137 epidemiologically-linked subtype C transmission pairs [7]. Although this finding has been shown for the *gag* and *env* genes independently, it has not been confirmed by full-length genome SGA from the transmitting partner's quasispecies [7,8]. We examined the selection bias for more consensus-like viruses by measuring the pairwise distance (branch length), of each viral variant to the LANL subtype C consensus node on the full-length nucleotide and amino acid phylogenetic trees (Fig. 1). TF variants had a significantly shorter pairwise distance to the subtype C consensus node than the median of their corresponding NT variants for both nucleotide (Fig. 2A; $p = 0.0156$) and amino acid (Fig. 2B; $p = 0.0469$) sequences. These transmission pairs confirm, as previously described, a selection bias for consensus-like amino acid and nucleotide sites across the viral genome during transmission.

Particle infectivity of TF and NT viruses

In a previous study, TF virions exhibited enhanced infectivity in comparison to chronic control viruses on TZM-bl cells [13]. To test particle infectivity within transmission pairs, we generated full-length IMC for 40 viral variants, including the 6 TF

variants and 3-8 NT variants from each chronically infected transmitting partner, as described previously [26]. We selected variants to represent the genetic diversity present in the donor near the time of transmission (Fig. 1), and confirmed that the IMC and amplicon sequences were identical by whole genome sequencing. We also excluded the rare sequences that contained gross genetic defects, such as large deletions and frameshift mutations in gene coding regions. For each IMC, we generated virus stocks by transfection of 293T cells.

We defined particle infectivity as the ratio of infectious units, as measured by the virus titer on TZM-bl cells, a standard reporter cell line whose permissivity correlates with that of PBMC [27], over total amount of virions, measured by reverse transcriptase activity of the virus stock. We confirmed that the particle infectivity of a subset of virus stocks generated from 293T cells and harvested 48 hours after transfection (for consistency, as particle infectivity decreased over time post-transfection, S1A Fig.) correlated with the particle infectivity of virus stocks generated from PBMC 8 days following infection (S1B Fig., $p < 0.0001$, $r = 0.9455$). Analysis of the particle infectivity of virus stocks produced from all of the infectious molecular clones showed that the particle infectivities of all viruses tested ranged from $7x10^{-5}$ to $1x10^{-2}$, and that there was also a wide range of particle infectivities within each transmitting partner's quasispecies (Fig. 3). In pair 3678, the TF variant was the most infectious virus compared to the rest of the transmitting partner's variants, while the TF from pair 3576 was the least infectious (Fig. 3). TF variants spanned the thousand-fold range of particle infectivities measured for all the viruses tested, as can be seen by the TF from pairs 3618 and 4473, which are found on extreme ends of the particle infectivity spectrum. Across all six transmission

pairs, we observed no significant selection for infectivity when comparing the TF to the median of the transmitting partner's quasispecies (Fig. 3; p = 0.6875). In these subtype C transmission pairs particle infectivity did not constitute a dominant determinant of transmission fitness.

Sensitivity of TF and NT variants to neutralization by antibodies present in the transmitting partner

We previously reported that Env glycoproteins derived from early viruses in acutely infected linked recipients were on average more sensitive to neutralization by plasma from the transmitting partner, compared to autologous Envs directly derived from the transmitting partner [1]. Antibody neutralization of SGA-derived genome length TF and NT variants, derived from the first month of infection, from heterosexual epidemiologically-linked transmission pairs, has not been examined to date. Using a previously described TZM-bl neutralization assay [1,28], we evaluated neutralization of full-length TF and autologous NT IMC by plasma from the transmitting partner near the time of transmission. Donor plasma (diluted 1:100) demonstrated relatively weak neutralization against the majority of viruses tested in each panel, with a median of 18% neutralization. The highest level of neutralization was seen in pair 4473 against the TF (51%) (Fig. 4A). Overall, TF variants were more efficiently neutralized compared to the medians of the transmitting partner's NT variants (Fig. 4A; p = 0.031). Additionally, greater neutralization negatively correlated with distance to the amino acid subtype C consensus (Fig. 4B; p = 0.011, r= -0.4995), suggesting a link between these two

measurements. Consistent with our previous findings, transmission did not select for TF variants with greater neutralizing antibody resistance to donor plasma.

In vitro replication of TF and NT viruses

Selection for consensus-like TF variants in these six transmission pairs may indicate a selection for viruses with greater *in vivo* fitness, as hypothesized from a study of 137 linked transmission pairs [7]. To determine whether this translated into a similar fitness advantage in activated CD4 T cells, we measured the replicative capacity (RC) of viruses *in vitro*. TF and NT IMC were tested for *in vitro* replication by infection of stimulated peripheral blood mononuclear cells (PBMC), at equal multiplicities of infection (MOI). Since the number of infectious particles to total particles varied greatly between all virus stocks tested, we based the amount of virus used for each replication experiment on a consistent MOI (0.01), rather than equal amounts of virus particles, in order to normalize for initial infectivity. We measured virus growth by reverse transcriptase activity of cell culture supernatants every 48 hours for ten days (Fig. 5A). RC scores were generated for each virus based on the area under the curve of virus growth, as described in the methods. TF viruses exhibited a wide range of RC among all the viruses tested, and the relative RC of TF as compared to NT viruses from the same donor also varied substantially (Fig. 5B). For instance, the TF from pair 3576 had the lowest RC when compared to the transmitting partner's quasispecies. Alternatively, pairs 3618 and 3678 had TF viruses with relatively high RC, although they were not the highest replicators from their transmitting partner's quasispecies (Fig. 5B). In total, we saw no significant selection for TF viruses having higher *in vitro* RC than the median RC

of the NT viruses tested (Fig. 5C; p = 0.219). Similar to particle infectivity, which correlated with RC over all the viruses tested (S2 Fig.; p = 0.0005, r = 0.5712), there was no evidence for a distinct replicative capacity profile associated with transmission. In addition, viruses closer to consensus typically had lower *in vitro* replicative capacities, since the pairwise distance to subtype C consensus correlated with *in vitro* replicative capacity (Fig. 5D; p = 0.0158, r = 0.4168).

Since dendritic cells have also been implicated as an initial target cell for establishment of HIV-1 infection in the genital mucosa [29], we examined the ability of the 6 TF and a limited set of 6 NT variants with similar *in vitro* RC scores, to productively infect and replicate in immature monocyte derived dendritic cells (MDDC) *in vitro*. We cultured MDDC by isolation and differentiation of blood-derived CD14+ monocytes from healthy donors and infected them with virus at a high MOI of 1. We assayed virus production by measuring the reverse transcriptase activity present in cell culture supernatants every 48 hours for twelve days. We found that the TF and NT variants studied did not significantly differ in their ability to replicate in MDDC (S3 Fig.; p = 0.87). Of the twelve TF & NT variants, six had detectable replication in MDDC (3 TF & 3 NT), suggesting that productive infection of MDDC is limited, even at a high MOI, and is not a requirement for transmission. Overall, these data suggest that HIV-1 transmission is permissive to TF variants with a wide range of *in vitro* replicative capacities relative to the transmitting partner's quasispecies.

Interferon-α resistance in HIV-1 subtype C transmission pairs

By conducting *in vitro* replication assays in cells pre-treated with exogenous interferon-α (IFN-α), previous studies found that subtype B and subtype C TF variants were relatively resistant to IFN-α compared to a panel of chronic viruses [13] or later variants from the same individual [1-4]. These studies suggested a selection during the HIV-1 transmission bottleneck for variants adept at escaping innate immunity, specifically the antiviral effects of IFN-α. However, these studies were not done in epidemiologically-linked transmission pairs, and thus were unable to directly compare TF viruses to related NT variants in the donor quasispecies near the time of transmission. To test whether the subtype C TF viruses investigated here exhibited relative resistance to IFN-α, as compared to NT variants derived from the transmitting partner's quasispecies, we assayed *in vitro* virus replication in PBMC in the presence and absence of IFN-α.

We assayed viral replication in activated CD8-depleted PBMC in the presence and absence of 5,000 U/ml of IFN-α, which was added 24 hours prior to infection in order to maximally inhibit viral replication, as described previously [2, 5]. Supernatant HIV-1 p24 antigen levels were measured every 48 hours for 10 days to assess the kinetics of viral replication. In the initial 21 variants tested, growth of virus in the presence of IFN-α was tightly correlated with *in vitro* RC scores in the absence of IFN-α (Fig. 6A; $p < 0.0001$, $r = 0.8844$), suggesting that *in vitro* growth in the presence of IFN-α was largely determined by viral replicative capacity.

In light of this, we attempted to delineate subtle differences in IFN-α resistance by performing further experiments with selected NT variants that exhibited relatively similar replication kinetics to the TF in each pair (to minimize the impact of replication differences). The replication of these selected viruses was assessed in activated PBMC in

the presence and absence of 1,000 U/ml of IFN-α (added 24 hours prior to infection), monitoring virus replication by reverse transcriptase activity in the supernatant. An example of such an assay for transmission pair 331 is shown in Fig. 6B. When compared to the tested NT variants, TF viruses did not differ significantly in resistance to IFN-α (assessed as the ratio of the RC score in the presence and absence of IFN-α) (Fig. 6C, $p = 0.219$). In pair 331 and 4473, the TF appeared to be more IFN resistant than the NT viruses from the same donor (Fig. 6C). In pair 3618 the TF was near the median of the NT variants, while in three pairs (3576, 3678, and 4248), the TF was the most sensitive to IFN-α. Overall, the IFN-α resistance of the TF viruses did not differ significantly from the median of the NT variants (Fig. 6C).

Because the TF viruses were not found to be more IFN-resistant than donor NT viruses, we validated the method used for analysis of IFN resistance with 3 subtype B TF and 6-month consensus virus pairs that had previously been demonstrated to differ in their IFN-α resistance [2, 6, 7]. As shown in Figure 6C, the 3 TF viruses were each confirmed to be more IFN-resistant than the matched 6-month virus from the same subject, verifying the ability of the methods used here to detect previously documented differences in viral IFN resistance. In the six subtype C epidemiologically-linked transmission pairs studied we also observed that IFN-a resistance correlated with the virus' ability to replicate (S4A Fig.). Although the RC and IFN-α resistance of the six subtype B TF and 6-month viruses was not statistically correlated, these subtype B TF viruses did have higher RC scores than their matched 6-month variants (S5 Fig.). Overall, these data suggest that a component of IFN-α resistance is the ability of TF and NT HIV-1 variants to replicate.

To confirm that this finding was independent of the amount of IFN used to inhibit viral growth, we measured replication at day 7 for four viruses with a representative range of RC scores using a range of IFN-α concentrations (0.5 U/ml - 10,000 U/ml). The relative sensitivity of these viruses was consistent across the range of IFN-α concentrations tested (S4B Fig.). Additionally, we tested a limited subset of TF and NT variants for their ability to induce IFN-a, which may have influenced IFN-a resistance measurements, and found that IFN-a levels above background were not detectable at day 8 in either PBMC or MDDC infected cultures (S4C Fig.). Hence HIV-1 transmission from these donors was not mediated by TF viruses that exhibited higher levels of interferon resistance than NT viruses, indicating that heterosexual HIV-1 transmission is permissive to viruses anywhere within the range of *in vitro* interferon resistance profiles observed in the donors studied here, and factors other than IFN-a resistance constituted the dominant determinants of transmission fitness in these pairs.

Discussion

The rapid within-host diversification of HIV-1 observed during chronic infection, which represents a primary obstacle to effective HIV prevention strategies, contrasts starkly with the viral homogeneity evident following transmission. The stringent genetic bottleneck is most pronounced in heterosexual transmission, where a vast majority of new infections are established by single viral variants. Correlates of transmission may become evident by studying the properties of these transmitted/founder (TF) variants, which, in turn, could help inform effective HIV-1 vaccine design. Studies of early and transmitted variants have found genetic and phenotypic signatures associated with transmission; however, none have examined full-length TF variants and corresponding non-transmitted (NT) variants present near the time of transmission from epidemiologically-linked transmission pairs.

In this study, we applied new molecular techniques to investigate the requirements of HIV-1 transmission in six subtype C transmission pairs. We amplified, sequenced, and generated infectious molecular clones (IMC) of matched full-length TF viruses very early after infection (Fiebig stage II) and near full-length NT variants from 22-45 days following the estimated date of transmission. Technical limitations associated with amplifying full-length virus from genital tract samples required us to amplify from patient plasma. Despite this limitation, we previously showed in eight epidemiologically-linked transmission pairs that the TF was most highly related to NT variants that were absent from the predominant genital tract subpopulations, and were found in both blood and genital tract of the donor partner [2, 5, 6, 8-19]. Consistent with this, in pair 331 we

observed a NT variant in the plasma of the transmitting partner with only three amino acid differences from the TF across the entire proteome (Fig. 1B).

In this study, we generated IMCs from the diverse donor quasispecies with great sequence accuracy and selected variants in an unbiased fashion. Five of the six pairs were female-to-male, the route by which the most stringent bottleneck occurs [3, 8, 20, 21]. Since high donor viral load and the presence of genital ulcers and inflammation (GUI) in the recipient can, to a certain degree, mitigate selection bias in the bottleneck, it is important to note that these six pairs include three donors with viral loads >100,000 RNA copies/ml, as well as one recipient with a reported GUI in the twelve months prior to seroconversion [1, 4, 8, 12, 16, 18, 19]. Despite these caveats, single variant transmission was observed in all six pairs.

Consistent with our previous findings [8, 11, 22], we observed selection during transmission for variants with more consensus-like amino acid and nucleotide DNA sequences from the available quasispecies present in the donor at the time of transmission, across the full viral proteome and genome, respectively (Fig. 2). It has been shown that HIV-1 within-host diversity during chronic infection is greater than between-host diversity, suggesting conservation of certain genetic elements during transmission [9, 23, 24]. In conjunction, studies of subtype A and D heterosexual transmission pairs demonstrated transmission of more ancestral viral variants, by measuring distances of each variant to their most recent common ancestor (MRCA) on a phylogenetic tree of Env sequences [9, 24-26]. In the current study, the LANL subtype C consensus node falls near the subtype C MRCA highlighting the equivalence of these two measurements (Fig. 1). Thus, HIV-1 transmission consistently selects for variants that more closely resemble

ancestral and consensus-like viruses, indicating that evolution in the host decreases transmission potential.

The viral diversification observed during chronic infection due to adaptive immune pressure targeted specifically against HIV-1 is likely driving viral evolution away from consensus [1, 8, 27-29]. We have previously shown that acquisition of resistance to antibody neutralization comes with a transmission fitness cost [1, 8]. We similarly found that TF viruses were more sensitive to neutralization by donor plasma acquired near the time of transmission when compared to the corresponding NT variants. It should be noted, however, that a limitation to this finding is that NT variants were cloned and tested with plasma from approximately four weeks after the estimated date of infection, although the two pairs with the largest time gap between transmission and sampling did not show the greatest neutralization of the TF. These data also reaffirm that TF variants are generally not resistant to antibody neutralization [1, 8, 30]. As expected, donor plasma tested against contemporaneous viruses (TF or NT) demonstrated limited neutralization capacity. Moreover, neutralization sensitivity correlated with the distance to consensus over all the viruses tested. Considering these observations, it is reasonable to propose that selection of antibody sensitive variants during transmission is a side effect of the transmission cost associated with non-consensus adaptations in general, and not an underlying mechanism of transmission itself.

In order to address the role of viral fitness in transmission, we measured the *in vitro* fitness of a subset of viruses from six transmission pairs. Although a previous study found that TF viruses were more infectious than chronic control viruses [2, 14], we found no bias towards increased infectivity when comparing the TF to the corresponding NT

variants. Particle infectivity in TZM-bl cells correlated with replicative capacity in PBMC, suggesting that entry into TZM-bl cells is representative of a component of viral replication in primary cells. Viral replicative capacity in activated PBMC, a fundamental measure of *in vitro* fitness, was also not higher for TF variants in comparison to the corresponding NT variants, and none of the TF variants exhibited the highest replicative capacity from among the tested NT variants. We found that more consensus-like variants, which are typically those that transmit, had lower *in vitro* replicative capacities over all the variants tested, indicating that higher *in vitro* replicative capacity is not linked to transmission. TF variants were also not observed to have enhanced replicative capacity in monocyte-derived dendritic cells, an *in vitro* model for dendritic cells, which may act as an initial target cell for establishment of HIV-1 infection. These findings argue against the original hypothesis that consensus-like variants would have higher *in vitro* replicative capacities. Thus, *in vitro* RC in activated PBMC or MDDC may not reflect *in vivo* transmission fitness, potentially because replication in stimulated PBMC may recapitulate the inflammatory environment that occurs some time after transmission and during chronic infection rather than conditions initially encountered at initial sites of virus replication. We cannot rule out the possibility that replication assays in cell types more representative of mucosal transmission, such as tissue resident CD4+ T cells or Langerhans cells, may yield different results. However, consistent with our observations, previous studies found a significant negative correlation between similarity to consensus and *in vitro* RC in a larger number of patients in differing cohorts using gag-chimeras [31-33]. Transmission of low *in vitro* fitness variants may seem counterintuitive; however, full-length TF IMC as well as over 200 transmitted Gag chimeras have been

shown to exhibit a wide range of *in vitro* replicative capacities [3, 5, 32, 34, 35], as we found for our six TF viruses. A recent theoretical model of HIV transmission predicted that variants with lower replicative capacity via increased latency would exhibit a greater transmission potential *in vivo* [8, 36], and it is therefore possible that modestly lower *in vitro* replicative capacity is an advantage during transmission.

A potential selection factor during mucosal transmission is the early innate immune response to HIV-1. Innate antiviral cytokines including IFN-a are induced at initial sites of HIV-1 replication in the mucosa and draining lymph nodes [8, 9, 37, 38], hence HIV-1 variants that are more resistant to the antiviral effects of IFN-α may have an advantage during transmission. Indeed, cross-species transmission of Simian Immunodeficiency Virus (SIV) to humans required escape from the interferon stimulated APOBEC3 restriction factors by enhanced Vif antagonism [14, 39]. A recent in-depth study using the rhesus macaque model also found that IFN-α treatment prior to intrarectal SIV_{MAC251} inoculation reduced the number of transmitted variants and increased the number of challenges necessary to initiate infection [31, 40]. Consistent with the hypothesis that type 1 IFNs contribute to the transmission bottleneck, previous studies using HIV-1 found that TF variants are generally more resistant to IFN-α *in vitro* than viruses present during early chronic infection [2, 14, 41]. Fenton-May et. al. [1, 2] found that TF viruses from both subtype B and C infected subjects were more resistant to IFN-α when compared to matched variants generated from the same individual six months post-infection or during early chronic infection. Parrish et. al. [1, 14, 42] found that TF viruses are more resistant to IFN-α than viruses from unmatched chronic controls, though this was true only for the subtype B and not for the subtype C variants they studied. In six

subtype C transmission pairs studied here we did not observe that TF viruses exhibited enhanced resistance to IFN-α compared to NT viruses. TF variants did not replicate to higher levels in the presence of IFN-α, nor did they have higher ratios of replication in the presence versus the absence of IFN-α.

These differing results could be due to differences in experimental protocols, as well as difficulties in separating inherent replicative capacity from interferon resistance. We therefore tested the IFN-α resistance of previously studied TF and 6-month viruses and confirmed that these TF variants were more resistant to the effects of IFN-α, consistent with previous observations. In addition, we found that the TF variants had higher replicative capacities than the 6-month consensus variants, although for this group of viruses IFN-a resistance did not directly correlate with viral replicative capacity. The influence of viral replicative capacity on measures of interferon resistance is not fully understood. The impact of multiplicity of infection on measured interferon resistance has been noted previously [8, 43], so in the current studies we utilized a low multiplicity to ensure adequate target cell availability even for the higher replicating viruses. We chose a MOI of 0.01 for our assays since it represented an input virus dose at which we were able to measure both replication differences between viruses, as well as IFN-α resistance differences (S4D Fig.). For the viruses tested from the six Zambian transmission pairs, we found that *in vitro* replication in the presence of interferon correlated with replication in the absence of IFN-α, such that the rank order of virus replicative capacities from lowest to highest was similar in the presence or absence of interferon. Even when a subset of viruses with more closely-matched levels of replication were studied so that we

were better able to observe IFN-α resistance differences, we found that TF variants were not IFN-α resistant compared to the matched NT variants.

The lack of difference in the IFN resistance of TF and NT viruses in these transmission pairs may be due to the length of time for which the chronically infected viral donors had been infected prior to viral transmission to their partners and derivation of the viruses studied. Fenton-May et. al. showed that while IFN-α resistance decreased over the first 6 months following infection, it subsequently increased in different subjects at time points from 2-7 years post-infection [2, 44]. Edlin et. al. and Kunzi et. al. further showed that viruses isolated from individuals who had progressed to AIDS were more IFN resistant than viruses from asymptomatic chronically-infected individuals [14, 45, 46]. Likewise, Parrish et. al. proposed that their observation of differences in IFN resistance between TF IMCs and IMCs from unmatched chronically-infected subjects in a subtype B-infected cohort, but not in a subtype C-infected cohort, may have been due to the subtype C-infected donors being sampled at later time points in chronic infection [14]. However, it should be noted that we did observe a range of interferon sensitivities across the six transmission pairs, with greater than a 100-fold difference being observed between TF viruses. In future, it would be of interest to determine whether chronically-infected donors in the Zambian discordant couples cohort who failed to transmit infection to their partners harbor more IFN-sensitive viruses than those present in the virus-transmitting donors studied here. However on the basis of the current results it seems likely that IFN-a does not make a major contribution to the HIV-1 transmission bottleneck, or may do so only in some transmission scenarios.

Transmission selection for consensus-like and more neutralization-sensitive TF variants suggests that within-host evolution of HIV-1 in response to human adaptive immune responses may cause a loss of fitness required for the establishment of infection in a naive host following transmission. We show that relatively high *in vitro* replicative capacity and preferential IFN-α resistance were not selected for during transmission of subtype C HIV-1 in the six pairs studied here. Thus, the *in vitro* assays of HIV-1 replication employed here may not be measuring some of the key determinants of transmission fitness, and other models of HIV transmission, such as low dose intravaginal challenges of humanized mice, or human genital explant cultures, may be needed to determine the phenotypic requirements of HIV-1 transmission that genetic differences are pointing to.

Materials and Methods

Study subjects

The six HIV-1 subtype C transmission pairs investigated in this study were enrolled in the heterosexual discordant couple cohort at the Zambia-Emory HIV Research Project (ZEHRP) in Lusaka, Zambia. Human subjects protocols were approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board. HIV-1 serodiscordant couples in this cohort were provided counseling and testing on a monthly basis prior to the negative partner becoming HIV-1 positive. The recipients were enrolled in the International AIDS Vaccine Initiative (IAVI) Protocol C early-infection cohort. Epidemiological linkage was defined by phylogenetic analyses of HIV-1 *gp41* sequences from both partners [47]. All individuals in this study were ART naive during the time of sampling.

Viral RNA extraction and PCR amplification

Viral RNA extraction and near full-length genome single genome amplification were performed as described in Deymier et al. 2014 [31]. Briefly, viral RNA was extracted from 140µl of plasma using the QIAamp Viral RNA mini kit (Qiagen) and was used for cDNA synthesis carried out with Superscript III (Life Technologies) and an anchored Oligo$(dT)_{18}$ primer. The cDNA was used immediately for PCR amplification. Near full-length single genome PCR amplification was performed by serially diluting cDNA, followed by two rounds of PCR amplification, so that ~30% of wells became positive. Both rounds of PCR were performed in 1x Q5 Reaction Buffer, 1x Q5 High GC Enhancer, 0.35 mM of each dNTP, 0.5 µM of primers and 0.02 U/µl of Q5 Hot Star

High-Fidelity DNA Polymerase (NEB) in a total reaction volume of 25 µl. First round primers were, 1U5Cc and 1.3'3'PlCb, and second round primers were 2U5Cd and 2.3'3'plCb [48]. Cycling conditions for both reactions are 98^{o}C for 30s, followed by 30 cycles of 98^{o}C for 10s, 72^{o}C for 7.5min, with a final extension at 72^{o}C for 10min. PCR reactions were run on a 1% agarose lithium acetate gel at 300 V for 25 min in order to determine the presence of a 9 kb band.

Sequencing

Positive ~9kb single genome amplicons were gel-extracted using the Wizard SV Gel and PCR Clean-Up System (Promega). Purified ~9 kb PCR amplicons were sent for sequencing to the University of Alabama Birmingham (UAB) sequencing core for Sanger sequencing.

In conjunction, multiple amplicons from recipient 3576 were sequenced by single-molecule nucleic acid sequencing (Pacific Biosciences), to confirm the TF. Briefly, SMRTbell libraries were constructed according to the manufacturer's instructions for 10kb amplicons. PCR reactions of DNA amplicons were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and mixed at equal concentrations to a total of 3ug DNA. Library preparation quality was assessed on a Bioanalyzer and SMRT sequencing on the PacBio RSII was performed following primer annealing and P4 polymerase binding to the library preparations. The consensus of the reads, aligned to the HXB2 reference sequence, were then taken to form a TF sequence, which matched the Sanger sequence.

Sequence Analysis

All 9kb viral sequences were aligned in Geneious bioinformatics software (Biomatters, Aukland, NZ) using MUSCLE [49], followed by hand aligning. The Los Alamos National Database HIV Consensus/Ancestral Sequence Alignments were used as reference sequences (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Phylogenetic trees were generated using the DIVEIN web server (http://indra.mullins.microbiol.washington.edu/DIVEIN/) [50]. Phylogenetic analyses were performed by maximum likelihood parsimony under Phylogeny/Divergence/ Diversity. For nucleotide sequence analysis a general time reversible model was used, with a fixed gamma distribution parameter of 1, and performed with 100 bootstraps. Amino acid phylogenetic analysis was performed using the HIVw model of evolution, with 100 bootstraps [51]. Pairwise distances from each branch node to the subtype C consensus node were extracted from the distance matrices of the phylogenetic trees.

Generation of full-length IMC

HIV full-length genome infectious molecular clones were generated as described in Deymier et al. 2014 [31]. Briefly, linked recipient specific primers were generated in order to amplify the full long terminal repeat (LTR) from the linked recipient white cell pellet DNA. This LTR was cloned into a pBluescript vector, and the TF sequence of the LTR sequence was inferred as the consensus sequence from multiple clones. Subsequently, a three-piece DNA HD In-Fusion HD cloning (Clontech) ligation reaction using a reamplified clonable near-full length amplicon and two LTR pieces generated by PCR from the linked recipient LTR generated the full-length IMC. TF IMC were correct

for the entire genome, whereas NT variants were chimeric for only for the R region of both 5' and 3' LTR, which was taken from the TF of that transmission pair. IMC were sequenced in order to confirm a match to the sequence of the single genome amplicon from which it was derived.

Generation of virus stocks and particle infectivity

293T (American Type Culture Collection) cells were transfected with 1.5µg of plasmid DNA, using the Fugene HD transfection reagent (Roche) according the manufacturer's protocol. Viral stocks were collected 48 hours post transfection and clarified by centrifugation. These virus stocks were then titered for infectivity on TZM-bl cell, as described previously [52]. The virus stocks were also measured for reverse transcriptase (RT) activity using a radiolabeled reverse transcriptase assay [52]. Particle infectivity of each virus was determined as the ratio of titer (infectious units/µl) over RT signal (RT/µl) for 3 independent experiments. Particle infectivity over time was measured by sampling 8ul (0.4%) per time point over a 3 day period.

Replication in PBMC and interferon resistance

Frozen peripheral blood mononuclear cells (PBMC) from buffy coats were thawed and stimulated with 20 U/ml of interleukin-2 (IL-2) and 3ug/ml of phytohemagglutinin (PHA) in R10 (Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% defined fetal bovine serum (FBS), 1 U/ml penicillin, 1ug/ml streptomycin, 300ug/ml L-glutamine) for 72 hours at 37C. After 48 hours, 1,000 IU/ml of interferon-α2a (Sigma Aldrich, Product # SRP4594) was added to a portion of

cells 24 hours prior to infection. $1x10^6$ cells were then infected in 15ml conical tubes by 2 hour spinoculation at 2,200 rpm with an MOI of 0.01 based on the TZM-bl titer in triplicate. Cells were then washed twice in 13ml RPMI, resuspended in 500ul of R10 media and plated in a 48 well plate in triplicate. 50ul of supernatant was then sampled every 48 hours starting with a day zero time point taken 2-3 hours after plating to get a baseline reverse transcriptase activity for each infection well using the radiolabeled reverse transcriptase assay.

Where noted in the text, an alternative strategy for another independent experiment with CD8-depleted PBMC was used with a few differences: anti-CD3 (R&D Systems clone UCHT1; 50ng/ml working concentration) and anti-CD28 (eBioscience clone CD28.2; 100ng/ml working concentration) antibodies were used to stimulate MACS microbead (from Miltenyi plus the MACS LD columns) CD8-depleted PBMC from three separate donors in a mixed lymphocyte reaction and then infected at an MOI of 0.1 based on TZM-bl titer. $2x10^5$ cells were then infected in the presence and absence of 5,000 IU/ml of interferon-α2a (Peprotech) and cells were washed three times with 10ml of RPMI and supernatant tested by a modified ELISA assay using the AlphaLISA HIV p24 (high sensitivity) kit (Product # AL291C PerkinElmer) per protocol instructions, using the same media for the standard as in the sample and loading 5ul per well.

The replication score (RC score) for each variant was calculated using a normalized area under the curve. The median of the replicates were background subtracted using the day 2 time point, adjusted for sampling by a measured exponential decay correction, and area under the curves (AUC) were divided by the AUC for a standard lab adapted subtype C virus, MJ4, to compare across transmission pairs

analyzed on different days. Interferon-α2a resistance was measured in a similar fashion, followed by calculating the ratio of the RC score in the presence of interferon divided by the RC score in the absence of interferon.

Monocyte derived dendritic cell infections

Monocyte derived dendritic cells (MDDC) were isolated from two healthy blood donors by CD14 positive bead isolation (Miltenyi Biotec), followed by culture at 37^{o}C in R10, supplemented with 40 ng/ml IL-4 (Peprotech) and 20ng/ml GM-CSF (Peprotech) for 7 days. MDDC differentiation was confirmed by flow cytometry using the following antibodies and stains: α-CD14 PB (clone M5E2), α-CD11c APC (clone S-HCL-3), α-HLA-DR V500 (clone G46-6) (BD Biosciences), and the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies). The phenotype of MDDC after 7 days was CD14 low, CD11c high, and HLA-DR high, as expected. Cells were harvested, and 3x10^{5} MDDC were seeded in a flat-bottom 96-well plate. MDDC were infected in a volume of 250µl of R10 with virus at an MOI of 1 for 4 hours. Cells were then washed three times with RPMI, and cultured for 12 days in R10 supplemented with 40 ng/ml IL-4 and 20ng/ml GM-CSF. 50µl of culture supernatant was collected every two days and replaced with fresh media. The supernatant was then analyzed for virus production by the radiolabelled RT assay[52].

Interferon Elisa

IFN-a levels were measured by the VeriKine Human IFN Alpha ELISA Kit from supernatants 8 days after PBMC and MDDC infections with a subset of viruses from

pairs 331 and 3678. The negative controls included media from PBMC and MDDC uninfected cultures. The positive controls included IFN-a spiked media equal to the initial amount of IFN-a utilized in these infections, along with supernatant from an infection carried out in the presence of IFN-a.

Neutralization assay

IMC derived virus and plasma taken from the same time point in the transmitting partner (donor) along with the TF from the recipient, were used to test antibody neutralization of variants circulating near the time of transmission. The TZM-bl neutralization assay was adapted for use with IMCs, in a similar fashion to what has been published previously for IMC [53] and pseudoviruses [1, 42]. Briefly, heat inactivated plasma was serially diluted 5-fold starting at 1:100, and each dilution was then mixed with 20 IU/ul of virus at a 1:1 ratio. After incubation at 37^{o}C for 1 hour, the plasma and virus mixtures were used to infect previously seeded TZM-bl cells (24 hours prior to infection at 6x10^{3} cells per well in a 96-well plate). After a 40 hour incubation, the Promega Reporter Buffer was used to lyse cells according to manufacturer instructions and, following two freeze-thaw cycles, luciferase was measured with the Luciferase Assay System from Promega (Catalog # E1501) in the supernatants on a luminometer using the Gen5 2.00 software. Maximal percent inhibition (compared to the no plasma control) was calculated at a dilution of 1:00 after background subtraction and removal of variants with a signal less than three times background for cell only control wells. The data is averaged from each virus run in duplicate from two independent experiments.

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Figures

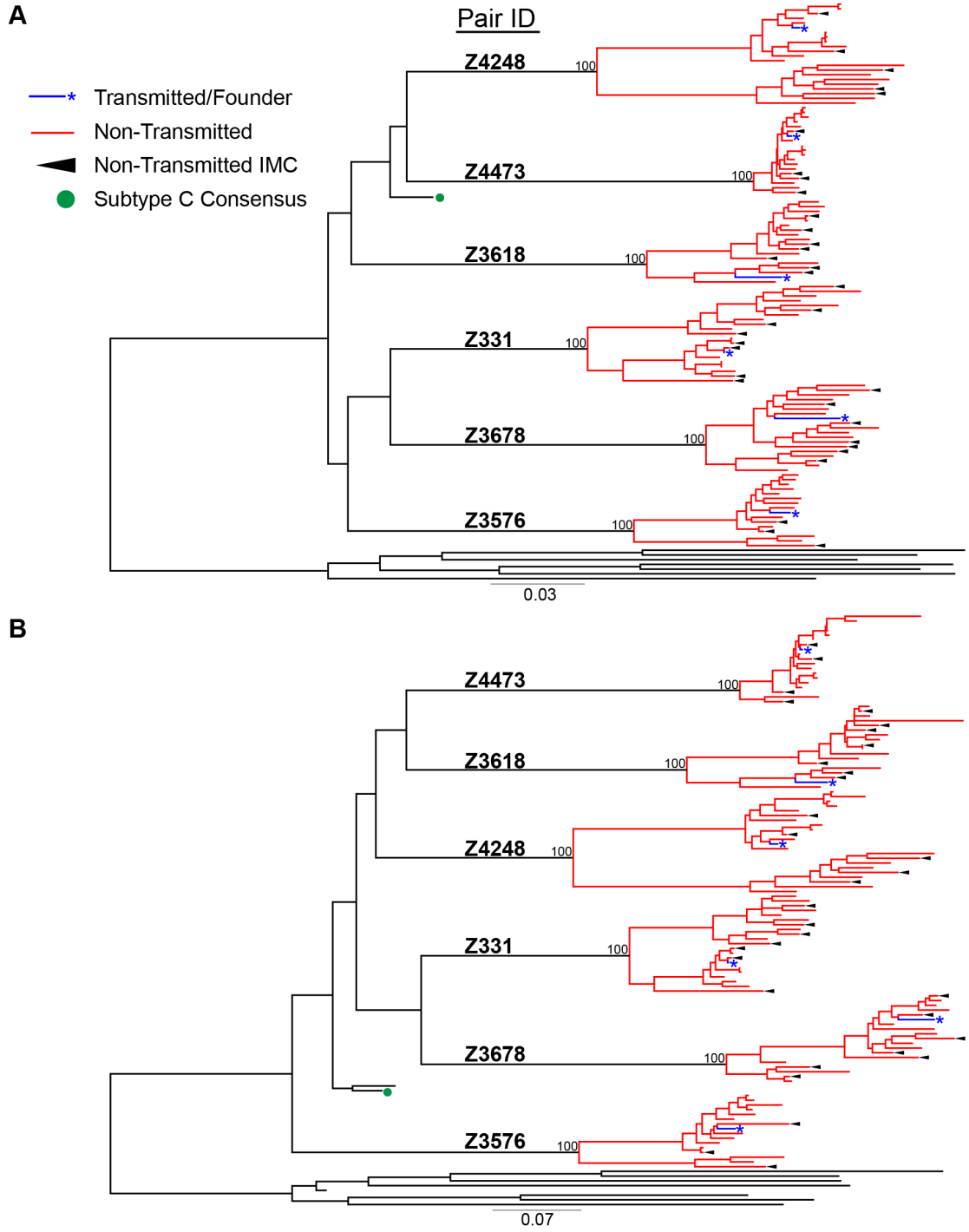

A
Pair ID
Transmitted/Founder
Non-Transmitted
Non-Transmitted IMC
Subtype C Consensus
Z4248
Z4473
Z3618
Z331
Z3678
Z3576
100
0.03
B
Z4473
Z3618
Z4248
Z331
Z3678
Z3576
100
0.07

Fig. 1. HIV-1 Full-length genome phylogenetic analysis of six epidemiologically-linked heterosexual transmission pairs

(A) Nucleotide sequences for all 115 single genomes amplified from six linked transmission pairs were aligned to the curated LANL consensus/ancestral alignment and a maximum likelihood tree was generated. (B) Single genome nucleotide sequences for each viral gene (*gag, pol, vif, vpr, vpu, tat, rev, env, &* ***nef***) were translated to their amino acids and then concatenated. These were aligned with LANL consensus/ancestral concatenated protein sequences and a maximum likelihood tree was generated. Transmitted/founder sequences from linked recipients are in blue, donor non-transmitted variants are in red, LANL database curated consensus/ancestral sequences are shown in black, where the LANL subtype C consensus is indicated by a green circle. Black arrows indicate virus variants from the donor quasispecies that were selected for generation of non-transmitted (NT) infectious molecular clone.

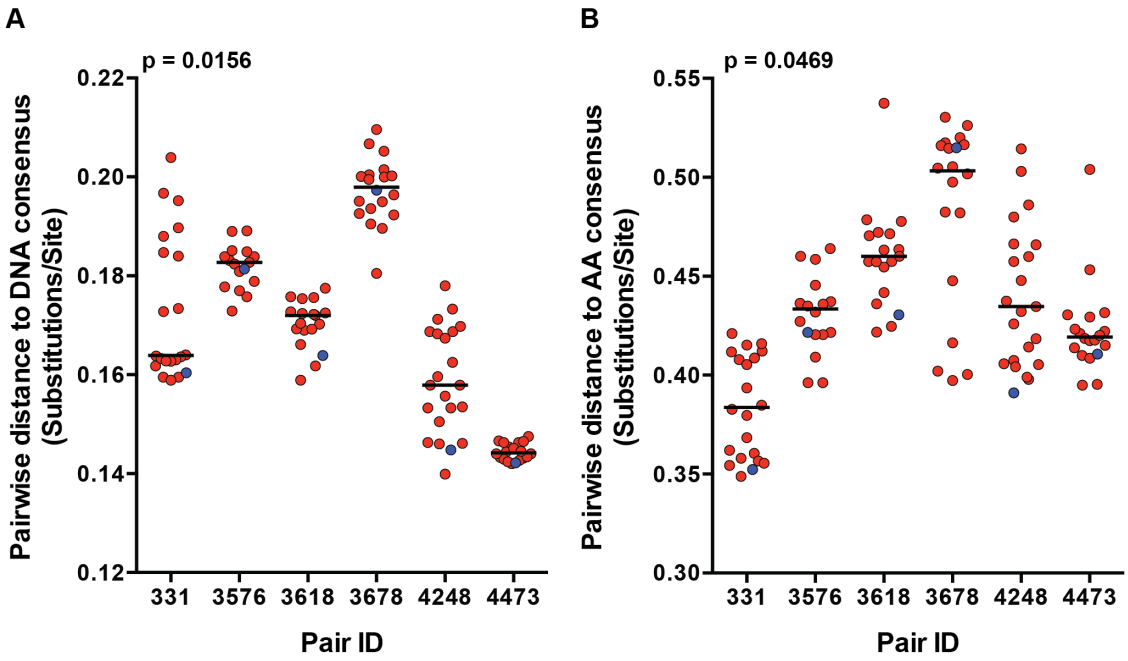


Fig. 2. Transmission selects for more consensus-like TF variants

The pairwise distance of each viral variant on the (A) nucleotide and (B) amino acid phylogenetic trees to the LANL subtype C consensus node were measured and plotted for each transmission pair. Transmitted/founder variants are in blue, and non-transmitted variants are in red. The median of the non-transmitted variants is designated with a black line. The statistical significance of the difference between TF and NT donor median values was analyzed using a one-tailed Wilcoxon matched-pairs signed rank test.

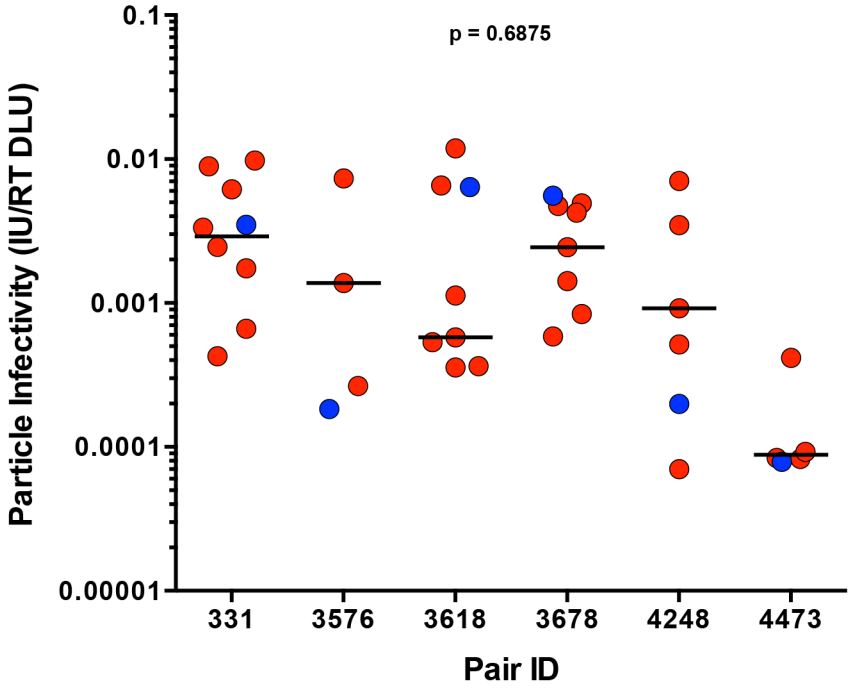


Fig. 3. Particle infectivity of TF and NT infectious molecular clones

293T cells were transfected with TF (blue) and NT (red) infectious molecular clones. Supernatants collected 48 hours post-transfection were titered on TZM-bl cells to define the number infectious units per microliter (IU/ul), while reverse transcriptase activity was measured simultaneously for total viral particles per microliter (RT DLU, reverse transcriptase digital light units). The particle infectivity (IU/RT DLU) of each infectious molecular clone is plotted for each transmission pair. The median of the NT variants is designated with a black line. The statistical significance of the difference between TF and NT donor median values was determined using a two-tailed Wilcoxon matched-pairs signed rank test ($p = 0.6875$).

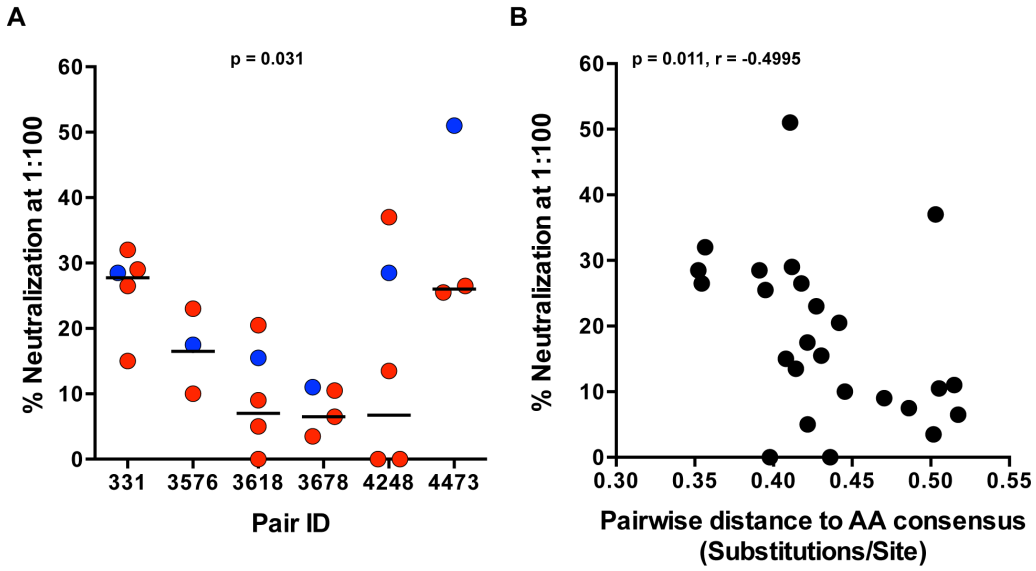


Fig. 4. TF are more sensitive to neutralization by donor plasma than NT

(A) Neutralization of TF (blue) and NT (red) IMC by donor plasma was measured for each pair in a TZM-bl neutralization assay. Percent neutralization by donor plasma (diluted 1:100) is depicted on the y-axis, and representative TF and NT viruses tested for each transmission pair are depicted on the x-axis. The median of the NT variants is designated with a black line. The statistical significance of the difference between TF and NT donor median values was determined using a two-tailed Wilcoxon matched-pairs signed rank test ($p = 0.031$). (B) Spearman correlation of the pairwise distance to the amino acid subtype C consensus and donor plasma neutralization described in part A over all the variants tested from 6 transmission pairs ($p = 0.011$, $r = -0.4995$).

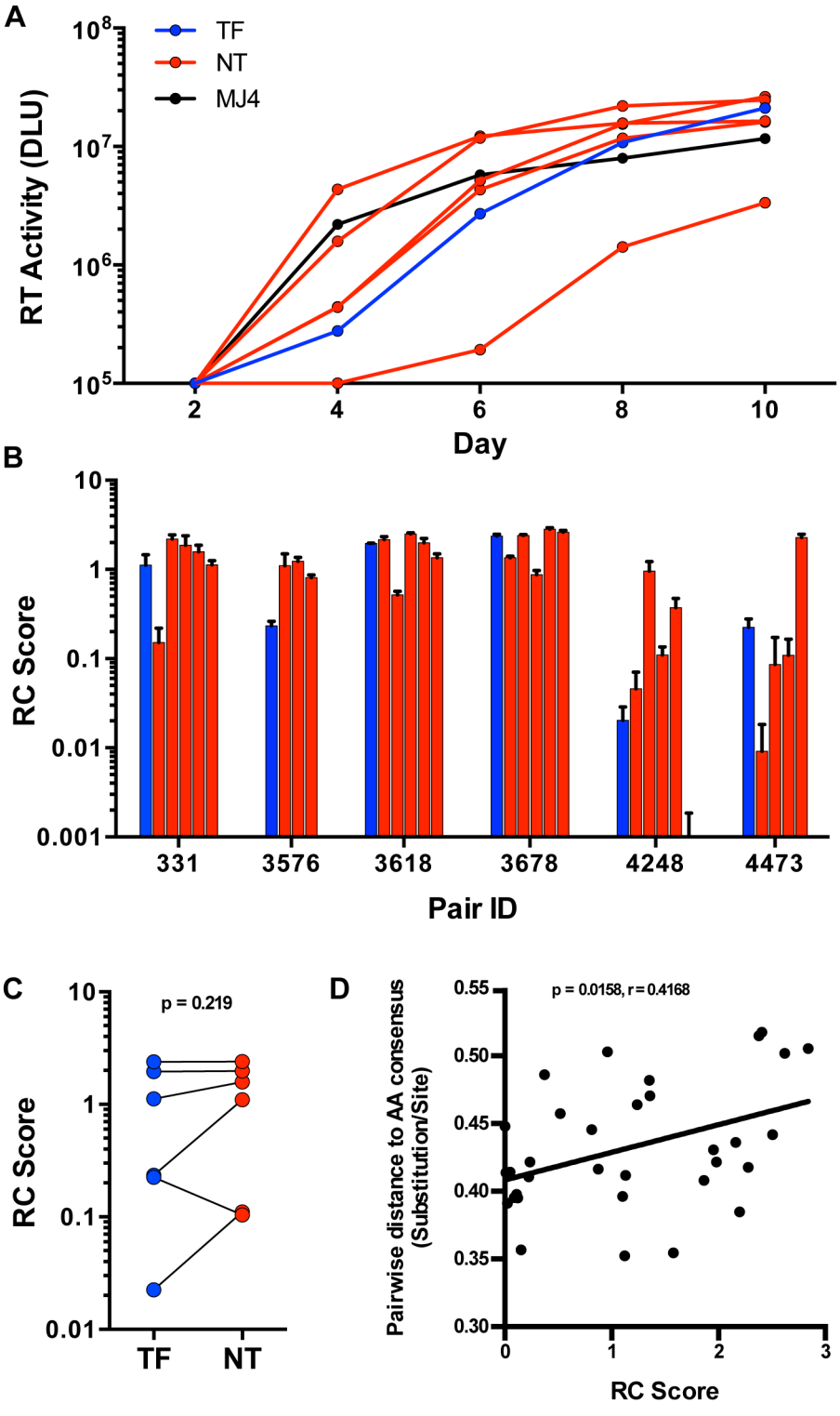

A
TF
NT
MJ4
RT Activity (DLU)
Day
B
RC Score
Pair ID
331
3576
3618
3678
4248
4473
C
p = 0.219
RC Score
TF
NT
D
p = 0.0158, r = 0.4168
Pairwise distance to AA consensus (Substitution/Site)
RC Score

Fig. 5. *In vitro* replication of TF and NT viruses in PBMC

(A) Virus growth over 10 days in PBMC culture as measured by reverse transcriptase (RT) activity (DLU = digital light units) of the TF (blue), NT variants (red) and MJ4 standard (black) for one representative transmission pair, 331. (B) Replicative capacity (RC) scores, based on the area under the curve relative to MJ4, of all tested TF (blue) and NT (red) variants from six transmission pairs. (C) RC scores of the TF (blue) compared to the median of the corresponding NT variants (red) (Wilcoxon matched-pairs signed rank test, two-tailed $p = 0.219$). (D) Spearman correlation of the pairwise distance to the amino acid subtype C consensus described in Fig. 2 and RC scores over all variants tested (Non-parametric Spearman $p = 0.0158$, $r = 0.4168$.) The linear regression line is shown for visualization purposes.

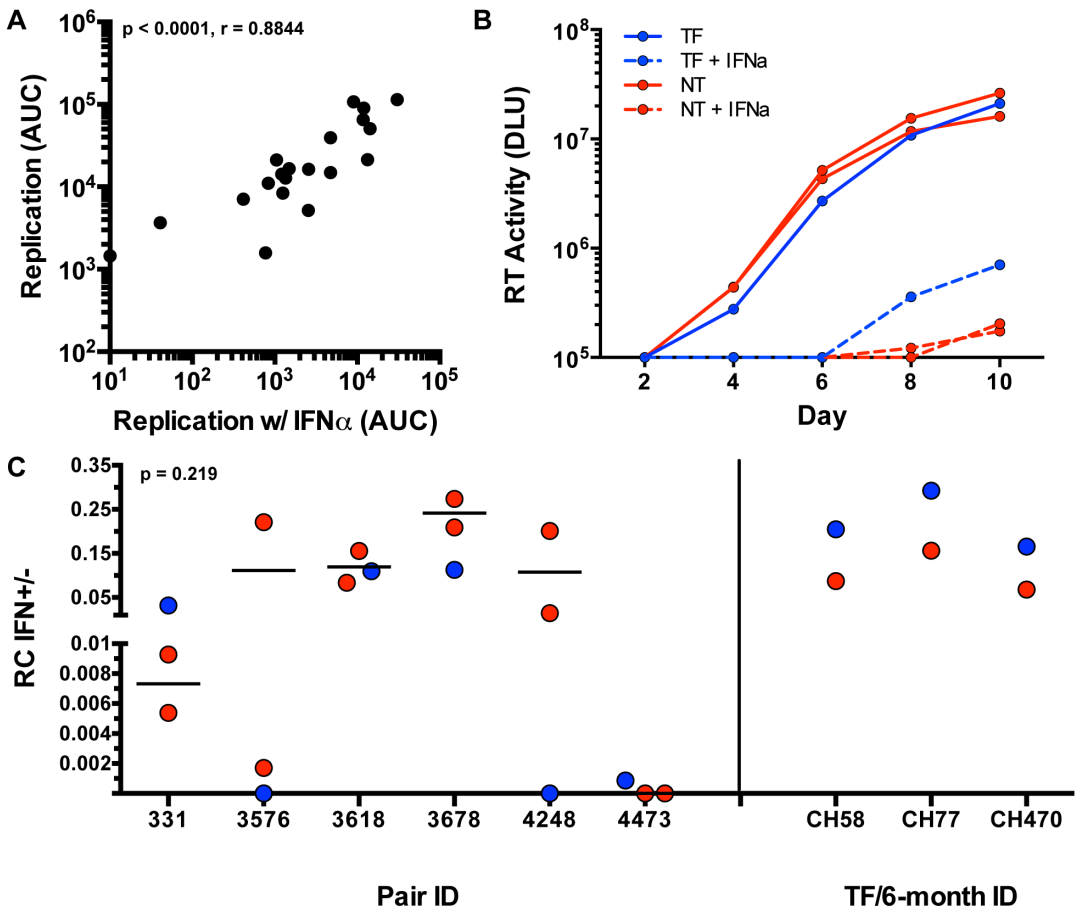
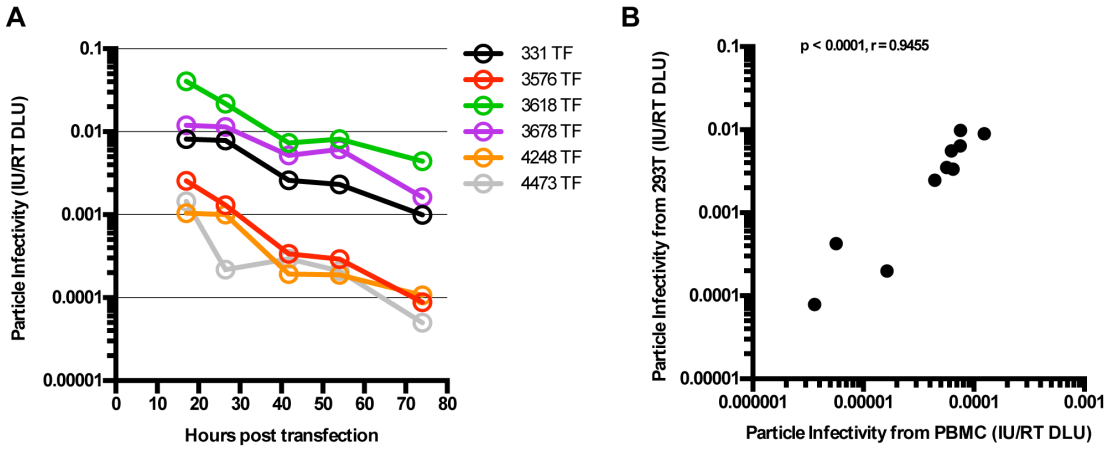


Fig. 6. Interferon-α resistance of TF and NT viruses

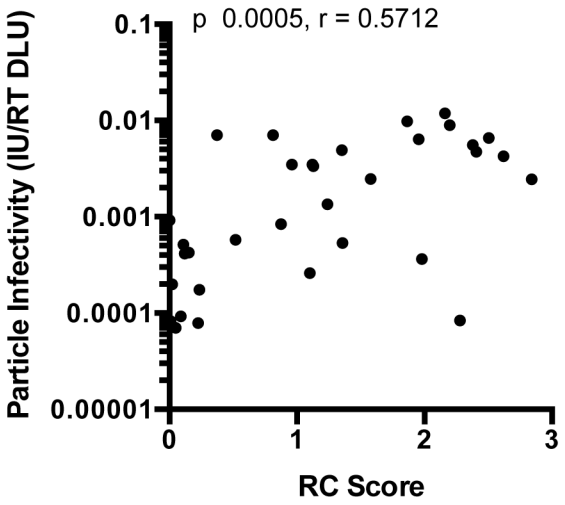
(A) Spearman correlation of the replication measured by area under the curve (AUC) (y-axis) of each tested variant (black dots) and the replication (AUC) in the presence of interferon alpha (x-axis) (Non-parametric Spearman $p < .0001$, $r = 0.8844$). (B) Virus growth over 10 days in culture as measured by reverse transcriptase (RT) activity (DLU = digital light units) of the TF (blue), and NT variants (red) in the presence of IFN-α (dotted) and absence of IFN-α (solid lines) in an example pair 331. (C) RC scores in the presence of IFN-α were divided by the RC score in the absence of IFN-α for TF (blue) and selected NT (red) viruses with similar replication kinetics (Wilcoxon matched-pairs

signed rank test, two tailed, p = 0.219). Subtype B TF (blue) and 6-month consensus (red) viruses are shown as controls on the right.



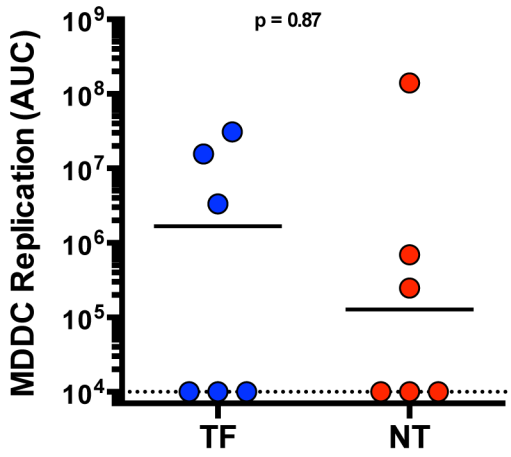
S1 Fig. Particle infectivity from 293T and PBMC derived virus

(A) Particle infectivity (TZM-bl titer divided by reverse transcriptase activity) of 293T cell derived TF virus stocks at different time points post transfection. (B) Correlation of particle infectivity assessed from day 8 of a PBMC infection and the particle infectivity from 293T derived stocks 48 hours after transfection of a subset of eleven viruses ($p < 0.0001$, $r = 0.9455$).



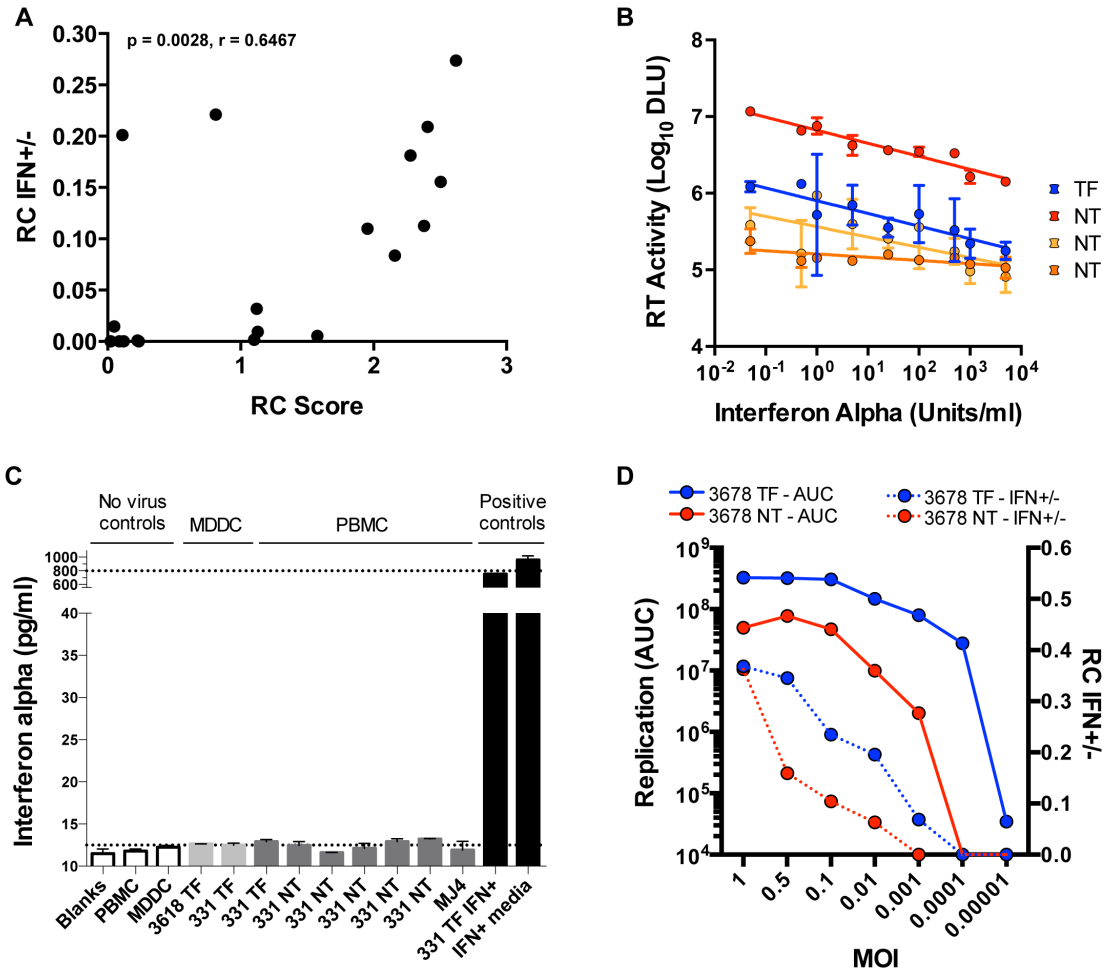
S2 Fig. Particle infectivity correlates with replicative capacity

Spearman correlation of particle infectivity and replicative capacity score of all TF & NT virus variants ($p = 0.0005$, $r = 0.5712$).



S3 Fig. Replication of TF and NT viruses in monocyte derived dendritic cells

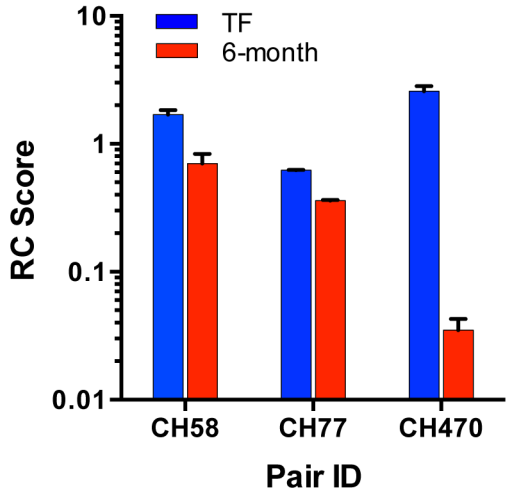
Virus growth in monocyte derived dendritic cells was measured by analyzing supernatant reverse transcriptase activity for 12 days following infection. Replication is depicted (y-axis) as the area under the curve for each virus variant. TF (blue) and NT (red) are presented with their group median. The difference between the groups was analyzed using a two-tailed Mann Whitney test ($p = 0.87$). Results are the average of replication in two healthy donors.



S4 Fig. TF and NT resistance to IFN-α

(A) Correlation of IFN-α resistance (RC IFN+/-) and RC Score of variants from figure 6C ($p = 0.0028$, $r = 0.6467$). (B) The TF (blue) and three NT (red, yellow, orange) variants from pair 331 with a representative range of RC scores were tested for replication in the presence of IFN-α concentrations from 0.5 U/ml - 10,000 U/ml. Supernatant reverse transcriptase (RT) activity at day 7 post-infection are shown. (C) Analysis of IFN-a levels in day 8 supernatants from PBMC and MDDC infected with a subset of viruses, to test for IFN-α induction *in vitro*. Negative controls are shown in white, MDDC infections in light gray, PBMC infections in dark gray, and positive

controls in black. (D) Area under the curve (solid lines) and IFN-α resistance ratios (dotted lines) from infections initiated at a range of MOI for the 3678 TF (blue) , along with an NT variant (red) with a different replicative capacity.



S5 Fig. Replication of TF and 6-month consensus infectious molecular clones

RC scores of three subtype B TF/6-month virus pairs (described in [2]) in activated PBMC.

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Chapter III: HLA class I downregulation by HIV-1 Variants From Subtype C Transmission Pairs

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Abstract

HIV-1 downregulates HLA-A and HLA-B from the surface of infected cells primarily to evade CD8 T cell recognition. HLA-C was thought to remain on the cell surface and bind inhibitory killer immunoglobulin-like receptors, preventing NK cell-mediated suppression. However, a recent study found HIV-1 primary viruses have the capacity to downregulate HLA-C. The goal of this study was to assess the heterogeneity of HLA-A, HLA-B and HLA-C downregulation among full-length primary viruses from six chronically infected and six newly infected individuals from transmission pairs, and to determine whether transmitted/founder variants exhibit common HLA class I downregulation characteristics. We measured HLA-A, HLA-B, HLA-C and total HLA class I downregulation by flow cytometry of primary CD4 T cells infected with 40 infectious molecular clones. Primary viruses mediated a range of HLA class I downregulation capacities (1.3-6.1-fold), which could differ significantly between transmission pairs. Downregulation of HLA-C surface expression on infected cells correlated with susceptibility to *in vitro* NK cell suppression of virus release. Despite this, transmitted/founder variants did not share a common downregulation signature and instead were more similar to the quasispecies of matched donor partners. These data indicate that a range of viral abilities to downregulate HLA-A, HLA-B and HLA-C exist within and between individuals, which can have functional consequences on immune recognition.

Importance

Subtype C HIV-1 is the most predominant subtype involved in heterosexual transmission in Sub-Saharan Africa. Authentic subtype C viruses that contain natural sequence variations throughout the genome are often not used in experimental systems, due to technical constraints and sample availability. In this study, authentic full-length subtype C viruses, including transmitted/founder viruses, were examined for the ability to disrupt surface expression of human leukocyte antigen (HLA) class I molecules, which are central to both adaptive and innate immune responses to viral infections. We found that HLA class I downregulation capacity of primary viruses varied, and HLA-C downregulation capacity impacted viral suppression by natural killer cells. Transmitted viruses were not distinct in the capacity for HLA class I downregulation or natural killer cell evasion. These results enrich our understanding of the phenotypic variation existing among natural HIV-1 viruses, and how that might impact the ability of the immune system to recognize infected cells in acute and chronic infection.

Introduction

Human leukocyte antigen (HLA) class I molecules loaded with peptides are downregulated from the surface of HIV-1 infected CD4 T cells[1]. Nef, an HIV-1 encoded accessory protein, mediates downregulation of HLA-A and HLA-B[2, 3], thus protecting against HIV-specific CD8 T cell recognition[4]. HLA-C was previously thought to be left on the surface of infected cells in order to inhibit natural killer (NK) cell-mediated viral suppression through binding of killer cell immunoglobulin-like receptors (KIR) as inhibitory ligands[3, 5-7]. However, a recent discovery changed this paradigm by demonstrating that Vpu, another HIV-1 accessory protein with functions that overlap with Nef, downregulates HLA-C - a finding previously obscured by wide usage of lab-adapted strains lacking such an ability[8]. This highlights the importance of studying patient derived uncultured viruses when possible.

HLA class I expression can impact upon NK and CD8 T cell recognition of virally infected cells, thus modulating adaptive and innate immunity. HLA-A and HLA-B present viral peptides primarily to CD8 T cells by interacting with their T cell receptors. Reducing surface expression of HLA-A and HLA-B prevents epitope specific recognition of infected cells by CD8 T cells which could otherwise suppress virus replication[4]. Strong experimental evidence supporting this phenomenon is demonstrated by the *in vitro* selection of viruses with efficient Nef-mediated HLA-A downregulation capacity, when passaged in the presence of Gag-specific CD8 T cell clones[9]. The *in vivo* relevance has been examined in experiments of simian immunodeficiency virus (SIV) infected rhesus macaques. SIV_{MAC239} *nef* mutants deficient in major histocompatibility

class I (MHC-I) downregulation revert early in infection[10] and SIV_{MAC239} infected rapid-progressors exhibited a 2-fold higher level of MHC-I downregulation on infected cells ex vivo, which was associated with lower peptide specific T cell responses[11]. Although, a recent study found little MHC-I downregulation on SIV_{MAC239} infected cells ex vivo using a pan-MHC-I antibody[12]. Peptide specific NK KIR interactions with HLA-A and HLA-B alleles also impact HIV infection[13, 14] and are associated with variations in viral control and disease progression[15-18]. Although the consequences of HLA-C expression are less well defined, both NK and CD8 T cell responses are impacted by HLA-C. Higher HLA-C expression in infected individuals correlates with slower CD4 T cell decline, increased CD8 T cell responses, and selection of HLA-C associated viral escape mutations[19]. NK KIR interactions with HLA-C can also drive HIV sequence based adaptations[20].

The downregulation of HLA class I molecules from the surface of infected cells may impact upon the establishment of infection. HLA class I allele sharing between heterosexual transmission pairs, and HLA class I homozygosity in mother to child pairs, increased the risk of infection, indicating a role for HLA class I molecules during transmission[21, 22]. Combinations of HLA-A, HLA-B and HLA-C alleles with specific NK KIR alleles have been associated with protection from HIV acquisition[23-27], and NK cells have also been implicated in the SIV macaque model, where elevated CD56 NK cell frequencies were associated with relative protection from SIV_{MAC251} challenge when interferon-alpha was pre-administered[28]. Thus, viral characteristics that modulate HLA class I expression may play a role in HIV acquisition and dissemination.

Acquisition of HIV-1 via the heterosexual route is characterized by a low chance of infection per mucosal exposure[29, 30], accompanied by a genetic bottleneck that leads to one or few viral variants establishing infection in a new individual[30, 31]. Understanding the forces that determine which viruses breakthrough has implications for prevention strategies, including vaccines. Although chance certainly influences which viral variants become transmitted/founder (TF) viruses, several studies have reported signature characteristics of transmitted/founder viruses[30-35]. A study of 137 subtype C infected epidemiologically-linked Zambian transmission pairs found that Gag, Pol and Nef consensus sequences are preferentially transmitted, a signature emphasized in Nef functional domains associated with HLA class I downregulation, suggesting *in vivo* viral fitness plays a role in the transmission bottleneck[36]. The genetic selection for consensus amino acids was confirmed across the entire genome sequence from six subtype C infected transmission pairs; however, virus-mediated HLA class I downregulation was not examined in that study [34].

Prior studies have not observed differences in HLA class I downregulation by subtype C viruses from acute and chronic infection[8, 37, 38]. However, viruses derived from heterosexual transmission pairs with the source quasispecies as a comparison were not available. Moreover, the three major HLA class I molecules HLA-A, HLA-B and HLA-C were not addressed together in primary cells. Most prior studies characterized HLA class I downregulation in cell lines with Nef expression vectors and chimeric viruses, which may alter physiologic expression levels, or exclude the effect of a co-evolved Vpu [37, 39-45]. Additionally, the extent to which there is within-host functional

diversity of HLA class I downregulation, which may reflect selection by the cellular immune system, remains to be established[9, 40, 45, 46].

In the study described here, we examined HLA-A, HLA-B and HLA-C downregulation using 53 HIV-1 variants: 40 infectious molecular clones previously constructed from plasma virus sequences from twelve individuals in six subtype C heterosexual transmission pairs, 12 subtype C Gag-MJ4 chimeras varying in replicative capacities, and a standard laboratory subtype B strain, NL4-3[34, 47, 48]. We also investigated the relationship between HLA expression and the ability to evade NK cell suppression *in vitro*. We found a range of HLA class I downregulation capacities (1.3-6.1-fold) of viruses from within and between individuals. The ability to downregulate HLA class I molecules, including HLA-C, was similar between source quasispecies and transmitted/founder viruses. Although replicative capacity influenced evasion of NK-mediated suppression, HLA-C downregulation was significantly associated with the susceptibility of variants to NK cell suppression. These data could be important to consider in cure and vaccine strategies aiming to induce CD8 and NK cell responses.

Results

Variation of Nef and Vpu proteins of subtype C viruses from transmission pairs

We previously sequenced and cloned 40 full-length infectious molecular clones from six subtype C transmission pairs from the Zambia-Emory HIV Research Project [34, 49]. To display the diversity of Nef and Vpu proteins in these viruses, we constructed phylogenetic trees of Nef and Vpu amino acid sequences, along with the laboratory strains NL4-3 and MJ4 (Fig. 1A and 1B). Viruses from within donor partners harbored a mean of 10 amino acid differences in Nef (range 0-35), whereas viruses from between different donor partners had a mean 37 Nef amino acid differences (range 21-59). Pair 4248 had two distinct Nef branches due to deletions and insertions in the N-terminal anchor domain (for amino acid alignments of Nef and Vpu sequences, see supplemental material Fig. S1). For Vpu, a mean of 5 amino acid differences were observed in viruses from within an infected individual (range 0-17), while between individuals a mean difference of 19 amino acids was observed (range 10-26). Non-transmitted variants differed to varying degrees from their cognate transmitted/founder virus in each pair in their Nef and Vpu amino acid sequences (Fig. 1C and 1D). The amino acid diversity found throughout Nef and Vpu between related and unrelated subtype C primary variants highlighted the potential for heterogeneity of HLA class I downregulation activities within a host quasispecies, between hosts infected with viruses of the same subtype, and across the transmission bottleneck.

HLA class I downregulation of subtype C variants from transmission pairs and laboratory strains

The extent to which authentic primary subtype C viruses differ in the capacity to downregulate HLA-A, HLA-B and HLA-C in primary cells, including those from transmission pairs, is unclear. To measure HLA-A, B and C downregulation simultaneously on primary cells by flow cytometry, we used commercially available serotype-specific antibodies for cells co-expressing HLA-A2 and HLA-B7, added an HLA-C specific antibody (DT9) to measure HLA-C expression, and included a pan-HLA class I antibody (W6/32) to measure total class I expression. For a majority of the *in vitro* experiments described here, we utilized leukapheresed cells from an individual expressing both HLA-A2 and HLA-B7 alleles. Primary cells were infected *in vitro* and stained at day seven, which enabled collection of data for both low and high replicative capacity variants. $Gag^{+}CD4^{-}$ T cells were gated and compared to $Gag^{-}CD4^{+}$ T cells since HLA class I downregulation could be robustly detected when CD4 was maximally downregulated and Gag expression was peaking (Fig. 2A), as has been described elsewhere[50]. Consistent with this, $Gag^{low}CD4^{+}$ cells overlapped in expression of HLA class I with $Gag^{-}CD4^{+}$ cells, and thus were not shown as a separate population. The median fluorescent Index (MFI) and percent HLA-negative cells correlated ($p<0.0001$) for HLA-A ($r=0.78$), HLA-B ($r=0.93$), HLA-C ($r=0.91$) and total HLA-I ($r=0.92$); thus, MFI was used to measure downregulation in subsequent analyses.

The extent of downregulation of HLA-A, HLA-B, HLA-C and total HLA-I on infected cells is illustrated in Figure 2B, where representative histogram plots from four variants display a range of viral phenotypes (Fig. 2B). The subtype B lab strain NL4-3

did not measurably downregulate HLA-C, as described previously[8], while the transmitted/founder from pair 3618 displayed the highest level of HLA-C downregulation of the variants tested (Fig. 2B). Variant 4248 16 showed relatively low levels of HLA-C downregulation, yet robust HLA-A and B downregulation, indicative of discrete functions of Nef and Vpu (Fig. 2B).

The mean fold-downregulation from multiple replicates and experiments is shown in a heat map in Figure 2C for HLA-A, HLA-B, HLA-C and total HLA-I (Fig. 2C). HLA-A2 was downregulated by the largest fold-change in expression on infected cells by median fluorescence intensity (MFI) compared to that on $CD4^{+}Gag^{-}$ T cells, with a range of 1.7-6.1 (3.2 median) (Figure 3A). HLA-C had a more limited range of downregulation by fold change (1.3-3.1 range;1.9 median), potentially due to the lower level of expression of HLA-C on uninfected primary cells (Fig. 2B, [6]). Downregulation of HLA-B7, which ranged from 1.3-5.1 fold (2.3 median), was highly correlated with that of HLA-A2 ($r=0.92$, $p<0.0001$; Fig. 3B). In contrast, there was no correlation between HLA-A2 and HLA-C downregulation ($p=0.61$; Fig. 3B), consistent with these molecules being downregulated by different viral gene products. Peripheral blood mononuclear cells (PBMC) from a different uninfected $HLA\text{-}A2^{+}/HLA\text{-}B7^{-}$ individual yielded similar results for the six transmitted/founder variants analyzed (Fig. 3C). Transmitted/founder variants generally exhibited lower downregulation for HLA-A ($p=0.34$) and HLA-B ($p=0.15$), though not significantly (Fig. 4A). Significant differences between quasispecies from different infected individuals were observed when univariate analyses were performed for HLA-A ($p=0.0003$), HLA-B ($p<0.0001$) and HLA-C ($p=0.007$) downregulation (Fig. 4A), demonstrating divergence of HLA downregulation capacity

between quasispecies. NL4-3, MJ4 and the different MJ4-Gag chimeras all exhibited low levels of HLA-I downregulation (Fig. 4A).

Since viruses from different infected individuals often displayed similar downregulation capacities, and related variants appeared by visual inspection of the heat map to have similar phenotypes (Fig. 2C), we performed hierarchical clustering to determine phenotypic linkages between variants using only their HLA functional footprint (Fig. 4B). Although transmitted/founder variants did not cluster or have a common signature, related variants often, but not always, grouped together. For instance, 4248 TF, 10 and 14, which appear on the same cluster by their Nef and Vpu sequences on the phylogenetic tree (Fig. 1A and 1B), also clustered by their HLA class I downregulation phenotypic signatures (Fig. 4B). Likewise, pair 331 TF, 6 and 14 have the most similar Nef and Vpu sequences from that transmission pair, and are clustered together by their HLA phenotype, despite having infectivities ranging almost 20-fold (331 TF=$3.5x10^{-3}$, 331 6=$4.3x10^{-4}$, 331 14=$2.5x10^{-3}$) and replicative capacities ranging more than 10-fold (331 TF=1.12, 331 6=0.15, 331 14=1.58) which were previously determined for these viruses[34]. Moreover, the lab strains NL4-3 and MJ4 clustered together with a pattern of low downregulation, despite their differences in sequence, subtype and co-receptor usage, which might reflect laboratory adaptation. Both convergence and divergence of phenotypes between quasispecies is evident from the interspersion of some variants throughout the clusters, as was the case for 331 11, for instance, which clustered next to 3618 15 (Fig. 4B). In 2/6 cases, groups of viruses from the same quasispecies were in two distant clusters (331 and 3618; Fig. 4B). However, in 4/6 cases, >75% of variants from a given quasispecies fell into just one of four major

clusters, demonstrating the general relationship between genetic and phenotypic characteristics as they relate to HLA class I downregulation (Fig. 4B).

Genotypic correlates of HLA class I downregulation

To identify amino acid positions potentially contributing to differential HLA class I downregulation, the highest and lowest performing terciles in HLA-A, HLA-B, HLA-C and total HLA class I downregulation were compared for amino acid signatures by Sequence Harmony analysis[51]. Significant positions with Z-scores less than or equal to -3 were found throughout Nef, though none were found in HLA-C related Vpu sites (Table 1). A number of amino acids identified here were previously reported to be involved in HLA class I downregulation, including Nef positions 84[9], 99[39], 106[37], 134[37], 136[9] and 203[7, 9, 39]. Sites matching the consensus sequence were not always associated with an increased capacity to downregulate HLA class I when grouped by HLA-A or HLA-B downregulation (Fig. 5). Furthermore, HLA-I polymorphisms linked statistically to donor or recipient partner HLA haplotypes were not associated with reduced Nef-mediated HLA class I downregulation (data not shown) as has been reported for early infection[40]. In addition, there was no correlation between the distance to the subtype C consensus in Nef, and HLA class I downregulation.

Natural killer cell suppression of subtype C variants and Gag-MJ4 chimeras in vitro

NK cells, as a component of innate immunity, act at the portals of entry and during the initial phases of viral dissemination. Whether the capacity of primary HIV-1 variants to downregulate HLA-C, which can act as an inhibitory ligand for NK cells,

influences NK cell susceptibility in primary cells has not been demonstrated. We therefore examined the ability of different variants to evade NK cell suppression *in vitro* within and between quasispecies as well as across transmission pairs.

To investigate the relationship between NK-mediated suppression of virus replication and HLA-C downregulation, we modified a previously published assay assessing NK-mediated viral suppression [52] by comparing viral strains rather than NK cell donors. The assay employs primary $CD4^+$ T cells infected *in vitro* to serve as targets, while autologous $CD56^+$ NK cells isolated in parallel serve as effectors. We examined a representative subset of 29 variants that included matched transmitted/founder and non-transmitted variants, as well as NL4-3, MJ4 and Gag-MJ4 chimeras with a range of HLA downregulation phenotypes and replicative capacities.

A range of NK cell mediated suppression of primary virus replication was observed at 7 days post infection, with between 1-42% residual replication (14% median; Fig. 6A) in the presence of NK cells. Laboratory strains NL4-3 and MJ4 were far less susceptible to NK suppression when compared to primary viruses from different quasispecies (Fig. 6B). Although there was overlap between viruses from different individuals, a significant difference was observed between pairs 331 and 4248 (Fig. 6B).

In primary viruses, the level of HLA-C downregulation significantly correlated with the level of NK suppression of virus release ($p=0.009$, $r=-0.47$; Fig. 7), and this association held when testing a different subset of variants in PBMC from a different uninfected person ($p=0.04$, $r=-0.60$). Therefore, transmitted/founder and non-transmitted variants, which varied in their ability to downregulate HLA-C, also varied in the susceptibility to NK cell suppression of virus release.

To determine the impact of replicative capacity on NK susceptibility, while controlling for variation in HLA-C downregulation, we assessed selected Gag-MJ4 chimeric variants, which contain identical Nef and Vpu proteins and exhibit different replicative capacities. Gag-MJ4 chimeras were relatively resistant to *in vitro* NK suppression (Fig. 8A). Replicative capacity and susceptibility to NK suppression, as measured by residual replication in the presence of NK cells, were correlated (p=0.01, r=0.77; Fig. 8B). Every 1 unit increase in replicative capacity score for Gag-MJ4 chimeras (replicative capacity is on a $\log_{10}$ scale) led to an 11% increase in residual replication in the presence of NK cells, indicating an impact of replicative capacity on *in vitro* NK susceptibility (Fig. 8B). However, sequence variation in Vpu in primary viruses is still the key factor given that the replicative capacity of primary variants did not correlate with NK resistance (p=0.23). This is illustrated well by viruses from pair 4248, which we previously showed exhibited lower replicative capacity (median RC = 0.08)[34], yet here we show have higher residual replication in the presence of NK cells compared to pair 331 (median RC = 1.35; p=0.02, Fig. 6A and 6B).

Discussion

HLA class I downregulation has not been previously investigated in primary cells with well-characterized full-length primary viruses derived from transmission pairs. Here, we investigated HLA class I downregulation *in vitro* with a previously described panel of 40 subtype C infectious molecular clones derived from near the time of heterosexual transmission. We also evaluated a panel of previously described Gag-MJ4 chimeras to assess the impact of replicative capacity, and added the commonly used NL4-3 subtype B laboratory adapted strain to compare our data to prior studies. We observed variation in HLA class I downregulation by HIV-1 both within and between individuals. HLA-C downregulation, mediated by Vpu, was in general less efficient than that for HLA-B, which was in turn less than HLA-A, though perhaps not less impactful on immune recognition since NK suppression correlated with the capacity to downregulate HLA-C. Despite the selection bias previously observed for consensus residues in the active site of Nef during transmission[36], transmitted/founder variants did not have a common HLA class I downregulation phenotypic signature; rather they reflected the quasispecies from which they were derived. This is surprising, since, even though transmission and establishment of infection may precede the adaptive immune response, one might have expected changes in HLA-I expression to modify susceptibility to the innate immune response..

Downregulation of HLA-A, HLA-B and HLA-C was common amongst the primary variants evaluated in this study, and examining all three simultaneously allowed their relationships to be analyzed. Previous studies have observed a relatively tight

correlation between HLA-A and HLA-B downregulation in CEM [39] and Jurkat cells [53] using expression vectors and chimeric viruses. We found very similar correlations (Fig. 3B) using authentic viruses in primary cells, which partially validates the use of HLA-A2 expression as a surrogate for HLA-A and HLA-B downregulation for future studies, as was done in the past with cell-lines and expression vectors[37, 39, 43, 54-57]. Nonetheless, there were differences between HLA-A and HLA-B, as HLA-A2 was generally downregulated more than HLA-B7 (Fig. 3A; HLA-A/HLA-B ratio, 1.35). This ratio is remarkably similar to what was found previously with primary Nef sequences cloned into NL4-3 in 721.221 cells and HLA-A24 and HLA-B35 downregulation (ratio, ~1.3)[7]. The data presented here does not take into account differences in downregulation that may exist between different HLA-A or HLA-B alleles, but is consistent with the notion that HLA-B is more resistant to downregulation compared to HLA-A, consistent with the primary role of HLA-B in the control of HIV viremia[7, 58]. HLA class I downregulation has been previously measured with a pan-HLA-I antibody[10, 38, 44, 50, 59-64], though fewer studies used primary cells[6, 54, 65]. We found total HLA class I correlated with HLA-A2 expression, which is consistent both with a previous study using Jurkat cells [53], and with the fact that surface expression of HLA-A and HLA-B is significantly higher than HLA-C[6]. Nonetheless, the pan-HLA class I antibody is useful in that it binds to the other HLA-A and HLA-B alleles besides HLA-A2 and HLA-B7. On the other hand, the results are confounded by the fact they reflect the sum of HLA-A, HLA-B and HLA-C downregulation, and the extent of HLA-A and HLA-B downregulation did not correlate with that of HLA-C.

A number of amino acid positions in Nef were significantly associated with HLA class I downregulation in our assays, and some of these were found in previous studies[7, 9, 37, 39]. However, Vpu sites associated with HLA-C downregulation were not identified by the same analysis. Using upper and lower quartiles instead of terciles gave only one significant Vpu position (position 13). Signatures associated with the pan HLA class I data yielded three Vpu sites, two of which were toward the N-terminus, similar to what was found in the initial study identifying Vpu mediated HLA-C downregulation[8]. Indeed, most of the diversity in the Vpu sequences under study here is in the extracellular and helical transmembrane domain at the N-terminus (see supplemental material Fig. S1). Viruses from within pair 3618 that differed substantially in HLA-C downregulation, differed most substantially at Vpu positions 2, 4, 18 and 79, although this was not significant. Mutagenesis studies of Nef and Vpu would be needed to validate sites not found in previous studies, and specifically to elucidate the mechanism of the interaction of Vpu with HLA-C.

The capacity of primary viruses to downregulate HLA-C correlated directly with *in vitro* susceptibility to NK suppression, consistent with the very recent results of Korner et al. with the lab-adapted strain JRCSF[66]. *In vitro* experiments have shown that HLA-C, when left on the cell surface, can inhibit NK cell killing of HIV infected cells by engaging inhibitory receptors[5]. However, given both the extensive diversity of the NK cell repertoire, which in a single individual includes as many as 30,000 populations of various activating and inhibitory receptor combinations[67], and the fact that other HIV proteins like Vpr influence NK cell activation and infected cell killing[68, 69], such a major contribution of a single, albeit crucial, NK ligand is not entirely predictable.

Why is HLA-C downregulation relatively common among primary HIV-1 variants, if it leads to NK recognition? The balance between evading NK and CD8 T cells may drive this phenotype. A reasonable hypothesis is that HLA-C downregulation is selected against in acute infection to avoid NK cells, and selected for during the adaptive immune response to avoid CD8 T cells, though this is not supported by the data presented here. Transmitted/founder variants were similar in the capacity to downregulate HLA-C (1.3-3.1 fold HLA-C downregulation) to the quasispecies from which they were derived, and perhaps as a consequence were not uniquely resistant to NK suppression. Apps et al.[8] examined nine transmitted/founder variants from subtypes B, C and D, three of which were subtype C variants with a 1.4-2.5 fold range of HLA-C downregulation in primary cells, suggesting future studies including more transmitted/founder variants may not detect a distinct ability of transmitted/founder variants to downregulate HLA-C and subsequently evade *in vitro* NK cell suppression. Romani et al. found a similar range of HLA-C downregulation using subtype A Vpu sequences from four patients at various stages of infection[70]. If non-specific NK cell evasion is not contributing to the HIV-1 transmission bottleneck, perhaps specific KIR HLA combinations of each transmission pair play a role, since HIV-1 mutations that lead to evasion of NK cells by peptide:HLA-C stabilization and subsequent binding of inhibitory NK cell ligands has been demonstrated [20, 71]. Such a mechanism would necessitate the transmission of preadapted viruses relevant to the recipient's HLA alleles, which is a phenomenon which has been demonstrated in a number of transmission pairs, and is a major determinant of viral load and disease progression in recipients[72, 73]. However, the impact on NK cells and risk of infection remains unclear, and future studies would need to use *in vitro*

systems that match the KIR and HLA genetics of each transmission pair from whom the viruses were derived.

HLA class I downregulation coincided with peak Gag expression *in vitro*, while cells with intermediate Gag expression exhibited little to no HLA-I downregulation (Fig. 2C). This observation is consistent with the results from a study that utilized anti-Gag antibodies and RNA probes to monitor the timing of events that lead to the disruption of surface HLA class I[50]. Interestingly, a recent study of SIV_{MAC239} infected cells ex vivo found little HLA class I downregulation at any stage of the virus life cycle defined by viral RNA transcripts in single cells, even though the levels other surface proteins were altered at different stages[12]. Nonetheless, the effect of timing of HLA downregulation indicates the potential to gauge the stage of viral protein expression by alteration of surface receptors, making virus producing cells identifiable without cellular fixation and permeabilization (i.e. for live cell sorting, RNA studies etc.). A study of the precise duration and timing of viral life cycle events coinciding with the alteration of cell surface molecules could benefit therapeutic cure strategies aiming to identify biomarkers associated with the transition from pre-bursting infected cells to virus producing cells.

The capacity to downregulate HLA class I was not generally associated with transmission fitness in this study. With the exception of positions 105 and 106 identified in the Sequence Harmony analysis, consensus residues, which are selected for during transmission[34, 36], were not necessarily associated with a particular level of HLA class I downregulation. Additionally, transmitted/founder viruses were not biased towards enhanced or reduced HLA class I (A, B, C or total) downregulation. Nevertheless, common downregulation signatures often grouped related variants, including

transmitted/founder viruses, consistent with the notion that transmitted/founder variants are more often phenotypically closer to their source quasispecies than other transmitted variants. Viral traits that impact disease progression, such as viral load, can be inherited across transmission [73-77], in part due to viral replicative capacity[48]. Thus, the heritability of other traits is to be expected. Genetic selection of viruses observed during transmission points to a bias that likely has an associated though yet undefined phenotype[31, 34, 36, 78-80]. However, the magnitude of the predicted phenotypic bias is unclear and is likely to be modest and specific to the circumstances of each transmission event, given the number of factors that influence infection risk and the genetic bottleneck. These include viral load[81, 82], HLA[21, 22, 83], circumcision status[81, 84, 85], route and direction of transmission[29, 86], presence of genital infections and inflammation[87-89], and the composition of the microbiome[90]. For these reasons, the dimensions of the genetic bias may vary significantly depending on the circumstances[36], and this appears to be reflected in the phenotypic data presented here and observed by others [33, 34, 38, 45, 91, 92].

Overall, our results show that quasispecies within and between individuals in the same subtype display a range of HLA class I downregulation capacities that influence immune recognition and may be shifted during transmission to some extent based on the transmitted/founder variant that establishes the new infection, even though no generalizable HLA signature appears to be selected. These data from primary viruses with the full complement of autologous proteins may be important when therapeutic or prophylactic strategies aimed at manipulating CD8 and NK cell responses are being considered.

Materials and Methods

Study Subjects and Samples

Infectious molecular clones of viruses from 12 subtype C infected, therapy naïve individuals in six transmission pairs in this study were derived as previously described[34, 49]. These individuals come from a heterosexual discordant couple cohort from the Zambia-Emory HIV Research Project, who signed informed consent forms agreeing to participate, as were the acutely infected individuals from which the Gag-MJ4 chimeras were created[48]. The University of Zambia Research Ethics Committee and Emory University Institutional Review Board approved the human subjects protocols for the couples in this cohort who were provided monthly counseling and testing prior to the HIV negative partner becoming positive. All human sample ID numbers were anonymized. Epidemiological linkage was determined by HIV-1 gp41 sequences derived from the couple[93] and newly infected recipients were enrolled in the International AIDS Vaccine Initiative (IAVI) Protocol C early infection cohort. Peripheral blood mononuclear cells from a limited number of healthy HIV(-) donors were obtained by leukapheresis by the Emory Center for AIDS Research Clinical Core in collaboration with the Emory Transplant Center following Emory IRB Protocol #45821 - "Phlebotomy of Healthy Donors".

Phylogenetic tree and sequence alignment

BioNJ phylogenetic trees from Nef and Vpu amino acid alignments were made using Geneious bioinformatics software (Biomatters, Aukland, NZ), ProtTest[94], and

DIVEIN software[95]. Full-length Nef and Vpu amino acid sequences were aligned by a MAFTT alignment with Geneious software, and then further hand aligned. ProTest software was then used to derive the best substitution model. The HIVb substitution model was used with 100 bootstraps and the best of NNI and SPR trees for improvements with optimized equilibrium frequencies, an estimated proportion of invariable sites, an estimated gamma distribution, and four substitution rate categories. The trees shown are rooted on the subtype B NL4-3 sequence as an outgroup, and the subtype C consensus for Nef and Vpu were derived from the Los Alamos National Database (LANL: https://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Comparisons in the text to the distance to the full-length subtype C consensus are based on calculations made with 115 full-length concatenated amino acid sequences from these six transmission pairs determined in a prior publication[34].

Generation of infectious molecular clones and Gag-MJ4 chimeras

Subtype C HIV-1 Infectious molecular clones [49, 96] and Gag-MJ4 chimeras were described in previous studies [34, 47, 48]. Briefly, 40 infectious molecular clones from 12 individuals in 6 transmission pairs from the Zambian cohort were derived from near-full length single genome amplifications (SGA) of autologous HIV RNA sequences of infected individuals 22-45 days away from the estimated date of transmission[34]. Gag-MJ4 chimeras were constructed from patient-derived gag sequences (and 142 nucleotides into pol) of subtype C acutely infected individuals in the Zambian cohort 33-49 days away from the estimated date of transmission, cloned into an MJ4 provirus backbone[47, 48, 96], which is a subtype C R5-tropic laboratory strain[97]. NL4-3 is a

subtype B laboratory strain that is X4-tropic[98]. All replication-competent viruses were created by plasmid transfection of 293T cells (American Type Culture Collection). Titers were generated on the TZM-bl indicator cell-line (NIH AIDS Research and Reference Reagent Program) to normalize input virus for primary cell infections at a constant multiplicity of infection.

Infections of primary cells in vitro

Frozen peripheral blood mononuclear cells (PBMC), obtained through Emory IRB Protocol #45821, as described above, were thawed in a 37C water bath and then washed twice in R10 (Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 1 U/ml penicillin, 10% defined fetal bovine serum (FBS), 1ug/ml streptomycin, and 300ug/ml L-glutamine) containing DNase with 1,500 rpm spins for 7 minutes each. Then, total PBMC, or isolated $CD4^+$ cells (following the protocol of the EasySep^{TM} Human $CD4^+$ T cell Isolation Kit catalog # 19052), were stimulated with 2-3 ug/ml of phytohemagglutinin (PHA) and 20 U/ml of interleukin-2 (IL-2 reconstituted in phosphate buffered saline; Sigman, catalog # I7908-10KU) in R10 for 48-72 hours at 2-3 million cells per ml in a flask in an incubator at 37 °C with 5% CO2 and 95% humidity. Then cells were infected either in 15ml conicals or in 96-well plates at $7x10^5$-$1x10^6$ cells per well at a multiplicity of infection of 0.1-0.01 by TZM-bl titer from blue cell counts in 250ul-300ul per well. Virus levels in the supernatants were assessed by a radioactive reverse transcriptase assay described previously and in more detail elsewhere[34, 96], using P^{33} that detects digital light units (DLU) from a phosphoscreen, and thus RT DLU is reverse transcriptase digital light units. Area under the curve was calculated from mock

subtracted RT digital light unit values using Prism with a baseline and minimum peak height of zero.

Flow cytometry for HLA downregulation

Flow cytometry was performed on a BD Fortessa in the Emory Center for AIDS Research Immunology Core. HLA-A2^{+}/HLA-B7^{+} double positive primary cells were identified as explained in the text after screening a number of PBMC donors with anti-HLA-A2-Alexa700 (clone BB7.2, Biolegend catalog # 343317) and anti-HLA-B7-FITC (clone BB7.1, ThermoScientific catalog # MA1-82180) antibodies. One double positive (HLA-A2^{+}/HLA-B7^{+}) HIV- individual was identified and leukapheresis allowed for enough cells to perform multiple replicates and experiments.

Infections were done as described above and stained at day 7 days post-infection *in vitro*, which was previously shown to be near the peak of infection for most variants tested here[34]. Multiple infection replicates (2-4) were pooled together for flow cytometry using the following antibodies in multiple experiments: Aqua Live Dead (Amine reactive dye), anti-CD3-APC-Cy7 (SP34.2), anti-CD4-BV711 (OKT4), anti-CD8-QD605 (RPA-T8), anti-HLA-A2-Alexa700 (BB7.2), anti-HLA-B7-FITC (BB7.1), anti-HLA-C-APC (DT9; conjugated after buffer exchange; Abcam), anti-HLA-ABC-PECy7 (W6/32), anti-p24Gag-PE (KC57). A median of 2,070 cells (range: 50-12,339) were analyzed from 2-5 independent experiments, with a median of 3.6% infected cells (0.041-17% range) of live CD3^{+}CD8^{-} cells. HLA-I downregulation was defined as the expression of the HLA marker on singlet^{+}lymphocyte^{+}Aqua^{-}CD3^{+}CD8^{-}CD4^{+}Gag^{-} cells

divided by singlet^{+}lymphocyte^{+}Aqua^{-}CD3^{+}CD8^{-}CD4^{-}Gag^{+} cells. Thus, the difference is a fold change of median fluorescence intensity (MFI) of a population of cells.

Genotypic associations with HLA downregulation

Alignments of amino acid sequences in FASTA format were exported from Geneious software for Sequence Harmony analysis (http: //www.ibi.vu.nl/programs/shmrwww/). Weblogos were then created based on the Sequence Harmony results (http://weblogo.threeplusone.com/create.cgi). The lowest and highest terciles of HLA-A, HLA-B were compared by Nef sequences, and HLA-C was compared by Vpu sequences. HLA-I was compared with concatenated Nef and Vpu sequences. Z-scores represent standard deviations from the mean calculated from 100 random permutations, and significant values with a cut off of -3 were included in Table 1. For the estimation of viral adaptation to HLA (data not shown), TF were compared to the median of the variants from donor quasispecies and were compared by a paired Wilcoxon test. The estimation of viral adaptation to an HLA haplotype was performed as previously described[72]. Briefly, the degree of adaptation of each viral sequence to the HLA haplotype of the recipient within each transmission pair was defined as the proportion of positions in Gag, Pol and Nef that could be targeted by each individual according to their HLA alleles that harbor an adapted residue (either non consensus or consensus)

NK suppression of replication in vitro

NK suppression was measured as previously described in He et al. with minor modifications[52]. Briefly, isolated CD4 T cells (EasySep™ Human CD4+ T cell Isolation Kit) and NK cells (EasySep™ Human NK T cell Isolation Kit catalog # 17955) from the same PBMC donor were co-cultured after CD4 T cells were stimulated overnight with 2ug/ml PHA and IL-2 (100U/ml), while NK cells were cultured separately overnight with IL-15 (2ng/ml reconstituted in phosphate buffered saline; R&D systems catalog # 247-ILB-005). Before mixing NK and CD4 T cells at various effector to target ratios (1:1-1:10), 60,000 CD4 T cells were infected at a multiplicity of infection of 0.1 with a spinoculation, or 1 without spinoculation, in 15ml conicals, then washed with 14ml RPMI and subsequently cultured in v-bottom 96-well plates in 200ul overnight. The following day, the cells were spun at 2,000 rpm for 5 minutes and washed twice, and transferred to a round bottom plate for the addition of NK cells in triplicate in 300ul total with 50U/ml IL-2. Supernatants were sampled at days 3, 7, and 11, or just day 7 depending on the experiment. Values reported are from day 7 post infection, which were found to correlate with area under the curve of days 3 to 11 for those where multiple time points were taken. A radioactive reverse transcriptase assay was performed on supernatants to measure the level of virus, and reported as digital light units (DLU), as describe previously[34, 96].

Statistics

Statistics were done using Prism 7 software and JMP Pro 13.0. Non-parametric Spearman correlations were shown in the text and figures as an "r" value. The Wilcoxon-matched pairs signed rank test was used to compare transmitted/founder variants and

matched donor quasispecies medians. ANOVA was used where shown. Linear regressions were performed to compare to historical data and shown in the text as "r^2". Sequence Harmony analysis was performed to compare amino acids associated with high and low level HLA class I downregulation.

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Figures

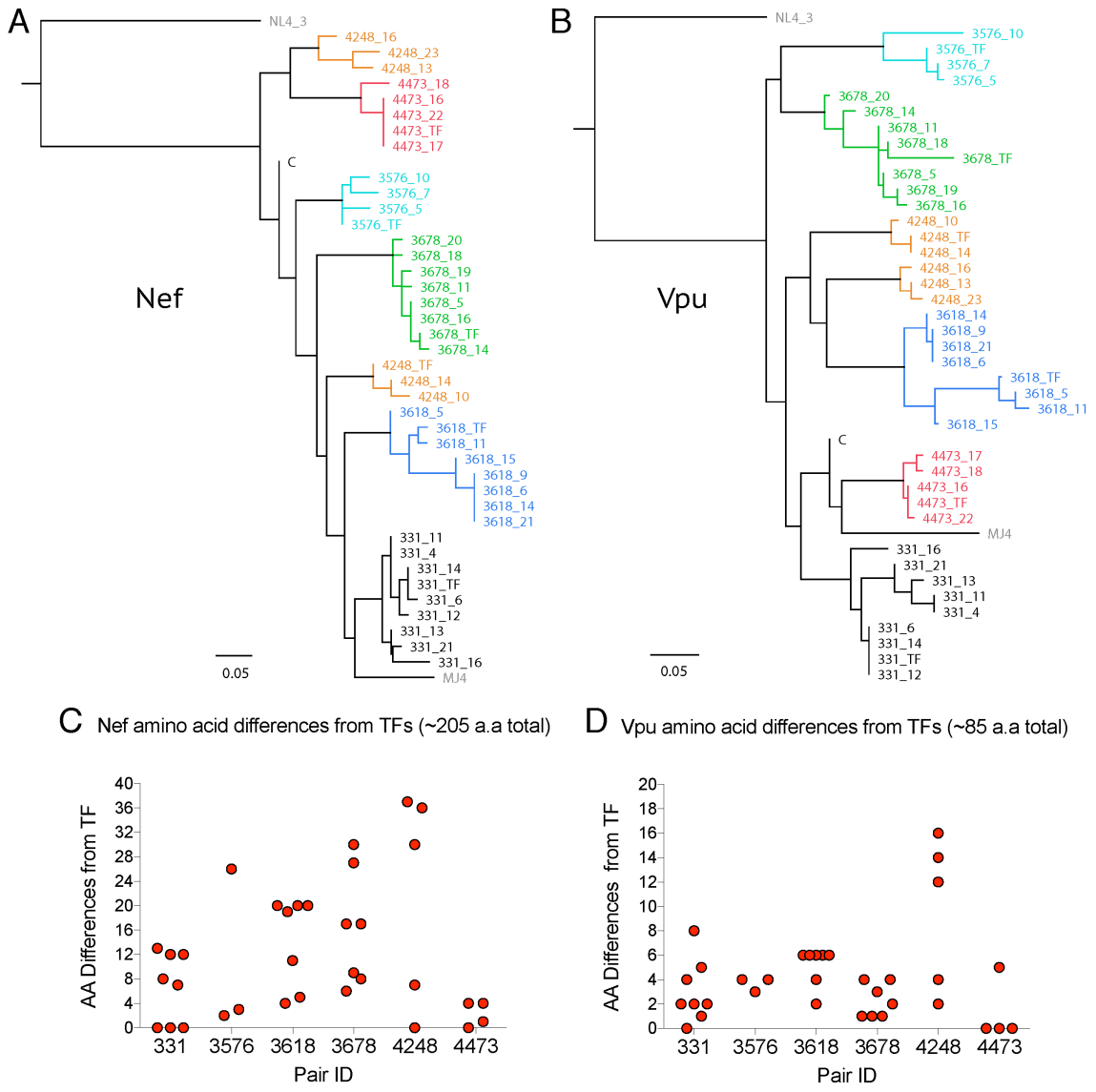


Fig. 1. Diversity of Nef and Vpu in characterized viruses

Viral variants from donors of transmission pairs are labeled as pair identifier followed by variant number, while recipient transmitted/founder viruses are labeled with TF. Each pair is indicated by color. The laboratory strains are in grey, while the subtype C consensus from the Los Alamos National Laboratory HIV Database (LANL) is labeled as

"C". (A) BioNJ tree of phylogenetic relationships of Nef and (B) Vpu amino acid sequences of TF and NT variants, along with one subtype C (MJ4) and one subtype B (NL4-3) laboratory strain. The LANL subtype C consensus, is included for reference. (C) Number of amino acid differences from the transmitted/founder for each non-transmitted (NT) donor variant from each transmission pair for Nef and (D) Vpu. C, LANL subtype C consensus; TF, transmitted/founder; NT, non-transmitted donor variant.

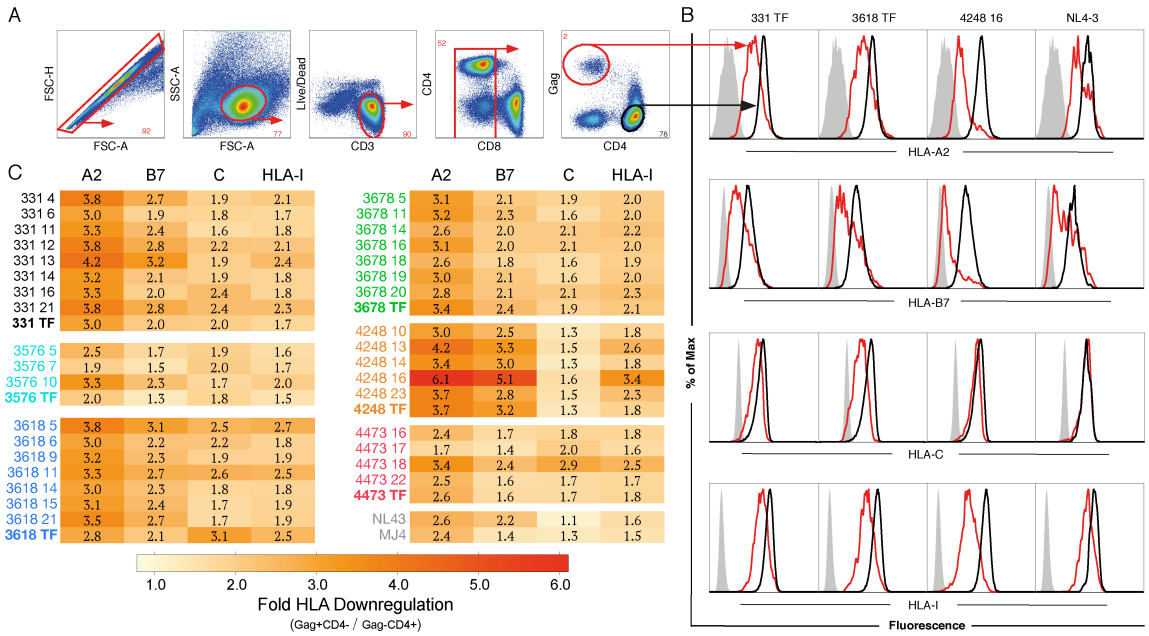


Fig. 2. HLA downregulation of infected cells *in vitro*

Infectious molecular clones from six transmission pairs along with two lab strains (MJ4=subtype C; NL4-3=subtype B) were assayed for the ability to downregulate HLA-I by flow cytometry with antibodies recognizing HLA-A2, HLA-B7, HLA-C and pan-HLA-I. (A) The gating strategy is shown as an example from 331 TF, and (B) histograms showing $CD4^{-}Gag^{+}$ (red), $CD4^{+}Gag^{-}$ (black) and HLA negative controls (grey) are shown for 4 variants that show a range of activities for HLA-A2 (top row), HLA-B7 (row 2), HLA-C (row 3) and HLA-I (bottom row) downregulation. (C) Mean values from pooled triplicates averaged from 2-5 experiments of the fold-downregulation of HLA by median fluorescence intensity (MFI) on infected T cells as in panels A and B, compared to uninfected $CD4^{+}$ T cells. Each viral clone is denoted with a pair ID followed by variant number when amplified from the donor partner, or TF for variants derived from the

recipient. HLA-A2, A2; HLA-B7, B7; HLA-C, C; pan-HLA-I (HLA-I); TF, transmitted/founder; NT, non-transmitted donor variant.

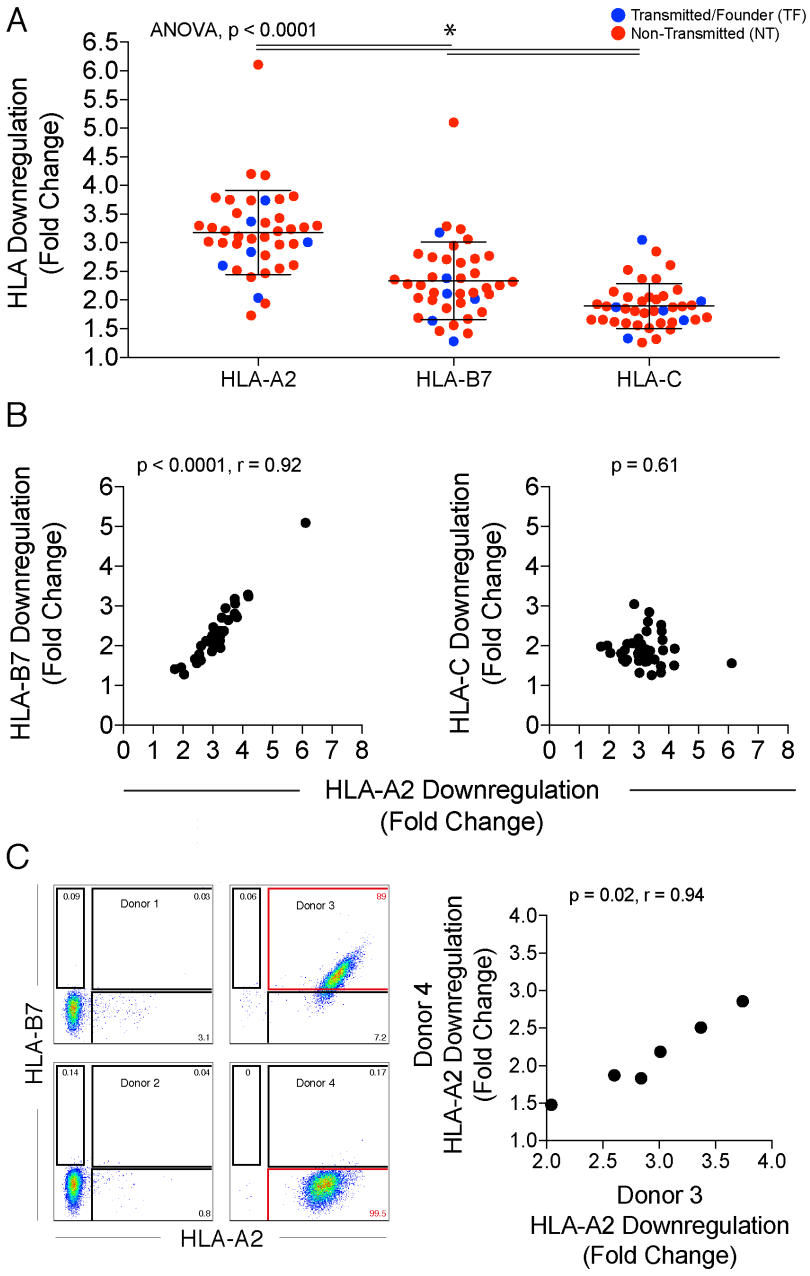


Fig. 3. Relationship of HLA class I molecules on infected cells

(A) Overall HLA class I downregulation for the tested primary variants showing the mean and standard deviation. Bars with asterisk above mark significant differences. TF are blue and NT are red (B) Spearman correlation analysis between HLA-A2 and HLA-

B7 downregulation (left) and HLA-A2 and HLA-C downregulation (right). (C) (Left) Identification of HLA-$A2^{+}$HLA-$B7^{+}$ and HLA-$A2^{+}$HLA$B7^{-}$ cells by flow cytometry by screening of 4 PBMC from different donors as examples and (Right) Spearman correlation of HLA-A2 downregulation in two different PBMC donors averaged from multiple experiments using TF variants. TF, transmitted/founder virus; NT, non-transmitted donor variant.

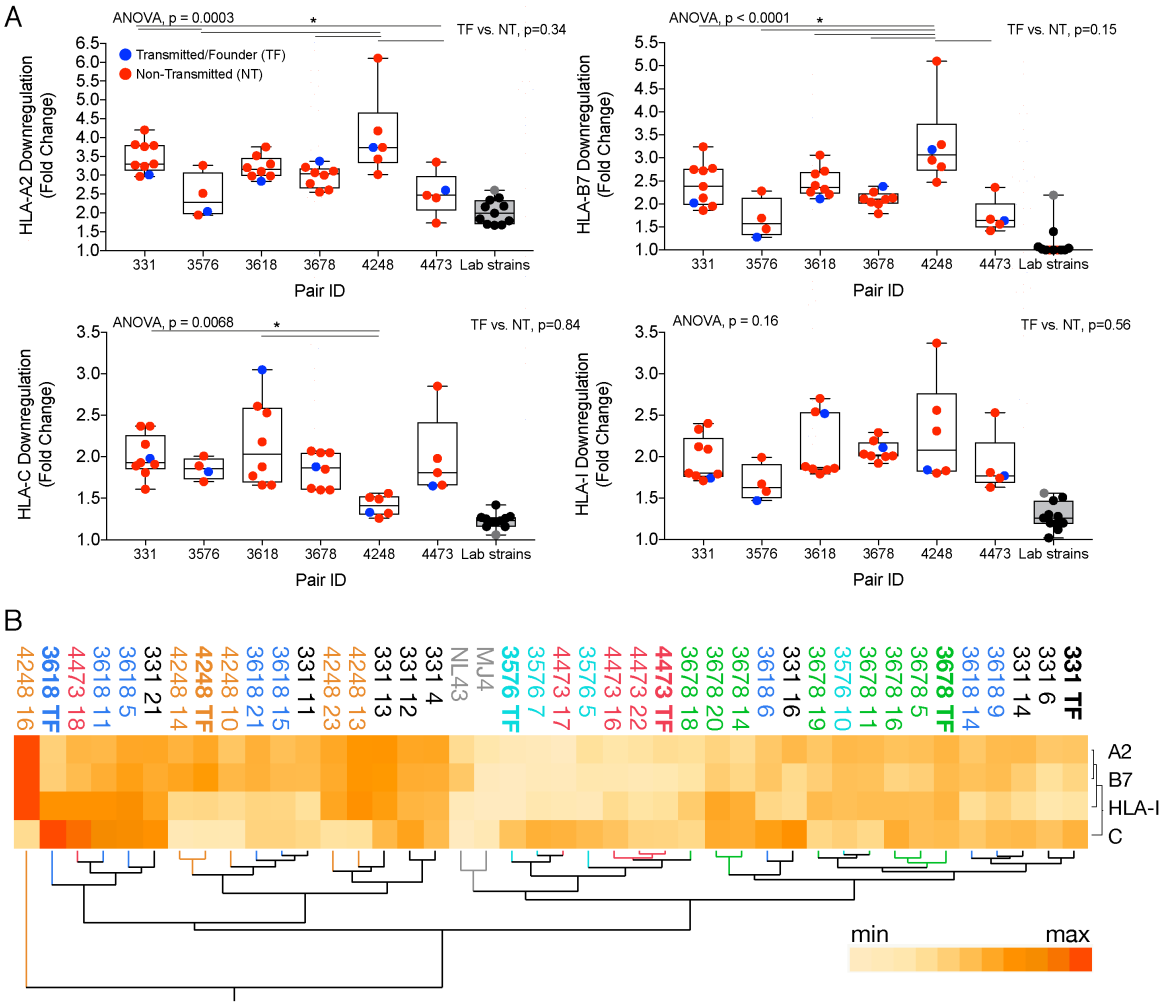


Fig. 4. Quasispecies level HLA class I downregulation and clustering by phenotype

(A) Box and whisker plots showing median and minimum to maximum per transmission pair (x-axis) downregulation of HLA-A2 (top left), HLA-B7 (top right), HLA-C (bottom left) and total HLA-I (bottom right). Transmitted viruses are in blue, non-transmitted variants are in red, NL4-3 is in grey, and in black is MJ4 and Gag-MJ4 chimeras. Lines and asterisks represent statistical significance at $p<0.05$ and all y-axes depict fold change of $CD4^{-}Gag^{+}$ divided by $CD4^{+}Gag^{-}$ MFI. (B) Hierarchical clustering analysis of variants by HLA downregulation level independent of their genetic relatedness. Each variant is labeled and colored per transmission pair as in Figure 2C.

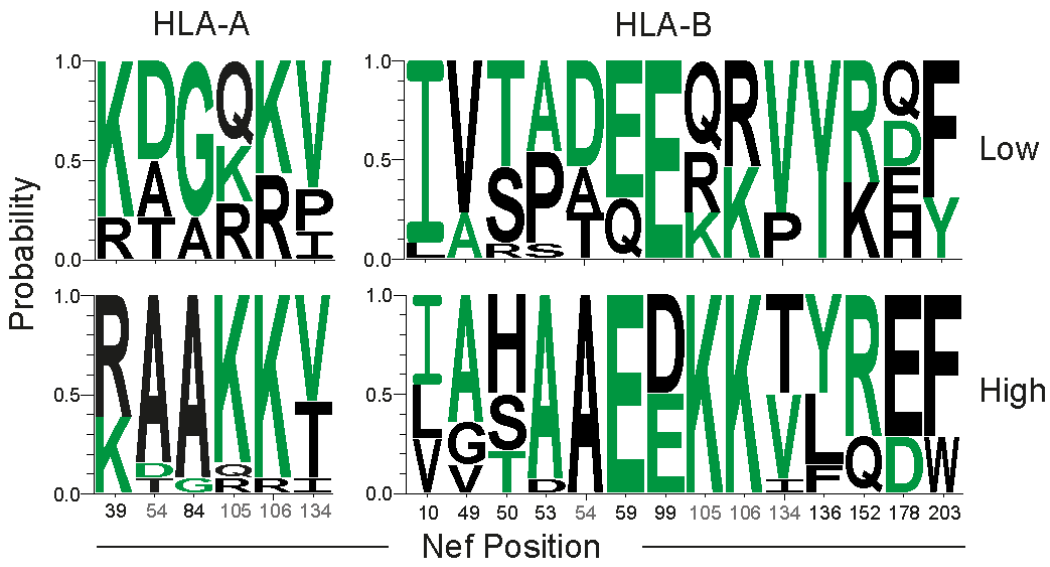


Fig. 5. Amino acids associated with HLA-A and HLA-B downregulation

Weblogos display the Nef amino acid sequence composition of primary viruses at the lowest and highest terciles for HLA-A and HLA-B downregulation. Sequence Harmony analysis determined the shown positions with Z-score below -3, a full listing of which is in Table 1. Green amino acids denote the subtype C consensus amino acid at that position, and grey numbers are Nef positions common to virus groupings by HLA-A and HLA-B. Position numbers based on the alignment in supplemental material (Fig. S1).

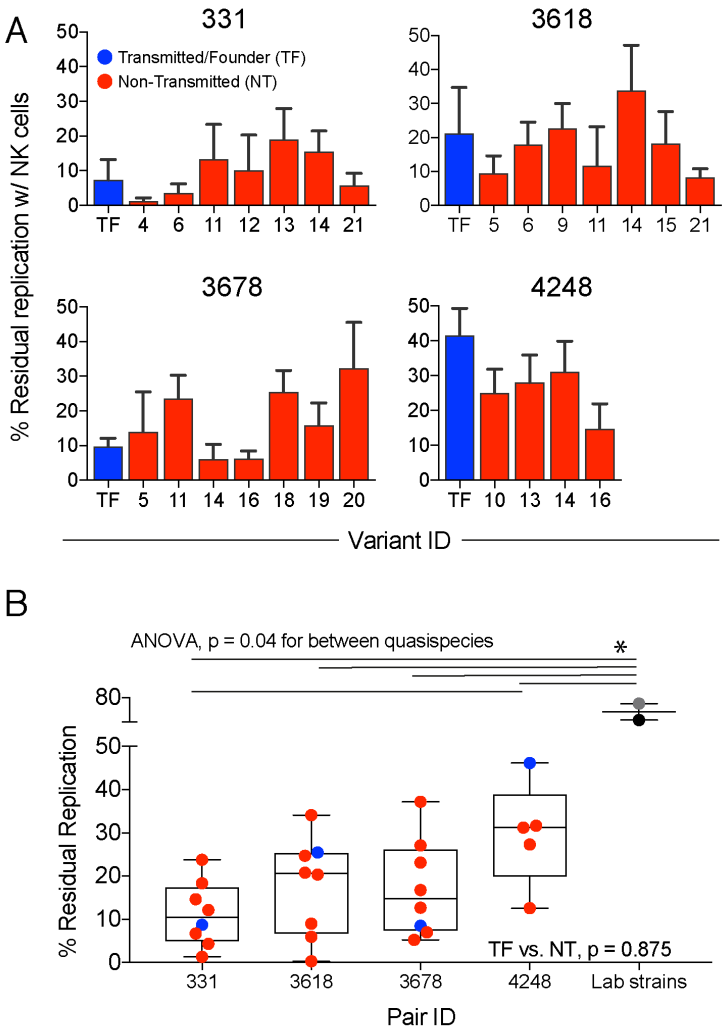


Fig. 6. NK cell suppression of virus growth *in vitro*

(A) TF (blue) and NT (red) residual replication in the presence of NK cells is shown for four pairs. Each variant is listed on the x-axis, and the y-axis is calculated as the percent of replication in the presence of NK cells compared to the no NK controls. (B) Mean quasispecies NK susceptibility, including lab strains NL4-3 (grey) and MJ4 (black). Lines and asterisks represent $p<0.05$ using ANOVA followed by Tukey's multiple comparison test. Wilcoxon test was done to compare TF and NT median over the pairs.

TF, transmitted/founder; NT, non-transmitted donor variant. TF, transmitted/founder; NT, non-transmitted donor variant.

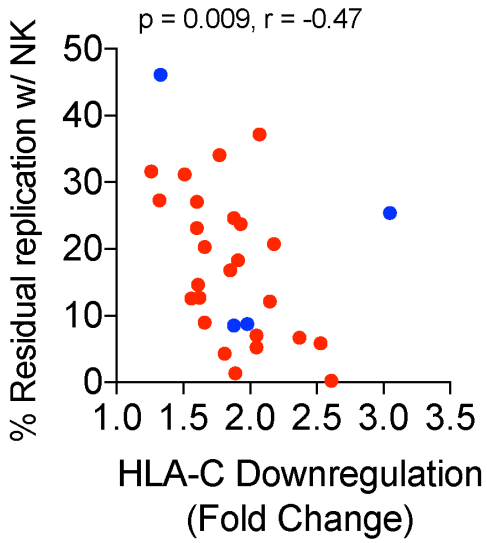


Fig. 7. Relationship between NK cell suppression and HLA-C downregulation

A Spearman correlation analysis of HLA-C downregulation on the x-axis against residual replication in the presence of NK cells. TF (blue) and NT (red) are colored. TF, transmitted/founder; NT, non-transmitted donor variant.

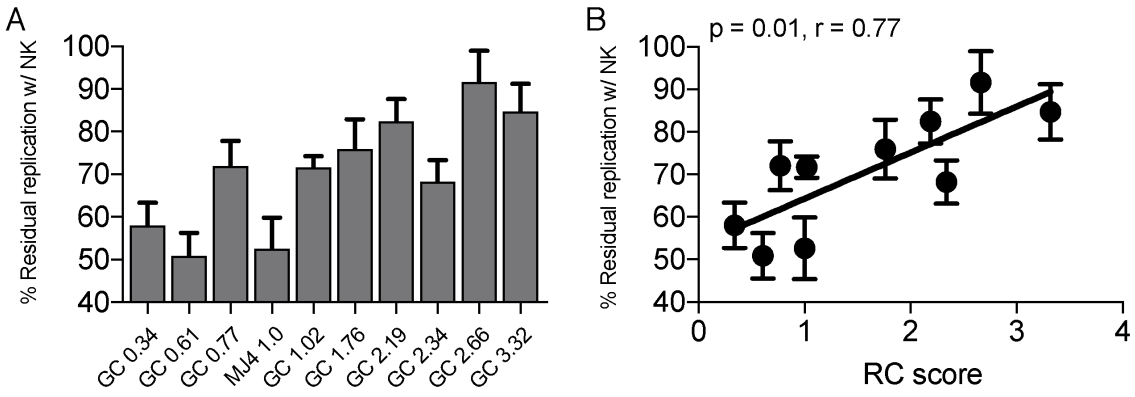
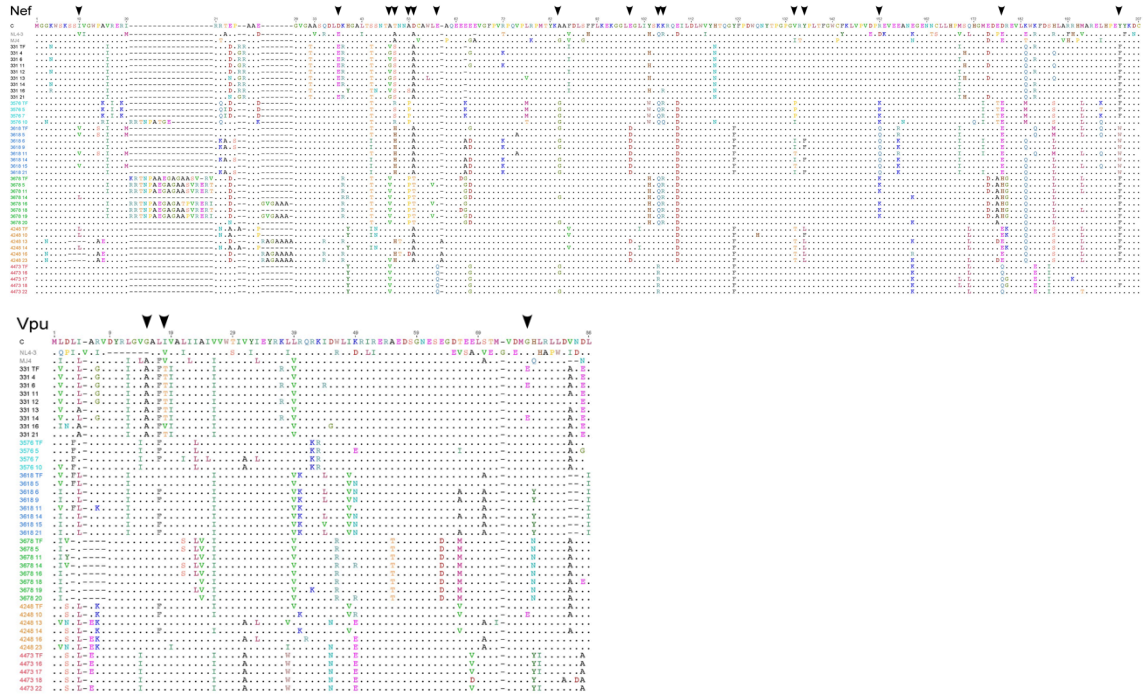


Fig. 8. Replicative capacity and NK cell suppression

(A) Residual replication in the presence of NK for Gag-MJ4 chimeric viruses (GC) ordered by replicative capacity scores. The x-axis contains names of each chimera by their replicative capacity score, such that variant ID GC 0.34, stands for gag chimera with a replicative capacity score of 0.34. (B) Spearman correlation analysis of replicative capacity versus NK suppression *in vitro*. TF, transmitted/founder; NT, non-transmitted donor variant; % of ctrl, percent of replication in the absence of NK cells; GC 0.34, Gag-Chimera with RC score 0.34; RC, replicative capacity.



S1 Fig. Supplemental Figure Nef and Vpu amino acid alignment of viruses phenotyped in this study

The alignment demonstrates substantial sequence variation. Numbering is based on the subtype C LANL consensus (shown as "C"), which is similar to HXB2 numbering. Amino acids that differ from the subtype C consensus are shown in color throughout the alignment, while dots are positions that match the consensus sequence. Dashes represent the absence of an amino acid at that position. Arrows point to significant positions found from Sequence Harmony analysis shown in Table 1, some of which are displayed in Figure 5.

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Chapter IV: Discussion

The studies described in this thesis were the first to examine full-length infectious HIV-1 molecular clones, unchanged by passage *in vitro*, from transmission pairs, in order to characterize the phenotype of the transmitted variant relative to non-transmitted variants. Although successful generation and examination of full-length transmitted/founder viruses was achieved in prior studies[1-4], we were the first to clone and assess genotypic and phenotypic characteristics of matched full-length viruses from the donor partner[5, 6]. We were able to accomplish this primarily due to sample availability from an established cohabitating couples cohort in Zambia, along with technological advances in cloning[5] and phenotyping. Subsequently, we challenged emerging concepts, added to previous studies, and made novel assays to examine and characterize primary subtype C HIV-1 variants.

Debate on Interferon Resistance and Transmission

A major paradigm we challenged in the field of HIV transmission virology was the resistance to interferon of transmitted/founder viruses. We made significant contributions at major conferences, and subsequently in the literature, by challenging the hypothesis that HIV variants are uniquely resistant to interferon. A number of studies performed in the same time frame reported results consistent with our findings[7-9], though other studies observed evidence in support of previous findings[10-12]. Interestingly, when the first data reporting transmitted/founder interferon resistance was

published, subtype C transmitted/founder variants were not different from chronic controls[1]. However, subtype differences were subsequently downplayed, since later studies showed higher interferon resistance levels for both subtype B and C variants[3, 10]. The recent comparison of viral isolates from 8 transmission events by Iyer *et al.* demonstrated striking support for the hypothesis that interferon is the major driver of the HIV genetic bottleneck[10]. In that study, all acute plasma isolates derived from outgrowth *in vitro* were strikingly more resistant to IFNα and IFNβ (up to 541-fold more resistant, based on residual replication in the face of maximal inhibitory doses for the latter) compared to those from the transmitting partner[10]. Nonetheless, an independent study of transmitted/founder isolates from 9 subtype B transmission pairs showed greater sensitivity to interferon[7]. Additionally, 12 subtype B and C acute and chronic viruses, when assessed for activities of accessory proteins Nef and Vpu, showed similar activity against interferon induced restriction factors *in vitro*[9]. Given that the data presented in this dissertation are in agreement with the latter studies and in stark opposition to results demonstrating a clear signal for interferon resistant transmitted viruses, it may be helpful to point out major differences in the experimental approaches used. In Iyer et al., viral isolates were grown for one month in $CD4^+$ T cells *in vitro* before assessing their phenotypic characteristics, the chronically infected partners all had very high VL (>100,000), and transmitted isolates had a uniformly higher particle infectivity than the viruses grown out from donor plasma. The replication and interferon assays were normalized by viral particles rather than infectious particles, and thus the effective multiplicity of infection of transmitted isolates was higher going into all replicative capacity and interferon assays. We would conclude therefore that interferon resistance

and replicative capacity could be a consequence rather than a cause of the striking viral phenotypes demonstrated. It is unclear why Iyer et al. observed higher particle infectivity in the acute isolates, while in the studies presented here we observed only 2/6 cases where the transmitted/founder had a significantly higher particle infectivity. Two speculative possibilities are: 1) *in vitro* recombination during viral isolation is different with highly similar variants from acute infection, compared to more diverse variants from chronic infection. 2) Particle infectivity, which we found to be somewhat dynamic over the course of hours to days from *in vitro* growth assays—likely due to Env stability[13] and the stoichiometry of Env trimmers needed for entry[14]—may have been optimized for acute isolates so that the highest level of virus stocks could be created. A recent article written as a spotlight for Iyer *et al.* in *Trends in Microbiology,* entitled "Interferon-I: The Pièce de Résistance of HIV-1 Transmission?" summarizes the main points of contention[15].

While testing our subtype C variants for interferon resistance, we observed a key issue in how interferon resistance is determined *in vitro*. Pretreatment of primary cells *in vitro* with interferon (the way the assays are performed in all of the transmission studies) changes the effective multiplicity of infection and thus alters viral growth kinetics[6, 7, 16, 17], making comparisons between viruses with disparate infectivities and replicative capacities difficult to interpret. Moreover, replication in the absence of interferon has been shown here, and by others, to correlate with replication in the presence of interferon[6, 7], suggesting *in vitro* replication in the presence of interferon may be primarily driven by replicative capacity rather than variability of primary viruses to effectively antagonize interferon induced restriction factors. Any viral antagonist tested

in vitro is likely to be impacted by replicative capacity. We found a similar result for example while examining NK mediated viral suppression (Chapter 3), and a collaborating laboratory showed that replicative capacity could influence resistance to some protease inhibitors as well[18].

We examined three previously published relatively interferon resistant transmitted/founder variants and their matched 6-month consensus viruses from the same person[3], and showed the transmitted/founder variants had higher replicative capacities than the 6-month consensus viruses, further indicating interferon resistance assays may be influenced by this difference[6]. Since then, a specific restriction factor associated with co-receptor usage, IFN-induced transmembrane protein 1 (IFITM1), was found to determine the resistance phenotype of the transmitted/founder and 6-month virus pairs[12], though Vpu:tetherin interactions have also been implicated as major determinants of resistance for these variants[11]. Regardless, their increased replicative capacity suggests they would have an advantage in most *in vitro* systems looking at inhibition.

Given the reduced selection pressure observed during transmission due to genital inflammation and ulcers in male recipients, gender of the recipient partner, and viral load in chronically infected partners, it seems unlikely that every infection will be equally influenced by interferon. These consequential variables need to be taken into account when comparing studies on any property of transmitted viruses, including interferon resistance. Of course, due to technological and practical limitations, in addition to sample availability, it has been difficult to assess enough transmission pairs with different variables to analyze specific circumstances, which is why most transmission pair studies

have looked at 1 to 9 pairs, and have tested transmitted viruses for traits that would be common to transmission events in general—even though such traits may not exist in every virus or may vary in magnitude, such that a large number of transmission pairs would be necessary for their discovery.

Influence of Adaptive Immunity on Transmission

A number of observations described here were not challenges to a paradigm, but rather extended previous results. We corroborated evidence for the preferential transmission of viruses closer to consensus, described in a larger study of viruses using *gag*, *pol* and *nef* population sequences, through a comparison of full genomes, from sequences derived by single genome amplification[6]. In a study of 137 transmission pairs, it was found that HLA-linked polymorphisms in donor virus sequences were less likely to be transmitted, suggesting that HLA-linked T cell responses were reducing transmissibility[19]. We also found an antibody linked phenotype, in that transmitted viruses had modestly increased sensitivity to donor plasma taken near the time of transmission[6], substantiating a previous finding [20]. This has at times incorrectly been taken to mean that transmitted viruses are more susceptible to antibodies in general, however, broadly neutralizing antibodies[21], pooled plasma[20], and donor plasma from one year after transmission (see Appendix B), do not differentiate transmitted viruses. Nevertheless, in a recent study of subtype B variants donor plasma was unable to neutralize either transmitted or non-transmitted isolates above the limit of detection[7]. Donor antibody sensitivity perhaps reflects a surrogate marker of a different phenotype, such as mutational escape away from consensus, which in our study inversely correlated

with donor antibody sensitivity[6]. Interestingly, the original study reporting this phenotype with patient derived *env* pseudoviruses appears to show enhanced neutralization for variants closer to the most recent common ancestors by visual inspection[20]. In total, these findings suggest that specialization of viral sequences to a specific host adaptive immune environment reduces transmission potential to some degree. Variants may perhaps retain the characteristics necessary for more efficient transmission by establishing infection in a reservoir that is produced sporadically or with a slow turnover rate, which would also lead to their presentation as minor variants in the host. This scenario is consistent with the speculative hypothesis that mechanical stress on mucosal surfaces from sexual exposure leads to shedding of the epithelial layer, local microbial translocation, activation of infected tissue resident memory $CD4^+$ T cells through toll-like receptor binding of microbial products, and production of highly infectious freshly produced archived viruses.

Viral Fitness and Transmission

What characteristics are viruses losing when mutating to escape adaptive immunity? A common presumption was that viruses must be losing replicative fitness or infectivity[22]. However, when we compared replicative capacity and infectivity in transmission pairs, we did not observe a higher level of replicative capacity or infectivity for transmitted/founder viruses, or viruses closer to consensus. These observations are unlikely due to the details of our *in vitro* system, given that we find consistency in a number of assays impacted by replicative capacity and infectivity in different settings, such as growth in the presence of interferon, growth in in resting T cells and in cells ex

vivo from different uninfected individuals. Other studies have shown variability in the relative infectivity and replicative capacity of transmitted/founder variants between cohorts and between viral variants[1, 2, 4, 5, 7, 10, 23]. Studies of transmission pairs usually have not observed selection for more infectious transmitted/founder virus envelopes or plasma isolates [6, 7, 24], though contrasting evidence has been reported[10]. For subtype B and C Env pseudotyped[7, 24] and subtype B and subtype C full-length viruses[1, 6], a 50-100 fold range of infectivities was found, similar to that seen in the non-transmitted variants, suggesting that there is wide latitude of *in vitro* infectivity for viruses that establish infection. A similar 100-fold range of replicative capacities has been observed for full-length transmitted/founder [1, 2, 4, 6, 7, 25] and chimeric viruses[26, 27]. However, a recent study of 8 subtype B and C transmission pairs found that early isolates replicated somewhat (1.4-fold) higher than variants from matched donor partners[10]. We observed moderately lower replicative capacities for viruses closer to consensus, which supported previous work looking at Gag-MJ4 chimeras[27]. Together these observations raise the possibility that modestly lower replicative capacities confer an advantage during transmission. Whether these *in vitro* systems recapitulate aspects of the environment that viruses face in the genital mucosa *in vivo* is unclear. However, *in vivo* translation from replication assays has been observed, either from correlations to HIV^+ patient attributes like viral load, CD4 counts, viral DNA load and immune activation[26, 27], or from SIVsmE543-3 infected macaques, where *in vitro* susceptibility and replication (by tissue culture infectious dose) in macaque cells from pre-infection (which was used to normalize viral challenge) tightly correlated with *in vivo* peak viral loads in those same macaques[28].

Bottlenecks and Virus Evolution

Creating artificial bottlenecks *in vitro* through serial passage of individual plaques has been shown to reduce rather than enhance fitness of RNA viruses[29-31]. Does the HIV genetic bottleneck serve the same purpose *in vivo* to reduce fitness, given that consensus-like viruses tend to transmit and have lower replicative capacities? HIV[32], like other RNA viruses including vesicular stomatitis virus[33], is reduced in fitness through *in vitro* passage with bottlenecks. Loss in fitness through bottlenecks could be due to a loss in viral diversity which decreases the potential to subsequently adapt. Alternatively, loss in fitness during transmission could be accomplished in a manner consistent with the model of Muller's Ratchet[34]. Muller theorized that genetic drift adds mutations at a higher rate than are reverted in asexual virus progeny, and thus selecting any one variant of many may result in the random loss of the least mutated virus thus resetting the baseline for future mutations in a new host (a good discussion of which can be found in Bergstrom et al. [35]). However, Muller's ratchet does not apply when recombination, reversion and compensation occurs due to rigorous selection pressures[34], which is certainly the case for chronic HIV infection *in vivo.* Nevertheless, assuming the *in vivo* HIV transmission bottleneck reduces fitness by Muller's ratchet or a loss of diversity, what would be the implication? HIV may have evolved to make use of the bottleneck in order to reset its baseline level of diversity and mutational load, perhaps to limit its virulence once inside a somewhat defenseless a naïve host. If the total diversity was transmitted, more rapid evolution and virulence could in theory limit the chances of viral transmission by reducing the time which an infected host

is infectious and asymptomatic. Indeed, even transmitting a few variants rather than one is associated with faster disease progression in HIV-1 infection (see Introduction, *Impact of the TF Phenotype on Disease Progression*). An alternative way to increase transmissibility is to increase *in vivo* viral load, in part a function of viral replication fitness[27], which would subsequently increase the infectious dose in the transmission fluid. Different viral variants are likely to transmit by different strategies, and long term virus survival in a population will determine which modes of action would be dominant.

Other viruses besides HIV, including influenza virus[36, 37], Hepatitis C virus[38] and Venezuelan equine encephalitis virus[39] go through population bottlenecks *in vivo*, suggesting shared mechanisms not unique to HIV may be at play. For influenza virus, viral determinants of bottlenecks were found in the surface hemagglutinin glycoprotein when grown *in ovo*[37]. Interestingly, in a ferret model using a 1918-like avian influenza virus, minor variants transmitted through airborne exposure and lacked a replication advantage[40], which is similar to what was found in interspecies transmission of a different H7N9 avian influenza from chickens to ferrets where a stringent bottleneck led to a fitness cost and limited virus adaptation to ferrets[41]. However, when utilizing viruses that did not differ in amino acid sequence, a stochastic yet strong bottleneck was detected due to donor variant frequency, recipient-level outgrowth, and route of exposure (contact infection vs. aerosol) in guinea pigs and ferrets[37, 42]. Thus, as has been proposed for HIV in a recent review[43], bottlenecks can occur at multiple stages of infection, necessitating a confluence of multiple factors that include both chance and virus specific traits. Perhaps these bottlenecks serve as a broadly evolved mechanism to ensure that the most extreme mutations, including those

that result in very rapid disease, are selected against. Overall, higher replicative capacity, as a trait measured *in vitro*, and enhanced viral infectivity, do not appear to be the characteristics associated with transmission fitness.

HLA class I and CD4 downregulation

Given that there are likely a number of factors involved, there is still reason to believe viral properties play a role, potentially in HIV-1 accessory genes, some of which have been associated with initial interspecies infections between different primate species and into humans[44]. Genetic data comparing *gag*, *pol* and *nef* functional regions pointed to an area in *nef*, an HIV-1 accessory gene, that seemed to be the most selected against during transmission[19]. The signal indicated that perhaps a Nef trait beneficial for transmission was being altered by mutational escape away from consensus. We postulated that Nef-mediated downregulation of HLA-I was perhaps dispensable and/or deleterious in circumstances like those occurring during transmission and initial viral dissemination, where CD8 T cell responses have not yet formed, and NK responses are dominant. We also considered that perhaps another Nef function like CD4 downregulation, which is aided by Vpu, would be important to foster virus release. As we began to investigate, a study was published showing that Vpu was responsible for downregulating HLA-C, while Nef was responsible for HLA-A and HLA-B[45], further supporting the usefulness of characterizing viruses with co-evolved Nef and Vpu proteins, which have distinct and parallel functions. Moreover, combinations of certain HLA-A, HLA-B and HLA-C alleles with specific NK KIR alleles have been associated

with protection from HIV acquisition[46-50], and thus transmission may be impacted by HLA class I interactions with Nef and Vpu.

We found that if there is a bias during transmission for Nef and Vpu associated functions in these transmission pairs, the effect would have to be subtle, since in six transmission events, very few common signatures were observed between transmitted/founder variants and those from the transmitting partner. Nevertheless, we did find a small but statistically significant increase in the efficiency of transmitted/founder viruses to downregulate CD4 on the surface of infected cells, which was consistent with the genetic bias found during transmission in Nef. Yet, the increase in efficiency was small, making concrete suggestions about the implications of this difference difficult without a more detailed biochemical study of CD4 downregulation with greater numbers of transmission pairs. Follow-up studies will be undertaken to determine the impact of this bias, and whether it extends to more viruses from transmission pairs. For HLA class I downregulation, we were able to move the field forward by adding to growing evidence about the importance of HLA-C downregulation, while cataloging the diversity within and between quasispecies of infected individuals for authentic primary viruses. We found that HLA-A, HLA-B and HLA-C were downregulated by most primary viruses tested to varying degrees, and that HLA-C was a sensitive determinant of *in vitro* NK cell suppression—a finding recently corroborated by the laboratory that discovered Vpu-mediated HLA-C downregulation using a lab-adapted strain JR-CSF[51].

This raises the following question: if HLA-C downregulation increases infected cell susceptibility to NK recognition, why do many virus variants still downregulate

HLA-C? Consistent with the current hypothesis for HLA-A and HLA-B downregulation, perhaps it is the pressure from HLA-C mediated CD8 T cell responses. HIV adaptations to HLA-C:KIR interactions have been observed[52], and higher HLA-C expression, rather than downregulation, correlates with slower disease progression, increased CD8 T cell responses, and increased selection of HLA-C associated viral escape mutations[53]. In a situation where the rs9264942 single nucleotide polymorphism is present, which enhances HLA-C expression[54, 55], modest downregulation of HLA-C may not impact NK responses. Thus, biochemical individuality may determine selection pressures in a given T cell TCR:HLA or NK KIR:HLA environment, highlighting the pitfalls of broad conclusions about HLA-C downregulation, or any viral trait which is impacted differentially by a the specifics of a given immunogenetic environment.

Primary Viruses as Reagents

During the course of our investigations, we also created new tools and methods for assessing primary viruses *in vitro*. We created full-length virus clones in a novel way[5], along with viral stocks, which the field can use for various studies. Using primary strains instead of lab-adapted strains can help the field in that primary strains differ in a number of ways from the standard lab-adapted strains used ubiquitously in HIV virological research. First, they tend to grow slower than lab-adapted strains, like NL4-3 and MJ4, which were selected for *in vitro* growth. This makes them more difficult to work with *in vitro*, though optimization should be achievable in most assays utilizing HIV primary variants. Second, these viruses infect different CD4 T cell subsets. NL4-3 infects many naïve like cells along with effector memory cells, MJ4 infects mainly

central memory and effector cells. The primary viruses however infect effector memory cells much more efficiently than naïve-like or central memory cells, which could be tied to the reason why primary variants are difficult to grow in cell-lines like CEM cells, which by definition retain the capacity to proliferate, similar to central memory-like cells. Third, HLA-C downregulation was obscured because of differences between most lab-adapted strains and primary strains[45, 51]. Such differences in the behavior of primary viruses, led us at times by necessity to create growth assays, interferon assays, NK suppression assays and flow cytometry assays. These differences provide a good rationale for utilizing primary strains in as many model systems as possible. We have already provided these reagents to collaborators for studies in humanized mice, dendritic cell cultures, tissue culture explant models of foreskin and vaginal mucosa, and others.

Summary

Much has been learned over the past two decades about the details of transmission at the genital mucosa. It is clear that during, what in general is an inefficient process, one or a limited number of genetic variants are transmitted from the transmitting partner and that this genetic bottleneck involves both stochastic and selective aspects that define the virus that ultimately establishes systemic infection. While animal models, particularly the macaque-SIV model, have shed light on the earliest transmission events, they are still limited by the need to have high infection rates that do not reproduce the much less frequent transmission rates seen in heterosexual transmission—which represents the bulk of transmissions world-wide. The importance of rapid viral dissemination from the genital mucosa, versus a more localized amplification of virus prior to the establishment

of systemic infection, remains to be determined. Similarly, questions still remain about the phenotypic requirements for transmission, i.e. interferon resistance or infectivity, and whether the selective bias for consensus residues imposed on the transmitted/founder variant results in discrete traits, such as *in vivo* fitness, beyond the well-established CCR5 tropism. Understanding in greater depth these key features of transmission will be essential to the development of effective interventions for HIV prevention.

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Appendix

Introduction

The figures included in here in the Appendix are part of the foundation of this dissertation, yet in themselves do not make a complete story from which to derive a full manuscript. They are the basis of technical advances, new insights about HIV-1 primary viruses, and future scientific directions, and some of referenced in the discussion. Each figure has a discussion of the results prior to the figure, a figure and legend, and a brief description of the methods. The figures are broken up into three major sections.

The first section is on patient characteristics, which include linked recipient viral load, $CD4^+$ T cell counts and NK KIR ligand groups for each transmission pair. The section is made up of one figure which can be useful to reference when considering data from Chapter 2 and Chapter 3.

The second section contains experiments that further elucidate viral traits. This section includes an analysis of an outgrowth assay of viruses from the plasma (plasma isolates) and the stability of viral infectivity over time of transmitted/founder and non-transmitted infectious molecular clones. The susceptibility of viruses to donor plasma antibody neutralization with plasma from near the time of transmission was included in Chapter 2, while this section includes an assessment of neutralization with plasma from one year post-transmission to see how the antibody response has changed with respect to viruses for which viral traits like replicative capacity have been measured. This section also includes an assessment of viral properties related to Nef and Vpu, that being virus

growth in unstimulated T cells, and the downregulation of CD4 and CD62L on infected cells.

The third section includes controls and key technical experiments, including the attempt to infect with primary viruses a commonly used HIV susceptible cell line, the CEM lymphoblastoid $CD4^+$ T cell line. The dynamics of uninfected $CD4^+$ T cell phenotypic markers in culture are assessed following stimulation and included in this section, along with a comparison of virus containing supernatants quantitated by the TZM-bl susceptible cell line titer or by the radioactive reverse transcriptase assay, the latter of which was used to measure viral growth throughout the experiments in this dissertation. Finally, experiments relevant to HLA class I downregulation are described, including an analysis of Nef reading frame translation and HLA-A downregulation, a time course of *in vitro* HLA-A downregulation, and a ΔNef control for CD4 and HLA-A downregulation.

Appendix A: Patient Characteristics

Patient Characteristics

Since the viruses derived from transmission pairs assessed in this dissertation come from infected individuals for which viral loads, CD4 counts, and HLA haplotypes relevant to NK KIR binding were available, they are include here for reference.

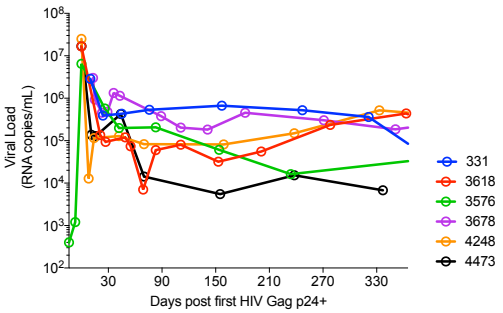
The average follow up for these individuals is 2.3 years and ranges from approximately 1-4 years, which is why only the first year can be shown for all six newly infected individuals focused on in this dissertation (Fig. 1A & 1B). The viral loads over time demonstrate that sequences amplified from the earliest time points were derived from near the viral load peak of all newly infected recipients (Fig. 1A). For one individual, 3576, the pre-peak time points were captured (green line in Fig. 1A). The linked-recipients under study had a range of viral load set-points (approximately $10^4 – 10^6$; Fig. 1A) and bounced between 200 and 800 CD4 T cells per microliter (Fig. 1B).

This was a diverse patient group in terms of NK KIR ligands for HLA-B and HLA-C since no two individuals in a pair when combined were jointly homozygous for any one HLA-B or HLA-C ligand (Fig. 1C). Pair 3678 was the only pair with HLA-B matching KIR types at both alleles, while pair 4248 was the only pair with completely mismatched HLA-B and HLA-C types (Fig. 1C). Interestingly, pair 4248 had the highest levels of HLA-A and HLA-B downregulation, and the lowest HLA-C downregulation levels, leading to the highest resistance in NK cell suppression (see Chapter 3 Fig. 2C, Fig. 4A, and Fig. 6). Of note, the transmitted/founder virus for pair 3618 had the highest

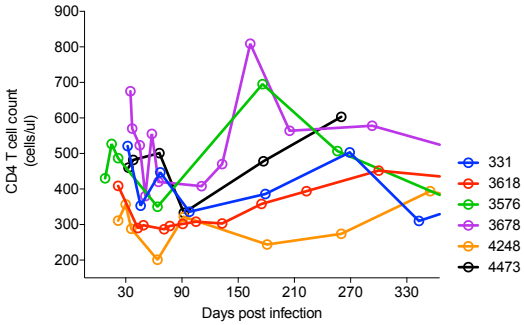
HLA-C downregulation level (see Chapter 3 Fig. 2C and Fig. 4A), and was mismatched for the HLA-C type (Fig. 1C).

The characteristics shown here turn the attention back to the situation as it stood *in vivo*. They are a helpful reference to questions that may come to mind while thinking about the data presented in this dissertation.

A Viral Load



B CD4 counts



C

HLA ligand groups for NK KIR

ID	HLA::B1	HLA::B2	HLA::C1	HLA::C2
Z331M	Bw6	Bw4-80I	C1	C2
Z331F	Bw6	Bw6	C1	C1
Z3618M	Bw6	Bw6	C1	C1
Z3618F	Bw6	Bw4-80I	C2	C2
Z3678M	Bw6	Bw4-80T	C2	C2
Z3678F	Bw6	Bw4-80T	C1	C1
Z4248M	Bw6	Bw6	C1	C1
Z4248F	Bw4-80T	Bw4-80I	C2	C2
Z4473M	Bw6	Bw4-80I	C2	C2
Z4473F	Bw6	Bw6	C1	C2
Z3576F	Bw6	Bw6	C2	C2
Z3576M	Bw6	Bw4-80I	C1	C2

Fig. 1. Viral load, CD4 counts, and HLA ligand groups for NK KIR binding of transmission pairs

(A) Viral loads and (B) CD4 counts for newly infected linked recipients from where the transmitted/founder viruses were derived for these studies. (C) Table of HLA ligand groups for NK KIR binding. Linked recipients were all denoted M for male, except for 3576 where the F for female is the recipient.

Method: The viral loads and CD4 counts were derived from the Hunter lab database and collated but not generated by the author of this dissertation. The NK KIR binding serotypes were derived from HLA haplotypes from the lab database, and then categorized based on the assigned allele.

Appendix B: Virus Traits

Introduction

A number of viral properties were examined in this section that were not included in a full manuscript because their relevance to a complete story are not well understood at this point. However, these observations are likely to be the basis for future studies examining viral traits, and provide a foundation from which to further investigate the characteristics of genome-length authentic primary viruses.

Plasma isolates

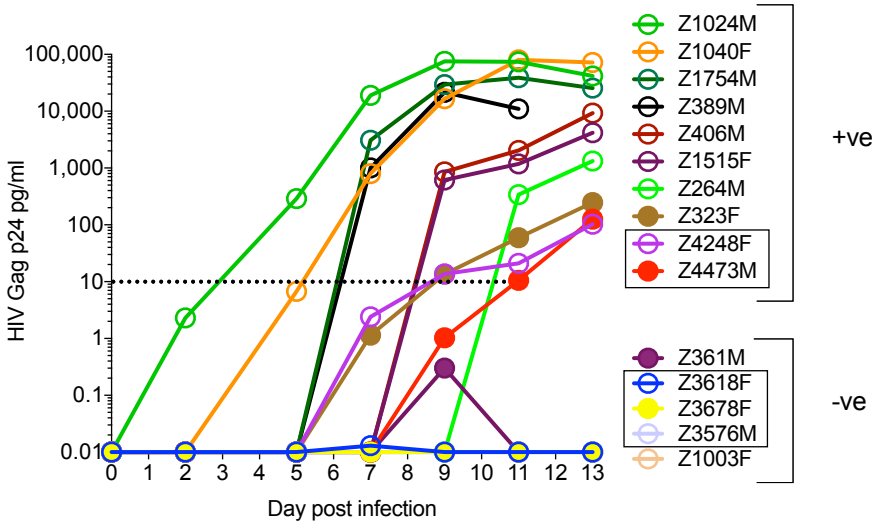
Most of the experiments shown in this dissertation were performed with viruses derived by amplification directly from plasma, and then subsequently cloned and regenerated *in vitro*. However, another method to generate viruses from infected individuals is by using their plasma to infect another individuals uninfected $CD4^+$ T cells (Fig. 2A). Initially, the goal of the outgrowth assays included here was to create viruses from not-yet transmitters and compare them to viruses from individuals that had already transmitted virus. Viral defects that could impact whether or not transmissions were occurring could become evident by examining these viruses and their outgrowth characteristics. The not-yet transmitters were matched for viral loads to the transmitters, and all of the not-yet transmitters produced HIV^+ outgrowth (Fig. 2A). Plasma viral load, and the amount of viral copies added to the culture, rather than transmission status, determined outgrowth (Fig. 2B and 2C).

The pace and success of virus outgrowth was driven primarily by the amount of virus added to the cells, mirroring findings that viral load is a major risk factor for HIV transmission[1]. Deep sequencing would be necessary to fully determine whether or not this model represents true bottlenecking of viral sequences or whether a dominant species well suited to conditions are growing out. Interestingly, the two plasma samples that gave positive cultures in this assay (Fig. 2A; donors for pairs 4248 and 4473) generally harbored low replicative capacity variants (see Chapter 2 Fig. 5B) compared to two of the samples which gave no positive cultures (3618, 3678). The absence of a positive culture could simply have to do with how the plasma was frozen down originally and thawed rather than viral properties, however, the positive cultures from 4248 and 4473 were relatively slow growing compared to other positive cultures (Fig. 2A), which mirrors the characteristics of these quasispecies generated directly from plasma amplification and cloning.

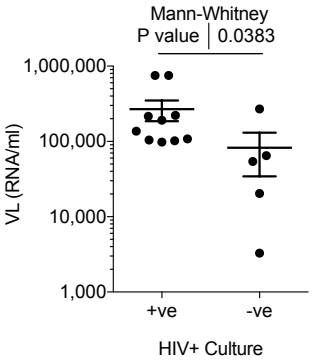
Virus isolation from plasma led to the outgrowth of viruses that clustered by sequence on one part of a phylogenetic tree which included sequences derived directly from plasma, suggesting there may have been a subgroup of fresh infectious particles, or viruses best suited to conditions, that initiated infection in the culture (Fig. 2D and 2E). This was despite the available diversity determined from direct amplification of virus from plasma ex vivo (Fig. 2D and 2E). Nonetheless, transmitted/founder viruses did not cluster with these isolates, suggesting their advantage during transmission is not reflected by outgrowth assays, consistent with findings presented in Chapter 2. Alternatively, the delay in the timing of the

donor plasma compared to the estimated date of infection may be impacting these results.

A



B



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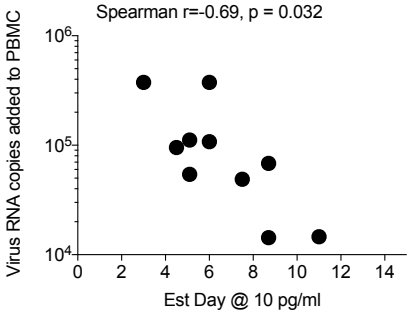
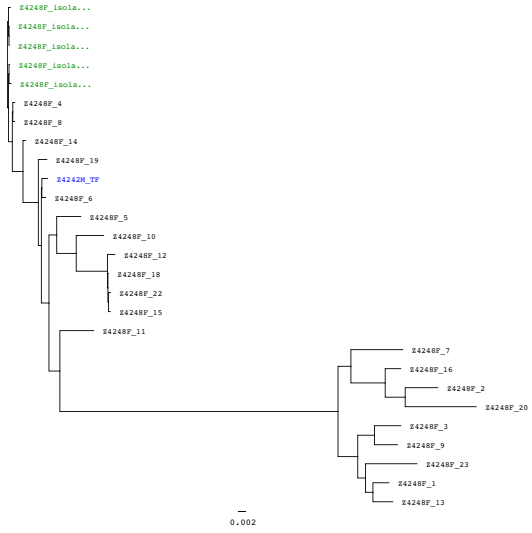


D Pair ID 4248



E Pair ID 4473

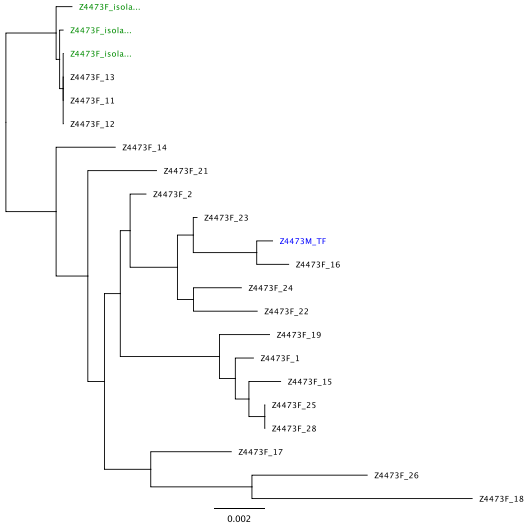


Fig. 2. Plasma isolation of viruses from patient plasma

(A) Infected patient plasma from 15 chronically infected individuals were used to infect peripheral blood mononuclear cells and HIV Gag p24 ELISA was performed on supernatants. 7 of the 15 of which were not-yet transmitters all in the group that grew virus with these ID numbers: 1769, 2153, 2753, 1645, 2028, 1839, 1996. Five individuals studied in depth in this dissertation were boxed. 10 out of a total of 15 plasma sample yielded positive cultures. (B) Viral loads of patient plasma impacted the success rate and (C) pace of HIV outgrowth. (D) Plasma isolates were sequenced from donor patient 4248 and (E) 4473, and put on a phylogenetic tree in green with sequences amplified directly *ex vivo* from patient plasma in black. The transmitted/founder sequence amplified from the recipient is in blue. Gag sequencing gave the same results (data not shown).

Method: Beads coated with anti-CD44 antibodies which aid infection were added to 500ul of patient plasma from the same time points where viruses were amplified from, and added to prestimulated (with CD3/CD28 antibodies) peripheral blood mononuclear cells. Beads and plasma were spun in a centrifuge per manufacturer instructions and then cultured for 13 days, and tested every 2-3 days for p24 positive supernatant. Sequences for *env* were derived from 1,922 nucleotides of gp120 variable loop 3 (V3) to gp41 with primers kindly given by the laboratory of Cynthia Derdeyn. Sequences were MAFFT aligned using Genious software, and FastTree was used to make the trees, which were rooted on isolate sequences for easy visualization closest neighbors. Gag sequencing gave the same results

Dynamics of viral infectivity

Viruses that are able to traverse the physical barriers of mucosal surfaces without losing infectivity may have an advantage during transmission due to the time that they are infectious. Therefore, we measured the stability of infectivity in culture media (which will likely differ from bodily fluids) over time using TZM-bl cells to test whether transmitted/founder viruses are more stable in infectivity. The initial concentration did not impact infectivity over time and significant differences between individual variants was apparent by differences in the slope of the line of decay on a semilog plot (Fig. 3A). Thus, we measured all variants in six transmission pairs. The results showed that stability of infectivity is not a trait differentiating transmitted/founder viruses from other viruses (Fig. 3B). Interestingly, some viruses lose a log of infectivity in approximately one day, while others lose that amount in two days, demonstrating a noteworthy variability in the stability of infectivity (Fig. 3B). Viruses from pair 3576, the only male to female transmission pair in the group, had significantly faster decay rates ($p<0.001$, Mann-Whitney) than viruses from the other pairs as a whole, suggesting a potential gender bias (Fig. 3B). More viruses from males would have to be assessed to further investigate this finding.

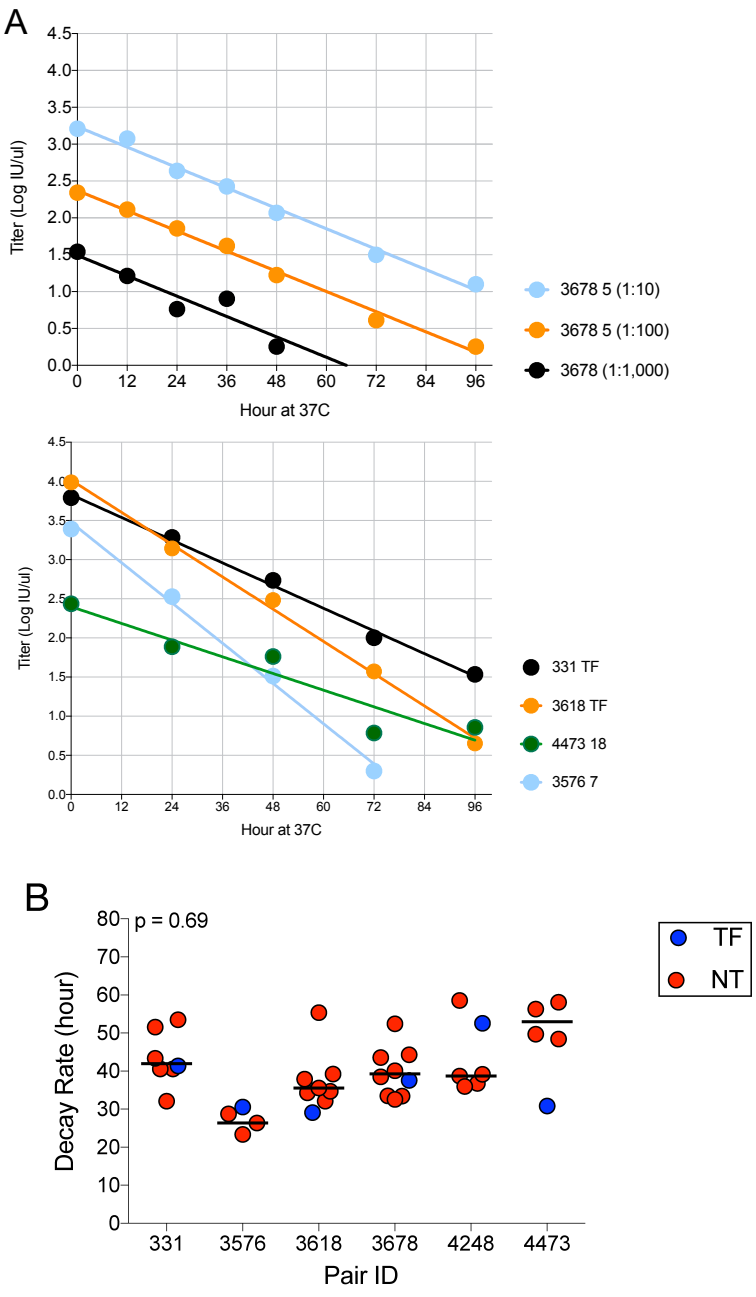


Fig. 3. Rate of viral titer decay over time for primary viruses from transmission pairs

(A) Dilutions of virus 3678 5 (top) were placed in culture at 37C, sampled every 12 hours, and titered by TZM-bl blue cell counts. This demonstrates that the same virus at

different concentrations from 10-1,000 fold diluted will produce the same rate of decay. This assay was then performed to assess viral stability of infectivity over time for other viruses (bottom) which differed in the stability of infectivity. (B) The summary of all the viruses tested from each transmission pair is shown. The decay rate on the y-axis represents the amount of hours it takes to lose one $\log_{10}$ of infectivity. Thus, the higher the decay rate in hours on the y-axis, the more stability of infectivity for that viral variant.

Method: Titers on TZM-bl cells were taken every 12 or 24 hours for 4 or 8 days and decay rates were measured as the reduction in $\log_{10}$ titer over time by linear regression on a semilog plot. The analysis was performed on Prism software. The slope of the line produces the rate per hour that one $\log_{10}$ infectious unit is lost per microliter. Thus for 331 TF in blue, which is also shown in the bottom of A in black, it took 40 hours to lose one log of infectivity by titer.

Virus susceptibility to donor plasma antibody neutralization

Transmitted/founder viruses tend to be more sensitive to donor antibodies from near the time of transmission[2], as confirmed in Chapter 2. Whether transmitted/founder viruses remain more neutralization sensitive at any time point after transmission in the donor, and whether viral characteristics like replicative capacity impact subsequent susceptibility is unclear. We therefore measured the donor plasma from one year after transmission to test the change of neutralization susceptibility of well characterized viruses, some of which went on to become transmitted/founder viruses. The results showed that transmitted/founder viruses did not remain neutralization sensitive one year after transmission to donor plasma antibodies, compared to non-transmitted viruses (Fig. 4A). This is consistent with other studies that show no impact of pooled plasma[2], indicating transmitted/founder viruses are not generally sensitive to antibody neutralization. As expected, neutralization of viruses near transmission was more potent with plasma from one year after that time point compared to contemporaneous plasma (Fig. 4B).

Higher replicative capacity variants tended to be harder to neutralize by donor plasma from both time points (Fig. 4C; 1 year post transmission, Spearman, $p=0.01$, $r=-0.46$; Near transmission $p=0.02$, $r=-0.48$). Higher replicative capacity also associated with increased potential N-linked glycosylation sites in these viruses (Spearman $r=0.51$, $p=0.003$; not shown), which could link these two observations to antibody escape. Indeed, increased neutralization of viruses with donor plasma

from one year after transmission correlates with less potential N-linked glycosylation sites in these viruses (Spearman r= -.46, p=0.01, not shown).

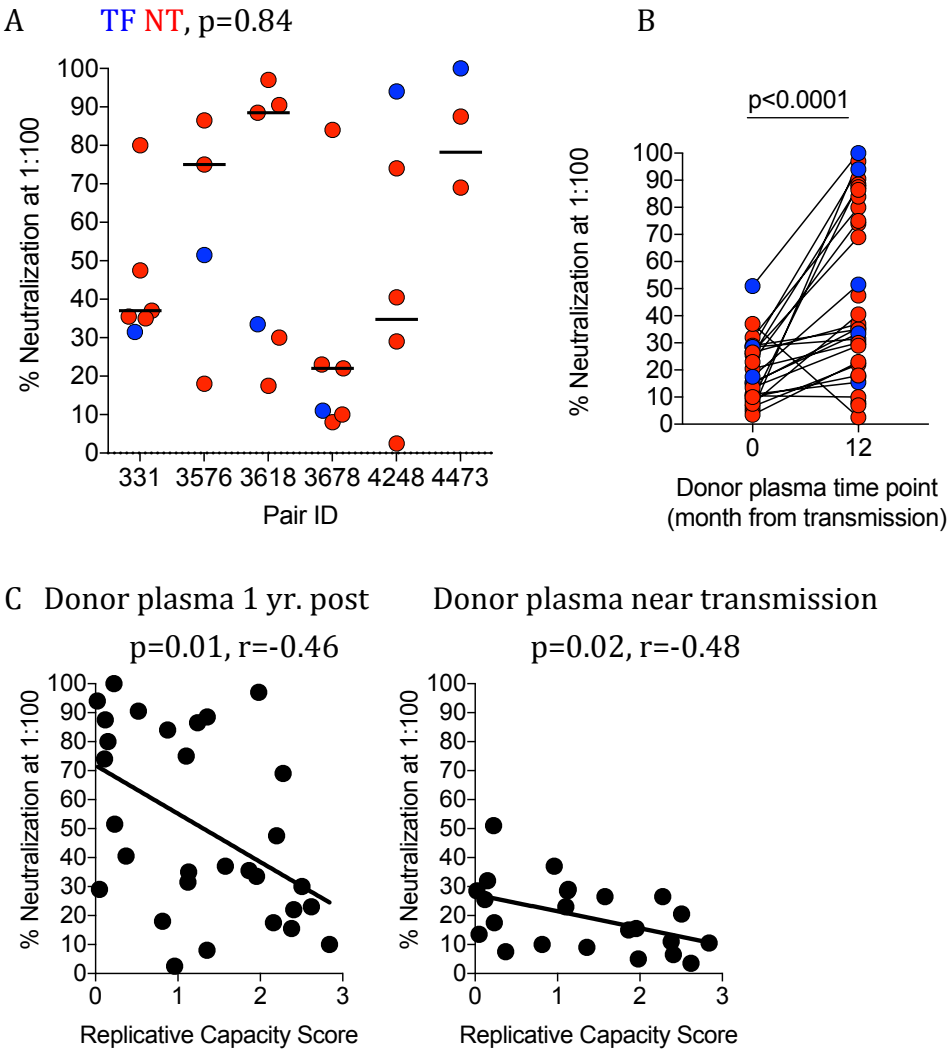


Fig. 4. Donor plasma neutralization of transmitted/founder and non-transmitted viruses one year after transmission

(A) Donor plasma from one year post transmission tested against transmitted/founder and non-transmitted donor viruses derived from near the time of transmission (TF vs. NT, p=0.84, Wilcoxon). Neutralization at the plasma dilution of 1:100 is shown. (B) Donor plasma from near the time of transmission compared to donor plasma one year later against the same viruses in A. (C) Correlation of

replicative capacity against donor plasma neutralization of donor viruses and transmitted/founder viruses at 1 in 100 dilution.

Method: See Chapter 2 methods section on plasma antibody neutralization. These experiments shown here were done with plasma samples from approximately one year post-infection

Replication in unstimulated primary T cells

Induction of virus replication from quiescent primary CD4 T cells is inhibited when Nef is deleted[3, 4]. To determine the relationship between infection of quiescent cells and other viral characteristics, we examined a subset of 16 primary variants from two transmission pairs, along with 4 Gag-MJ4 chimeras, for the ability to replicate following infection of unstimulated peripheral blood mononuclear cells (PBMC).

Infections of resting PBMC, which were then stimulated 3 days after infection, were compared to infections of stimulated cells infected in parallel to compare differences in replication within the same experiment. For each variant, replication in stimulated cells outpaced growth in quiescent PBMC as expected (Fig. 5A and 5B). Replication by primary strains in stimulated cells reached a median of 7.6 $\log_{10}$ DLU by area under the curve (AUC) over a 10 day culture, while over the same time period, unstimulated PBMC infections exhibited areas 2.3 log10 lower ($p<0.0001$; Fig. 5C). In general, those variants that replicated efficiently in stimulated PBMC, were able to generate a spreading, albeit delayed, infection in unstimulated cells. Similarly, those variants that replicated less well were frequently undetectable during the 10 day culture (Fig. 5B). Pair 3618 variants, unlike 331 variants, were all detectable by day 10, consistent with modestly higher quasispecies replication in the stimulated cell controls ($6.3x10^7$ vs. $1.6x10^7$ median, $p=0.01$). To further assess the impact of replicative capacity on resting cell infections, Gag-MJ4 chimeric viruses with the same Nef and Vpu from MJ4, yet different replicative capacities, were utilized. The replication of the Gag-MJ4 chimeras demonstrated a link to replicative capacity, since replication in resting cells ranked in the order of their RC scores (GC 2.6=66%; GC 2.1=60%; GC 1.0=15%),

despite having identical Nef proteins. Finally, replication of primary variants from resting cells correlated with RC ($p=0.01$, $r=0.70$; Fig. 5D), and particle infectivity ($r=0.69$, p0.004), but not with other Nef characteristics such as downregulation of CD4 ($p=0.68$), CD62L ($p=0.45$), HLA-A ($p=0.12$), HLA-B ($p=0.09$), or HLA-C ($p=0.65$), indicating that in this experimental system, replication from stimulated and unstimulated cells alike is primarily driven by characteristics generally associated with replicative capacity more so than a specific Nef trait.

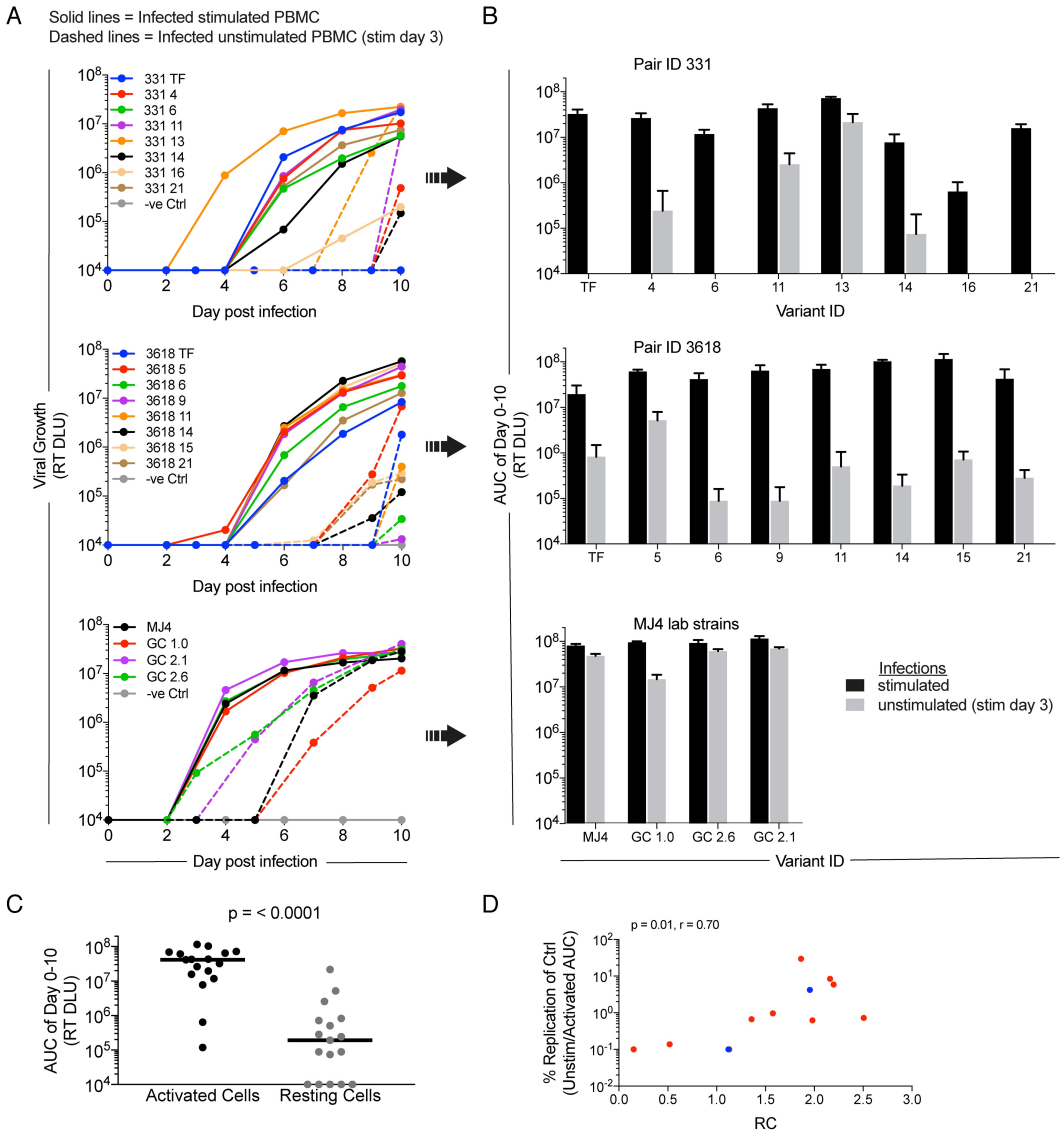


Fig. 5. Induction of replication from resting cells

Resting and activated PBMC were infected by a subset of lab strains and primary IMC from transmission pairs 331 and 3618. Stimulation of PBMC by PHA before and after infection was done to test variation in Nef-associated viral induction from infected resting cells which then become activated. (A) Replication curves of stimulated cells (solid lines) or unstimulated cells subsequently stimulated on day 3 post-infection (dashed lines)

broken up by transmission pair or laboratory strain, with arrows pointing to the (B) area under the curve calculated from the mean of triplicate infections (grey = resting cell infection, black = prestimulated cell infection). Variant ID is shown on the x-axis. (C) The overall reduction in viral growth over 10 days when inducing virus from resting cells using a Mann-Whitney test—zeros set at 10,000 RT DLU as limit of detection, and (D) a Spearman correlation analysis of RC and percent of replication in unstimulated compared to stimulated cells colored for TF (blue) and NT (red). TF, transmitted/founder; NT, non-transmitted donor variant; RT DLU, reverse transcriptase associated digital light.

Method: Infections were done similarly as to what was described for other assays, except for some notable differences explained here. Cells were thawed and either stimulated with PHA for 3 days as detailed above, or left unstimulated. Unstimulated cells, and PHA-stimulated cells 3 days later, were infected by each variant at a multiplicity of infection of 0.1 by spinoculation at 2,200rpm for 2 hours in 500ul total with $3x10^6$ cells in 15-ml conicals. Cells were then divided into 1 million cells per well in triplicate wells and put in 12-well plates in 2ml, and virus was sampled every 2 days for 10-13 days. Unstimulated cells were stimulated with PHA on day 3 post infection.

CD4 and CD62L downregulation

Phenotypic diversity with respect to CD4 and CD62L downregulation within quasispecies and across transmission pairs has not been examined for full-length IMC with matched Nef and Vpu sequences. We measured CD4 and CD62L downregulation on cells infected by 40 IMC from six transmission pairs, two laboratory strains, and 9 Gag-MJ4 chimeras[5, 6] using flow cytometry.

CD4 was nearly completely removed from the surface of infected cells once Gag expression peaked (Fig. 6A). Variation within a quasispecies significantly overlapped with between quasispecies variation (ANOVA p=0.15; Fig. 6B). However, TF viruses in all 6 pairs were more efficient at downregulation than the median of the matched donor's quasispecies (p=0.031; Fig. 6B). The extent of CD4 downregulation was not linked to the genetic bias towards consensus residues focused in the active site of Nef in TF variants in previous studies on this cohort[7, 8], with no correlation with the distance to the subtype C consensus (p=0.42). Additionally, CD4 downregulation did not correlate with potential downstream effects like replicative capacity (p=0.49) or particle infectivity (p=0.82) measured in Chapter 2[7].

CD62L downregulation on infected cells was also well conserved but not as dramatic as CD4 downregulation (Fig. 6C); nevertheless, a majority of CD4- infected T cells downregulated CD62L (69-84% range of CD62L- % of Gag+ cells; mean 76%; Fig. 6D). CD62L downregulation did not correlate with CD4 downregulation (p=0.72). Individual variants displayed relatively little variation in CD62L downregulation on infected cells, and the quasispecies differences fell within the variation of the Gag-MJ4 chimeras; though pairs 3618 and 3678 had significantly increased CD62L

downregulation compared to pair 331 (Fig. 6D). TF variants were not significantly higher or lower than the median of the quasispecies within each transmission pair (Fig. 6D; p=0.84). Thus, CD62L downregulation capacity is a relatively conserved function amongst primary variants and is conserved across transmission.

Most prior studies investigating Nef and Vpu properties, including those on CD4 downregulation capacity, have used vectors expressing Nef and/or Vpu in cell lines amenable to transfection, while a few others have cloned individual genes into a common backbone—most often NL4-3. We used primary cells with replication-competent viruses from transmission pairs and detected a small but statistically significant increase in the efficiency of TF viruses to mediate CD4 downregulation (Fig. 6A). Since CD4 downregulation could facilitate virus release[9, 10] and limit the impact of ADCC on infected cells[11], conservation of this function could be important for transmission related selection pressures found both in the donor and recipient, particularly in the context of mucosal tissues. Most previous studies have observed no signatures of Nef or Vpu mediated CD4 downregulation unique to acute infection[12, 13]. A study of subtype B and C viruses reported similar levels of CD4 downregulation for six TF viruses compared to six chronic viruses after transfecting CD4 expression vectors into 293T cells with either Nef or Vpu[12]. A comparable study investigating subtype C Nef from 56 individuals observed no consistent CD4 downregulation pattern in acute/early versus matched viruses from one year post infection in a transfected CEM cell line[14]. Likewise, a study of CD4 surface expression on transfected SupT1 lymphoblasts with patient derived Nef proteins, showed relative preservation of CD4 downregulation capacity in six MSM subtype B transmission events; although unusually weak CD4

downregulating Nef were found in two of the five donor quasispecies and not in recipients[13]. Interestingly, enhanced CD4 downregulation for TF variants was observed in a study examining Vpu, rather than Nef, from 10 subtype B TF viruses, which were compared to the NL4-3 Vpu in transfected Hela cells, although the differences were minor when comparing TF to chronic viruses[15]. It should be noted, however, that both Nef and Vpu play complementary roles in downregulating CD4 surface expression, and in the current study we were assaying the cumulative impact of both of these accessory gene products.

Variation in the ability to downregulate CD62L with different primary variants including transmitted/founder viruses has not been examined. Both non-transmitted and transmitted/founder variants effectively downregulated CD62L, and no consistent bias was observed for transmitted/founder variants compared to their matched donor quasispecies (Fig 6B). Given the role of this homing molecule in retaining $CD4^+$ T cells in the lymph node, efficient downregulation may facilitate circulation and systemic spread of virus both during acute and chronic infection.

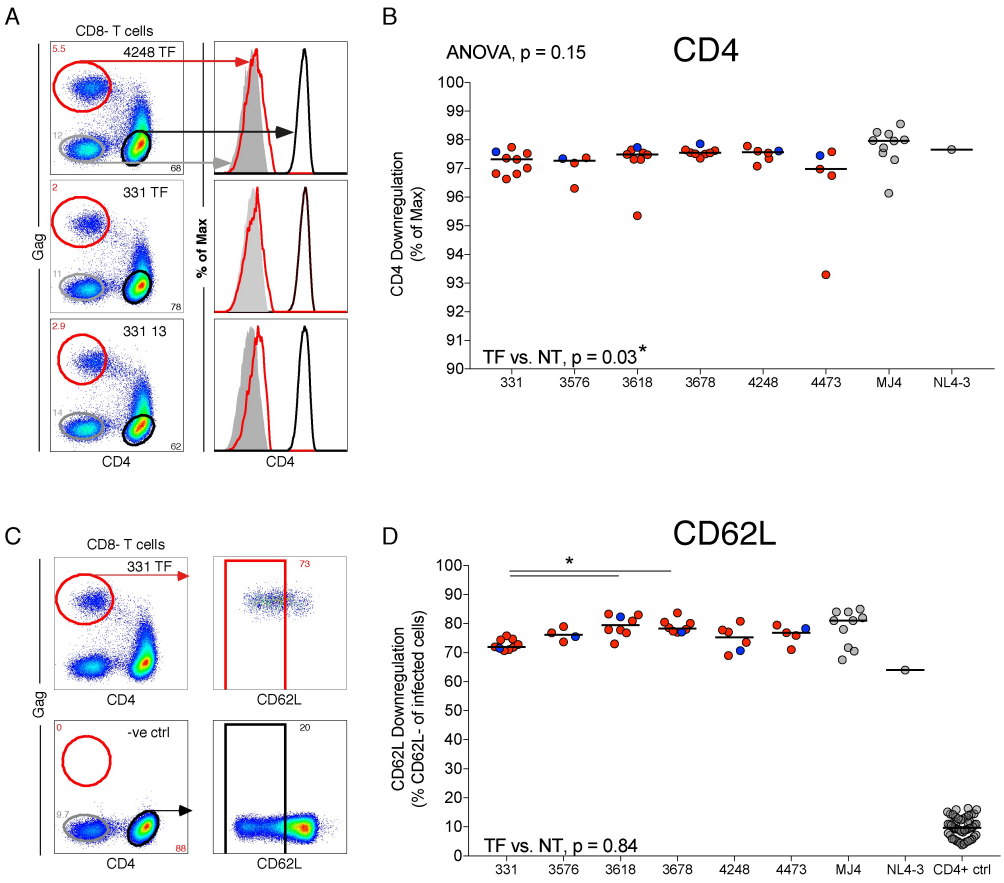


Fig. 6. CD4 and CD62L expression on infected cells

Infectious molecular clones (IMC) from six transmission pairs along with two lab strains (MJ4=subtype C; NL4-3=subtype B) were assayed for the ability to downregulate CD4 and CD62L by flow cytometry from pooled triplicates averaged from 2-5 experiments. (A) Example staining of CD4 from two TF and one non-transmitted (NT) donor variant. The left column shows CD3+CD8- T cells, with upstream gates as in Chapter 3 Fig. 2A, while the right column shows a histogram with Gag-CD4+ cells as positive controls (black), and CD4-Gag- cells as negative controls (grey) for each variant shown in that row. (B) Summary data of all tested variants. The y-axis shows the max possible

downregulation set at 100% as defined by the MFI of the CD4-Gag- population for each well to normalize staining variability (same as grey in panel A). On the x-axis is the variant or pair ID name, and CD4+ ctrl are negative controls (CD3+CD8-CD4+Gag-). (C) Example staining of CD62L from 331 TF. The left column shows CD3+CD8- T cells, and the right column shows CD62L gating of Gag+ (red) and Gag-CD4+ (black) cells. (D) Summary data of all variants averaged per transmission pair (x-axis). On the y-axis is the percent of CD62L negative cells that are Gag+CD4- as in the top right box in panel C. Bars and asterisks represent $p<0.05$ per quasispecies including the TF using an ANOVA and then Tukey's multiple comparison test, while the p value on the graph is a Wilcoxon paired analysis of TF vs. the median of NT variants over all the pairs for panels B and C. TF, transmitted/founder; NT, non-transmitted donor variant

Method: See staining and infection details in Chapter 3 as was done for HLA class I expression. Briefly, peripheral blood mononuclear cells were infected with each HIV variant separately in replicates and in multiple experiments and stained at day 7 with antibodies to CD3, CD4, CD8, CD62L. The rest of the relevant details are in the figure legend.

Appendix C: Technical Findings

Introduction

A number of observations in laboratory work do not get published because they do not fit well into a narrative. However, many of these experiments are crucial to understanding technical underpinnings of experimental decisions. These experimental details also provide the basis for interpretations of complex data that relies on an understanding of the experimental systems under study. This section includes data that is very relevant to the understanding of the experimental systems used throughout this dissertation, and therefore the understandings of the limits and boundaries of the findings herein.

Infection of CEM cells with full-length viruses

Gag-MJ4 chimeras and MJ4 infect CEM cells well, however, primary full length viruses derived from the same people where the Gag-MJ4 chimeras came from cannot infect CEM cells after 10 days of culture (Fig. 7) (when leaving cultures longer in other experiments, full-length viruses were found to grow by day 14 though they were mutated). Primary cells (PBMC) used for most experiments in this dissertation were readily infectable when using these and other full-length primary viruses

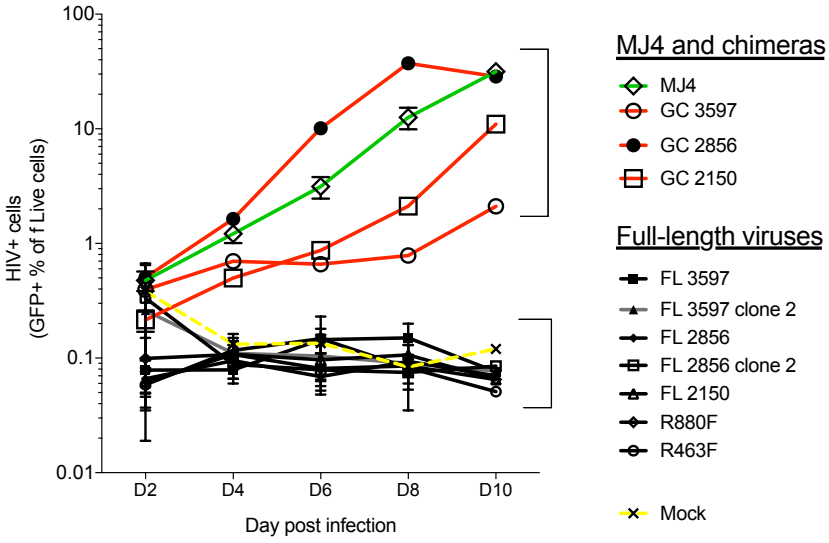


Fig. 7. Infection of CEM cell line with MJ4, Gag-MJ4 chimeras, and full-length primary viruses from four individuals

Growth of full-length primary viruses, MJ4 (lab-adapted subtype C strain), and Gag-MJ4 chimeras in CEM cells in a 10 day culture.

Method: CEM cells were infected with MJ4, Gag-MJ4 chimeric viruses, and full-length primary viruses at an multiplicity of infection of 0.05 based on TZM-bl titers. Green fluorescent protein (GFP) is produced upon HIV infection of these cells and is measured from days 2-10.

Dynamics of uninfected $CD4^+$ T cell phenotype during culture

To examine HIV relevant phenotypic dynamics of CD4 T cells before, during and after stimulation with two different stimulants that might impact HIV growth, we measured surface expression relevant markers with flow cytometry. Since memory cells are the primary cells infected by primary HIV strains , especially effector memory cells[16], the increase of memory cells shown here by day 6 can impact HIV replication (Fig. 8A). The increase of CCR5+ CD4 T cells in culture under different conditions may also impact the number of target cells in culture (Fig. 8B). Thus, it is important to think of HIV replication as a dynamic balance between changing amounts of both target cells and virus which infects and kills those target cells.

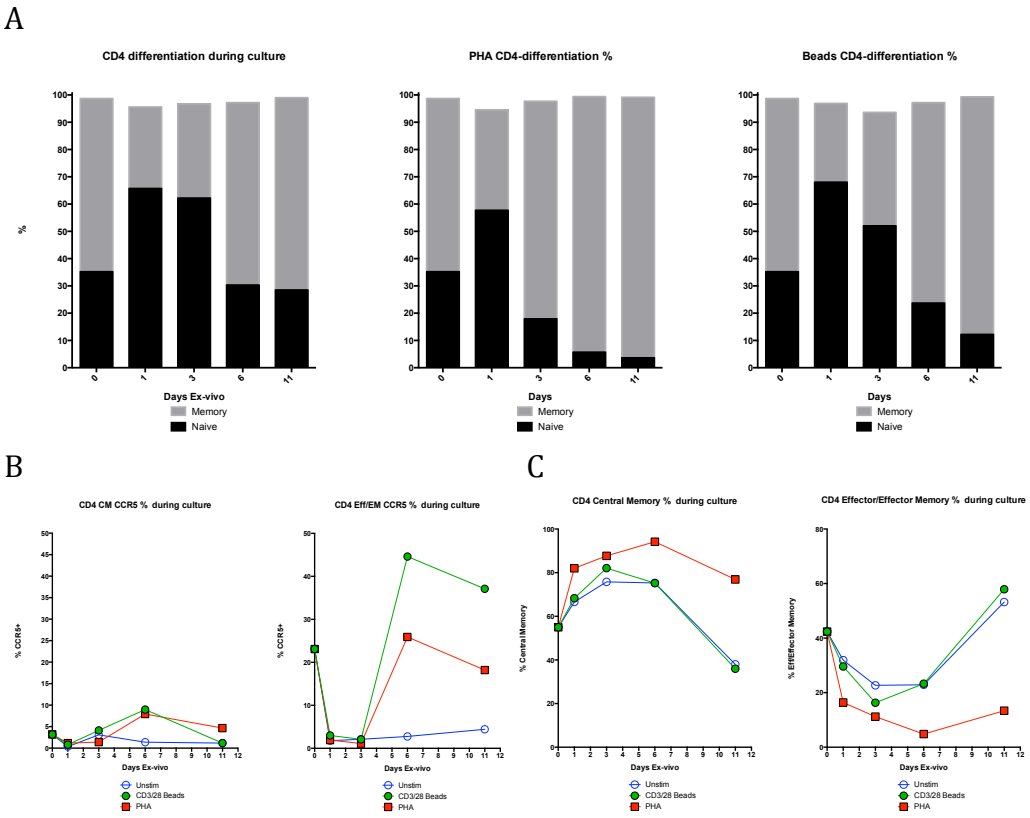


Fig. 8. HIV relevant phenotypic markers on CD4 T cells during culture following stimulation

(A) Percent of Naïve-like CD4+CD27+CD45RO-CCR7+T cells (black) compared to memory-like CD4+CD45RO+ and CD4+CD27-CD45RO- T cells (grey) in culture over time when unstimulated (left), stimulated with PHA (center), or stimulated with anti-CD3/CD28 beads (right). (B) Within the memory-like cells, central memory which express CCR7, and effector and effector memory-like cells over time in culture with different stimulations, and (C) the expression of CCR5 in those subsets.

Method: PBMC were stimulated for three days by CD3/CD28 beads, phytohemagglutinin (PHA) or left unstimulated for 11 days . Cells were stained ex

vivo, and at day 1, 3, 6, 11 with the following antibodies: Aqua Live/dead, CD3, CD4, CD8, CCR5, PD1, CCR7, CD27 and CD45RO. PHA was added at 3ug/ml, and CD3/CD28 beads were added at 1 bead per cell. Viability was 95% ex vivo before culture and cells were cultured at 1 million cells/ml with 330ul per well in 48 well plates.

Measuring the amount of virus present in the supernatant

We utilized RT activity in most experiments to test for virus growth in this dissertation. These data show that the titer from a susceptible cell line gives the same result in amount of virus present (Fig. 9), especially when taking the area under the curve of both measurements (Fig. 9, right). This finding adds to the confidence that differences in RT activity were unlikely to change the results shown in this dissertation.

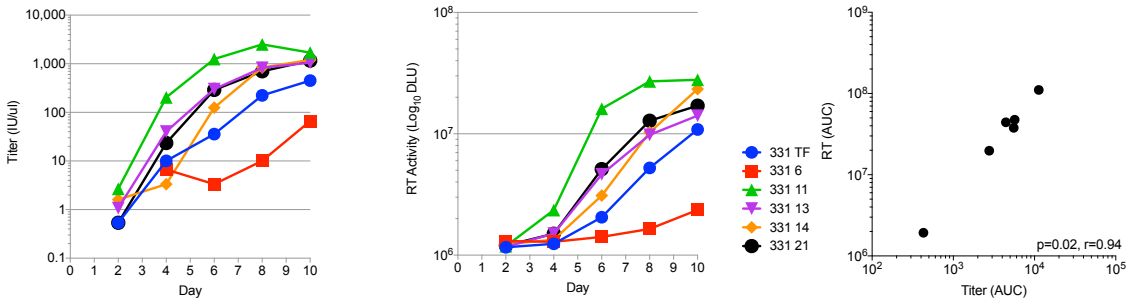


Fig. 9. Reverse transcriptase assay versus TZM-bl titer to measure virus in supernatant

(A) TZM-bl titer (left) over the course of an HIV growth assay compared to the same supernatants tested for RT activity with the radioactive reverse transcriptase assay (right). (B) Correlation between area under the curve values generated from TZM-bl titer or RT activity.

Method: Titers on TZM-bl susceptible cell line counted by blue cell counts, and RT activity in digital light units (DLU) performed as in chapter 2 of this dissertation on supernatants generated from a low multiplicity of infection of peripheral blood mononuclear cells with the indicated viruses from pair 331. Supernatants measured every 2 days for 10 days.

HLA-A downregulation short time course

We measured HLA downregulation for primary viruses derived from transmission pairs in Chapter 3. To determine the best time and cell population to gate, we performed a short time course to see if there were dynamic changes in HLA downregulation. This time course demonstrates that HLA downregulation is not immediate upon infection, and can change in a population of cells over time (Fig. 10). Because of this, kinetics of downregulation will vary between viruses with different replicative capacities and growth kinetics. Thus, it is important to know the growth properties of viruses tested for HLA downregulation to take that into account when designing downregulation assays in primary cells. This virus downregulated 40% of infected cells on average by day 7, as seen in Chapter 3 Figure 2, though the conditions shown here are not identical.

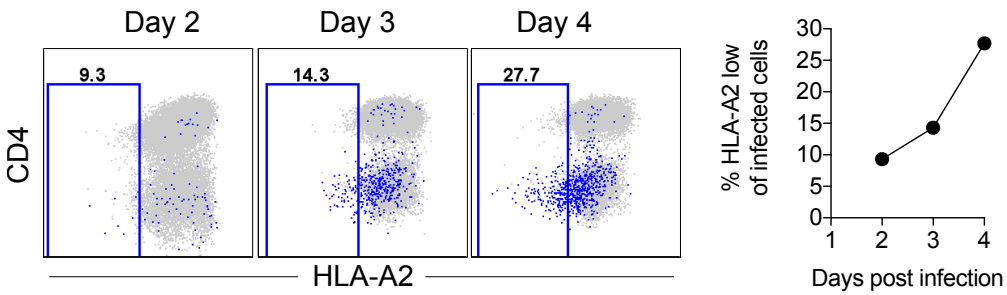


Fig. 10. HLA downregulation short time course

Shown is a flow cytometry plot of infected cells in blue overlaid onto uninfected CD8 negative T cells in grey. Days 2-4 of an infected culture are shown and the percent of infected cells in the blue HLA low gate were plotted (right).

Method: HIV-1 primary viral variant 331 TF (transmitted/founder virus) was used to infect peripheral blood mononuclear cells at a multiplicity of infection equal to 1. Cells were then stained at day 2, 3 and 4 with Aqua live/dead and antibodies to CD3, CD4, CD8, HLA-A2, HIV Gag-p24.

ΔNef downregulation of CD4 and HLA-A

While testing whether or not HLA downregulation could be detected in primary cells , a ΔNef virus was used a control. These experiments confirm that Nef is the protein responsible for the phenomenon of CD4 and HLA class I downregulation in our system, and that our system is able to detect HLA class I and CD4 downregulation (Fig. 11).

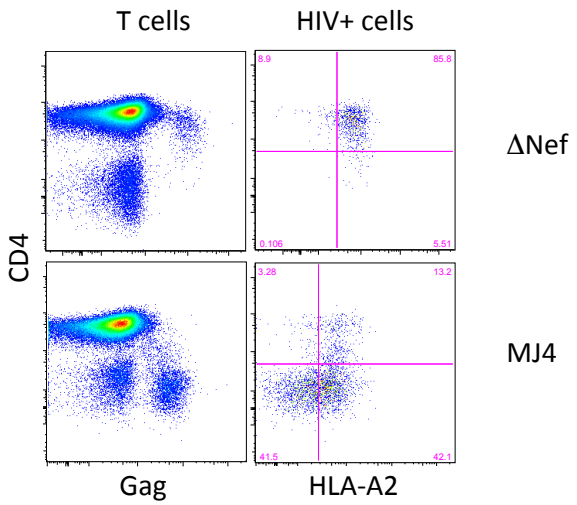


Fig. 11. Nef deleted virus does not downregulate CD4 or HLA class I

A flow plot of a Nef deleted virus tested for the ability to downregulate CD4 and HLA-A2 from the surface of infected Gag+ cells (top), along with a lab-adapted virus that has a functional Nef protein (bottom). The first column contains all T cells, while the second column is within infected cells only.

Method: An NL4-3 backbone where Nef was deleted and a subtype C primary virus envelope was added, along with a subtype C lab-adapted strain MJ4, were used to infect cells at a high multiplicity of infection. Cells were then stained with Aqua live/dead and antibodies to CD3, CD4, HLA-A2 on the cell surface and HIV Gag-p24 intracellularly.

Analysis of Nef reading frame and HLA-A downregulation

The expression of Nef could be impacted by the reading frame in which Nef is translated, thus leading to differences in the ability to downregulate surface proteins like HLA-A. However, these data show that the reading frame does not appear to impact HLA-A downregulation (Fig. 12).

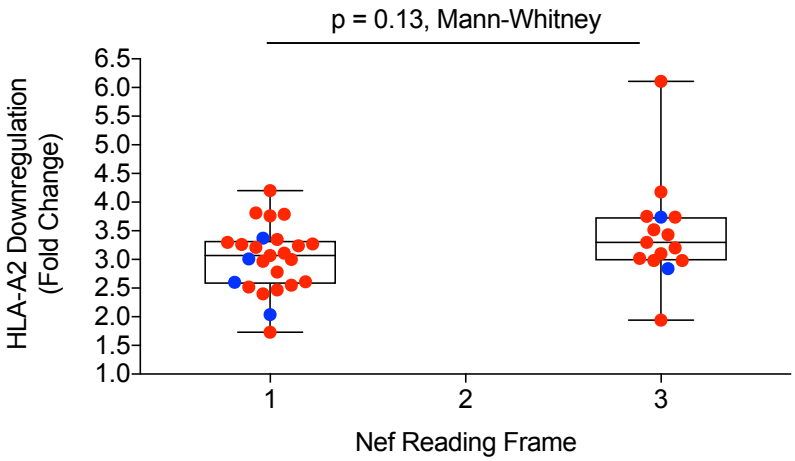


Fig. 12. Nef reading frame analysis and its impact on HLA-A2 downregulation

Variants with translated Nef reading frames at 3 do not show a statistical defect in HLA class I downregulation, represented here by HLA-A2 fold-downregulation.

Method: Genetic analysis for nucleotide translations were performed on Geneious software for translations. Statistic done by non-parametric Mann-Whitney test.

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