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# Determinants Of Hiv-1 Transmission Fitness

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**Abstract**
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HIV-1 is predominantly transmitted by mucosal routes and almost 80 percent of new infections are initiated by a single variant. The elucidation of the biological properties of transmitted viruses which distinguish them from non-transmitted variants are critical for the development of therapeutic interventions. To identify such properties, we characterized the biology of 300 limiting dilution-derived virus isolates from the plasma and genital secretions of eight HIV-1 donor and recipient transmission pairs representing the most prevalent subtypes (B and C). Recipient viruses were more infectious per viral particle as determined on a reporter cell line, replicated to higher titers and were released more efficiently from infected primary CD4+ T cells than the corresponding donor isolates. Recipient viruses were more resistant to the inhibitory effects of IFN-α2 and IFN-β evidenced as higher half-maximal inhibitory concentrations and higher replication at the maximal doses of IFN-α2 and IFN-β than corresponding donor isolates. Interestingly, pretreatment of CD4+ T cells with IFN-β, but not IFN-α2 selected donor plasma isolates that exhibited phenotypes similar to transmitted viruses. This suggests that transmitted variants are distinct and that the selective pressure imposed by type I interferons may in part be responsible for the bottleneck associated with mucosal transmission. We next wanted to assess the role of the interferon stimulated gene, tetherin in the antiviral state established by type I IFNs. Thus, we introduced mutations into the vpu gene of various HIV-1 constructs to specifically disrupt their Vpu-mediated tetherin antagonism, and determined the effect on replication and release from infected cells in the presence and absence of IFN-α2. Mutations at key residues in Vpu reduced the viral particle production and release from infected primary CD4+ T cells and this was particularly evident in IFN-α2-treated cells. Interestingly, transmitted HIV-1 variants were released to higher levels from infected cells than chronic control viruses, even in the absence of Vpu. Thus, the counteraction of tetherin resulting in efficient particle release is an important determinant of the interferon resistance of mucosally transmitted HIV-1.

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# DETERMINANTS OF HIV-1 TRANSMISSION FITNESS

Shilpa S. Iyer

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## ABSTRACT

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HIV-1 is predominantly transmitted by mucosal routes and almost 80 percent of new infections are initiated by a single variant. The elucidation of the biological properties of transmitted viruses which distinguish them from non-transmitted variants are critical for the development of therapeutic interventions. To identify such properties, we characterized the biology of 300 limiting dilution-derived virus isolates from the plasma and genital secretions of eight HIV-1 donor and recipient transmission pairs representing the most prevalent subtypes (B and C). Recipient viruses were more infectious per viral particle as determined on a reporter cell line, replicated to higher titers and were released more efficiently from infected primary CD4+ T cells than the corresponding donor isolates. Recipient viruses were more resistant to the inhibitory effects of IFN-α2 and IFN-β evidenced as higher half-maximal inhibitory concentrations and higher replication at the maximal doses of IFN-α2 and IFN-β than corresponding donor isolates. Interestingly, pretreatment of CD4+ T cells with IFN-β, but not IFN-α2 selected donor plasma isolates that exhibited phenotypes similar to transmitted viruses. This suggests that transmitted variants are distinct and that the selective pressure imposed by type I interferons may in part be responsible for the bottleneck associated with mucosal transmission. We next wanted to assess the role of the interferon stimulated gene, tetherin in the antiviral state established by type I IFNs. Thus, we introduced mutations

into the vpu gene of various HIV-1 constructs to specifically disrupt their Vpu-mediated tetherin antagonism, and determined the effect on replication and release from infected cells in the presence and absence of IFN-α2. Mutations at key residues in Vpu reduced the viral particle production and release from infected primary CD4+ T cells and this was particularly evident in IFN-α2-treated cells. Interestingly, transmitted HIV-1 variants were released to higher levels from infected cells than chronic control viruses, even in the absence of Vpu. Thus, the counteraction of tetherin resulting in efficient particle release is an important determinant of the interferon resistance of mucosally transmitted HIV-1.

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# CHAPTER 1

# INTRODUCTION

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## Section 1.1 – Mucosal transmission of HIV-1 and the associated genetic bottleneck

### HIV-1 Epidemiology and Transmission Routes

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus and the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). Phylogenetic analyses estimate that HIV-1 entered the human population in the early 20$^{th}$ century (1). Since its introduction into the human population HIV-1 has infected over 70 million people (2) and killed over 35 million. Despite widespread efforts to reduce transmission, 2 million people become infected by HIV-1 annually (2). HIV-1 is comprised of four groups – M (main), N (non-M, non-O), O (outlier) and P (3). These groups are the result of 4 independent zoonotic transmissions of a simian immunodeficiency virus (SIV) to humans (3) with groups M and N derived from an SIV infecting chimpanzees (SIVcpz) (4) and groups O and P resulting from an SIV that infects gorillas (SIVgor) (5, 6). Group M accounts for the majority (99%) of all HIV-1 infected individuals worldwide (3, 7) and, on the basis of genetic differences is divided into multiple subtypes namely A, B, C, D, F, G, H, J, K and circulating recombinants (8). Subtypes B and C account for most of the group M infected individuals with specific geographic distribution (9). Subtype B HIV-1 is predominant in Northern America and Western Europe (9), while subtype C infections account for half of all HIV-1 infections globally (10) and is predominant in India and Sub-Saharan Africa (9), where its prevalence is high (as high as 25% in South Africa, ref) (11) (ref).

Transmission of HIV-1 can occur by mucosal, perinatal and parenteral routes (7) and of these, mucosal transmission accounts for approximately 90% of all new infections worldwide (12) (7) (2). Heterosexual (HSX) exposure is the predominant route of transmission and is responsible for nearly 70% of all infections worldwide (7), with men who have sex with men (MSM) accounting for approximately 20% (2). Sexual transmission of HIV-1 is a relatively inefficient process and

studies estimate the probability of transmission per sexual exposure to range from 1 in 20 to 1 in 3000 (12-18) depending on the route of exposure. However, factors like increased donor viral load, concomitant sexually transmitted diseases (STD) with resultant inflammation and ulcers, altered mucosal microbiota, socioeconomic factors and gender of the recipient can influence transmission efficiency (17-36). HIV-1 infection causes the depletion of CD4+ T cells, a subset of T cells essential for the proper functioning of the immune system(37) (37-39), Decline in CD4+ counts below 200 cells/ul is AIDS-defining (40), results in immunodeficiency, opportunistic infections and ultimately death (41-44). Despite the advent of highly active antiretroviral therapy (HAART) and other promising intervention strategies (45), a broadly effective vaccine remains the most cost effective means to stem this public health problem (46, 47) (48).

**HIV-1 Entry and Early Events**

Infection by HIV-1 involves the interaction of the viral envelope glycoprotein (Env) with its receptor (CD4), a conformational change in Env facilitates binding to coreceptor and entry into susceptible target cells (49). C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) are the primary coreceptors used by HIV-1, of these CXCR4 usage is limited and typically occurs in the chronic stage of infection in certain individuals (50) (51), while acute viruses predominantly use CCR5 (52-54). For obvious reasons, studying the early events in HIV-1 transmission in humans is impractical, and thus much of what is known about transmission comes from observations of experimental infections in the simian model of HIV-1 infection (SIVmac infection of rhesus macaques) (55). In the next few paragraphs, results from the studies of HIV-1 and SIV will be used to describe the early events following exposure. In the SIV model, both cell-free (56) and cell associated (57) virus stocks are capable of initiating infection (58), using both high challenge doses and a low-dose escalation strategy. Multiple sites in the female

genital tract including the ectocervix, transitional zone, endocervix and the vaginal epithelium have been demonstrated to be potential sites of infection(59-61) (62-65) (66). Similarly, foreskin, penile tissue and rectum are sites of infection in males (61, 67-71).

While SIV RNA + cells are observed as early as 2 hours following exposure (62), the identity of these initial target cells is still a controversial subject. The main cell type that sustains viral replication is activated CD4+ T-cells. However, studies of SIV suggest that myeloid cells could be initially infected and transmit virus to underlying CD4+ cells (58, 66, 72-74). Alternatively, Haase and colleagues describe a model where resting CD4+ cells are the first cells to become infected (62, 65, 75, 76), although these cells are hard to infect *in-vitro* (77, 78). In a third model, predominantly described by Steinman and colleagues describe how dendritic cells (DCs) interact with HIV-1 and transfer infectious viral particles to CD4+ T-cells, without themselves getting infected (79-81).

Following transmission, a series of viral and host immune markers appear that are reproducible across individuals irrespective of virus subtype or route of transmission. This set of markers was first described by Fiebig and colleagues and is helpful to stage individuals during acute infection (82).

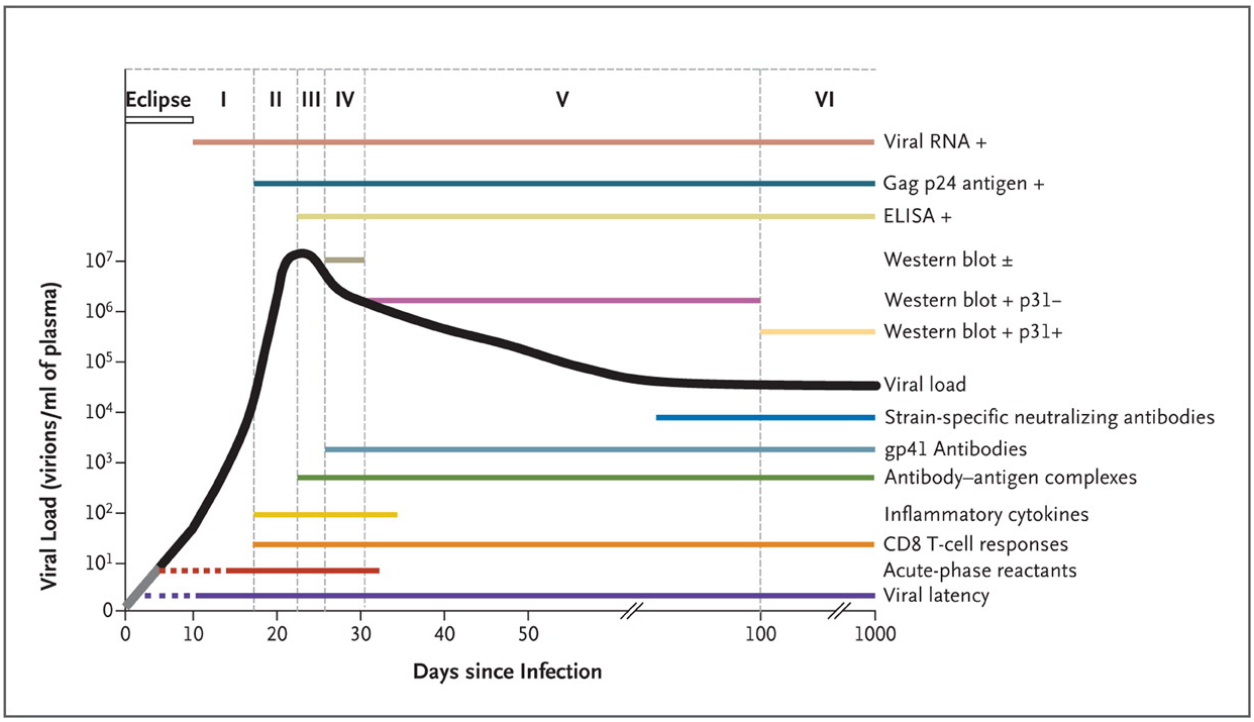


**Figure 1.1.** Stages of infection and the immunopathogenesis of HIV-1 infection. The phases of HIV-1 infection can be divided into six, distinct stages (82) based on the appearance of viral RNA, the gag antigen, antibodies for HIV-1 proteins, first detectable by ELISA and then by western blot, and these are indicated above the viral load curve. The lines beneath the viral load curve indicate early events and immune responses in the host, beginning with the establishment of viral latency, and the development of CD8 T-cell responses and binding and neutralizing antibodies. Figure from (32)

There is an initial eclipse phase before the first detection of virus in the blood, followed by the appearance of viral RNA (Fiebig I), viral p24 antigen (Fiebig II), virus specific antibodies detected first by ELISA (Fiebig III) and then by western blot (Fiebig IV-VI) (Fig 1.1) (82). Shortly after the eclipse phase, there is an exponential increase in viral RNA levels as the virus spreads from local expansion in submucosal CD4+ T cells to secondary lymphoid tissue like the gut

associated lymphoid tissue (GALT) (7). This exponential viral replication is characterized by a high reproductive ratio ($R_0$) of 8 (83). $R_0$ is a measure of the number of cells that become infected by virions produced by a single infected cell. Viruses with an $R_0 > 1$ will result in a spreading infection, while viruses with an $R_0 < 1$ will be extinguished (84).

**Bottlenecks during mucosal transmission**

Individuals with chronic HIV-1 infections harbor viral populations with extensive genetic diversity (85). These intra-patient virus populations are commonly referred to as 'quasispecies' because they consist of non-identical, yet related viral genomes subjected to constant variation, competition and selection pressure (86). In strong contrast, viral populations in most newly infected recipients are far more homogenous (7, 87-92). The narrowing in genetic diversity observed during transmission is termed the transmission bottleneck (7, 90). Single genome sequencing (SGS) allowed the determination of the extent of the transmission bottleneck, and the unambiguous inference of the viral genome that initiated infection, termed the transmitted founder (TF) virus (52, 93, 94). This method eliminates Taq polymerase induced errors, template switching and non-proportional representation of viral variants, all problems observed with bulk PCR followed by cloning and sequencing (95-103) (93). During early acute infection, viral evolution occurs in the absence of the adaptive immune response and thus during the earliest phases of infection, viral diversification occurs in a random manner (32, 52, 82).

The stringency of the transmission bottleneck depends to a certain degree on the route of transmission (30, 52, 104-107) (Fig 2). Using SGS, multiple studies have shown that 80% of all mucosal infections are initiated by a single viral variant (7) (Table 1.1) with HSX transmission (81% single variant) associated with a more stringent bottleneck than that observed with MSM (62% single variant) (Fig 2). However, even in HIV-1 transmission by injection drug users (IDU), half of all new infections are associated with the transmission of a single variant (106, 107).

Taken together, these results indicate that there are likely hurdles to viral infection beyond the mucosal surface.

| Risk group | Study | Subjects | Single variant | | Multiple variants | | Median | Range |
|---|---|---|---|---|---|---|---|---|
| HSX | Keele | 79 | 65 | 82% | 14 | 18% | 1 | 1-4 |
| | Abrahams | 69 | 54 | 78% | 15 | 22% | 1 | 1-5 |
| | Haaland | 27 | 22 | 82% | 5 | 19% | 1 | 1-6 |
| | Total | 175 | 141 | 81% | 34 | 19% | 1 | 1-6 |
| MSM | Keele | 22 | 13 | 59% | 9 | 41% | 1 | 1-6 |
| | Li | 28 | 18 | 64% | 10 | 36% | 1 | 1-10 |
| | Total | 50 | 31 | 62% | 19 | 38% | 1 | 1-10 |
| IDU | Bar | 10 | 4 | 40% | 6 | 60% | 3 | 1-16 |
| | Masharsky | 13 | 9 | 69% | 4 | 31% | 1 | 1-3 |
| | Total | 23 | 13 | 56% | 10 | 44% | 1 | 1-16 |

**Table 1.1** Stringency of HIV-1 transmission by different routes. Transmission by heterosexual (HSX) contact is the most stringest, with 80% of new infections initiated by a single transmitted variant. This is irrespective of the gender of the donor and recipient. Transmission among men who have sex with men (MSM) is less stringent, with close to 40% of new infections being

initiated by multiple variants, likely associated with the breach in mucosal barrier function. Transmission among injection drug users (IDU) is the least stringent with 45% of infections initiated by multiple variants, consistent with the absence of a protective mucosal surface. Data compiled from (30, 52, 104-107)

What are the reasons for the observed transmission bottleneck? The transmission bottleneck is a result of selective processes that impact various stages of the transmission process (108). (fig 1.2)

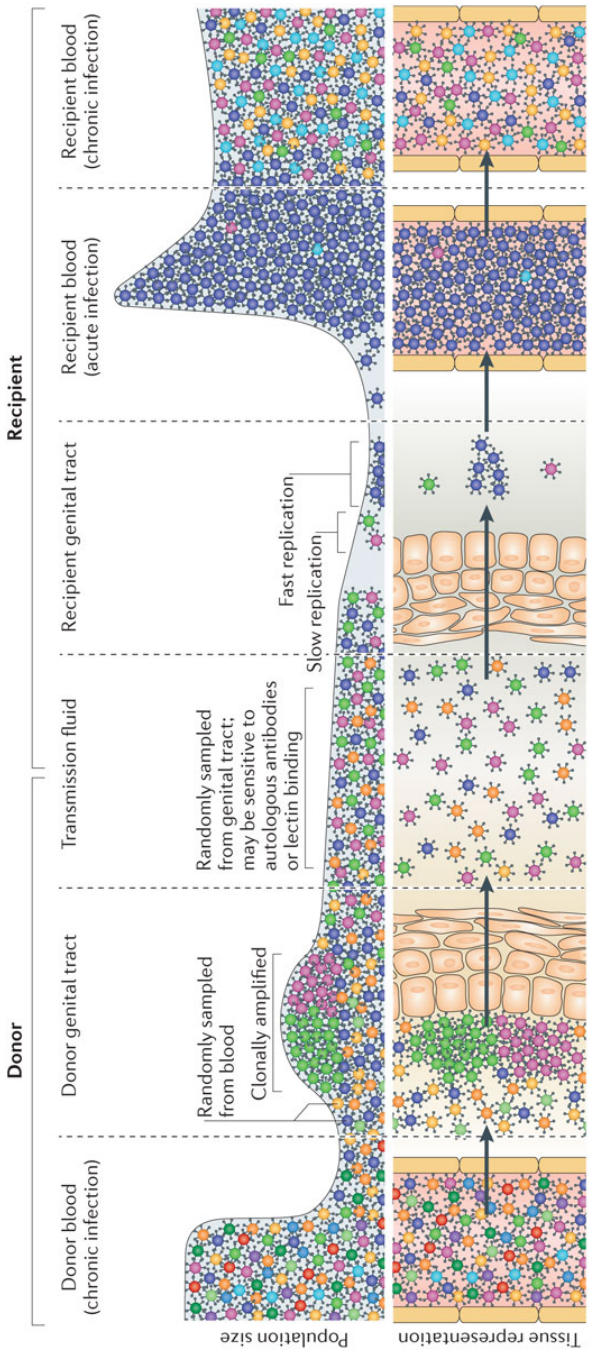


**Fig 1.2** Multiple genetic bottlenecks that can influence the Transmitted founder virus, Chronically infected individuals harbor extensive genetic diversity. Viruses from the blood seed the genital tract of the donor, potentially resulting in clonally amplified lineages which become genital tract-

specific. A minor variant from this population is selected for during transmission, resulting in a genetically homogenous founder population derived typically from a single viral variant. Evolution of the virus In the host, especially in response to the adaptive immune response results in the diverse, chronic quasispecies. Figure from (108)

Mucosal tissues can form physical barriers to transmission, and likely contribute to the low efficiency of HIV-1 transmission. In the female genital tract (FGT), the vagina and ectocervix are lined by multilayered squamous epithelium (61). This thick layer of tightly-packed cells prevents the contact of virus with underlying target cells. In contrast, the transition zone between the ecto and endo – cervix is lined by a single layer of epithelial cells and has an abundance of CD4+ target cells, and thus constitutes a site with increased susceptibility (109, 110). In contrast, the exposed part of the male genital tract (MGT) is lined by keratinized, stratified squamous epithlium and is likely more restrictive for transmission (7, 61, 111). The fact that male circumcision decreases the risk of transmission indicates that the penile foreskin is an important route for transmission (61, 68, 69, 112). In addition, mucosal cells can release microbial defensins that inhibit transmission (113, 114) and mucus that lines these surfaces – cervicovaginal mucus (CVM) or rectal mucus can retard viral diffusion (115-117). The importance of this first line of defense is underscored by the observation that ulcerative infections and microabrasions during sexual contact can mitigate mucosal barrier function(7, 30). The differences in the mucosal barriers that transmitted viruses overcome depending on the route of transmission can likely influence the phenotypes of these viruses. (31, 108, 118, 119).

In addition to these physical barriers, viral compartmentalization in different tissues of the body (blood, genital tract, lymph nodes) and the selection of specific variants to seed the genital tract can contribute to the observed transmission bottleneck (Fig 3). Evidence for genital tract-

specific viruses has been reported (87, 120-127). Interestingly, Boeras and colleagues have demonstrated that the TF is not drawn from the major genital tract viral variant and is in fact a minority variant, more related to viruses in the blood (121). Additionally, viruses that are sensitive to neutralizing antibodies present in either the semen or cervicovaginal fluid might be prevented from transmission (108).

Concomittant infections of the genital tract can contribute to higher rates of HIV-1 acquisition and increased frequency of multiple variant transmission by enhancing inflammation and immune activation, and providing increased activated target cells (30, 128-131). Beyond the mucosa, viral particles must locate and enter subepithelial CD4+ T-cells, undergo replication and dissemination to secondary lymphoid tissue(55). Overcoming the innate immune system is an important early step in the establishment of infection. Miller and colleagues have shown the induction of type I IFN in tissue regions which are SIV+ (132) and recent unpublished data from Jake Estes suggests the induction of type I IFN can extinguish early foci of viral replication. Thus the selective forces that act at transmission are multiple and might limit seroconversion and the numbers of transmitted variants(108).

## Section 1.2 The host immune response to HIV-1 infection

Exposure to virus, initiates a signaling cascade that involves the production of cytokines like CCL20, MIP1a and others by epithelial cells resulting in the recruitment of plasmacytoid dendritic cells (pDCs) and activated CD4+ T cells generating fresh target cells to fuel the new infection (55, 58, 61, 65, 133, 134). Borrow and colleagues measured the levels of 30 plasma cytokines and chemokines in sequential plasma samples from acutely infected blood donors during the eclipse and early exponential phase of infection (135). This study found that the acute phase of HIV-1 replication is characterized by a 'cytokine storm' with some cytokines like Interferon alpha (IFN-α) and interleukin 15 (IL-15) being produced early and transiently while others demonstrate larger (Interferon gama induced protein 10, IP-10) or more sustained increases (tumour necrosis factor alpha, TNFa and monocyte chemoattractant protein 1, MCP-1). Dendritic cells, both myeloid (mDC) and plasmacytoid (pDC) are responsible for the slower, sustained cytokines and rapid, transient cytokines respectively. This cytokine cascade can contribute to the control of viral infection including the activation of effector mechanisms like natural killer (NK) cells and priming of the adaptive immune response (136-138). Conversely, the production of immunostimulatory cytokines can enhance early viral replication through the provision of susceptible, target cells (132, 139).

### The innate immune response to HIV-1: Type I Interferons

HIV-1 Env-CD4 interactions result in the endocytosis of viral particles into pDCs. Viral nucleic acids, particularly RNA in the endosome stimulate toll-like receptor 7 (TLR7), which activates pDCs, inducing the secretion of type I Interferon (IFN) (140, 141) via myeloid

differentiation primary response gene 88 (MYD88) and interferon regulatory factor 7 (IRF7). Alternatively, in other CD4+ cells HIV-1 cDNA is sensed following infection. In these cells, reverse transcription products are sensed by cytoplasmic sensors like cyclic GMP-AMP (cGAMP) synthase (cGAS) and IFNg – inducible protein 16 (IFI16) (141-144). These sensors signal through stimulator of IFN genes (STING) and IRF3. Both of these signaling pathways converge on the production of IFN (shown in Fig 1.3). Compared to all the cell types present in the blood, pDCs produce 200-1000 times more IFN after microbial microbial exposure (145).

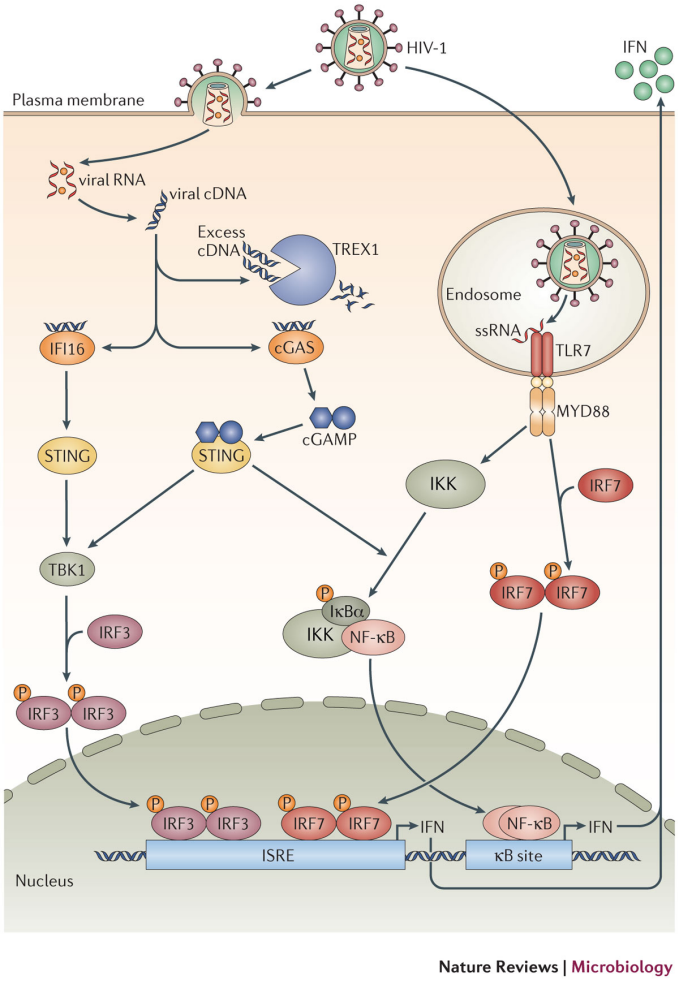


**Fig 1.3** Intracellular sensing of HIV-1 infection in an infected host. Following binding and entry into a cell, reverse transcription products are sensed by cGAS and IFI16 which activate a

signaling cascade ultimately resulting in the induction of type I interferons. In addition to cDNA, viral RNA can also be sensed by TLR7 in the endosomes of pDCs. Figure from (141)

There are three families of IFN (type I, II and III) of which, type I IFNs are primarily responsible for antiviral effects against HIV-1 (141, 146, 147). Type I IFNs consist of 12 alpha subtypes, IFN-β, IFNw, IFNe and IFNk (148, 149). These IFNs act in both an autocrine and paracrine fashion to signal through the heterodimeric interferon receptor (IFNAR) (150). This receptor is made up of two subunits (IFNAR1 and IFNAR2) and ligation of the receptor causes downstream signal transduction through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways (151) (ref). STAT1/2 dimers can bind to IRF9, which in turn binds to the IFN-stimulated response elements (ISREs) in the promoters of IFN stimulated genes (ISGs) (ref). There are over 300 ISGs with anti-viral, anti-tumour, anti-proliferative and immunoregulatory activities (152).

Type I IFNs bind and signal through IFNAR, yet different subtypes are reported to have distinct biological functions (153, 154). The IFN subtypes differ in their affinity for receptor subunits (152, 154-156), and this coupled with differences in off-rates are thought to be responsible for the variety of downstream effects (152). Consistent with this, different subtypes of IFN-α inhibit HIV-1 replication to different levels. A recent study (157) found that IFN a8, a14 and a6 potently reduced the number of infected cells and the production of infectious viral particles while subtypes a21, a1 and a2 had more modest effects. This is relevant and extremely critical to verify, as IFN-α2 is most commonly used in clinical trials (158-169). Veazey and colleagues demonstrated that the topical application of human IFN beta to vaginal tissue of rhesus macaques prevented the animals from simian human immunodeficiency virus (SHIV) acquisition (170). This protection was observed despite an increase in immune activation. In this study, the

authors mention that IFN b was the most potent at suppressing HIV-1 replication when compared to other alpha subtypes.

While clearly antiviral, recent studies addressing the roles of IFN during acute and chronic infection revealed complex and antagontistic effects. Sandler and colleagues found that the administration of IFN-α2 to rhesus macaques prior to challenge was protective, and associated with the increase in antiviral gene expression, while treatment with an IFN receptor antagonist resulted in accelerated progression to AIDS. Paradoxically, prolonged administration of IFN caused reduced response to IFN, reduced antiviral gene expression and more rapid loss of CD4+ T-cells (171). These findings are consistent with previous studies that found persistently elevated levels of IFN and ISGs are associated with increased viral loads (172, 173).

The levels of endogenous IFN fluctuate over the course of HIV-1 infection. After the rapid and transient elevation observed in acute infection, levels of IFN are thought to return to baseline, and are undetectable in the plasma of infected individuals (135). However, during chronic infection and particularly during progression to AIDS, IFN-α2 levels increase (174, 175), accompanied by an increase in viral load and CD4+ T-cell decline. Taken together, these results underscore the complex, fine balance between the protective, beneficial and antiviral effects of type I IFNs and the detrimental and inflammatory effects, especially observed with prolonged exposure. However, in acute infection, elevations in type I IFNs are clearly protective and associated with restricting transmission (171, 176, 177).

**The adaptive immune response to HIV-1 infection**

The cellular adaptive immune response to HIV-1 is well characterized and associated with a decline in peak viremia after acute infection (178). In fact, the speed of activation of HIV-specific CD8+ T-cells, visible in the first ten days after detectable viremia, and the magnitude of

this response govern subsequent immune control and acute phase resolution (179). This cytotoxic T-lymphocyte (CTL) response places selective pressure on the virus and is evidenced as viral evolution in sites of CTL pressure, outgrowth of viral escape mutants and the replacement of the wildtype infecting strain (180, 181). Importantly, the depletion of CD8+ T cells in the simian model of AIDS demonstrated the role that these cells play in early viral control and decline from peak viremia (182).

The humoral response to HIV-1 arises early in infection, resulting in the production of binding antibodies (Ab) with no discernible effect on viremia and that do not exert selective pressure on the acute virus (183). Neutralizing antibodies (Nab) are those that bind viral particles and prevent them from infecting target cells. These Nabs appear later in infection and are limited in their breadth of neutralization, that is, they can neutralize the virus in the individual in which they arose but cannot neutralize a virus from a different individual. These Nabs are potent enough however, to drive virus escape as evidenced by the selection of escape mutations (183, 184).

Adaptive immune responses drive viral evolution and can thus impact disease progression. Shortly after transmission, HIV-1 establishes a reservoir of latently infected cells (185, 186). These latently-infected cells harbor integrated proviruses, are generally not permissive for viral gene expression, are extremely long-lived and present the major hurdle to curing HIV-1 infection(185). Given this, the virus might be most vulnerable early in infection between the eclipse phase and peak viremia. This leaves a small window of opportunity for interventions (187) and hence it is of interest to determine the properties of the transmitted virus because interventions and vaccines aimed at blocking transmission would need to target any specific features of the transmitted virus.

**Section 1.3 Transmitted Variants: Genetic and Biological traits**

Single genome amplification followed by direct amplicon sequencing (SGS) made possible the inference and enumeration of transmitted variants (52, 93). The unambiguous description of the viral genome that initiated and founded clinical infection, allowed the comparison of genetic features of TF and non-transmitted variants. As Env is the first viral protein to interact with host cells, many early studies focused on this protein (118, 188-193). A functional Env is a trimer of heterodimers. Each heterodimer is made up of a surface unit (gp120) and a transmembrane portion (gp41). Surface unit gp120 is made up of both constant regions and variable loops, and is a heavily glycosylated protein (49). Env sequences from individuals with acute subtype C infections have been reported to have fewer potential N-linked glycosylation sites (PNLGs) and shorter variable loops when compared to Env sequences from respective donors or unmatched random chronically infected individuals (118, 190). However, many of the findings are not reproducible across cohorts or viral subtypes. Indeed, in studies of subtype B viruses, researchers have often found no differences in either the length of the variable loops or PNLGs (188, 192, 194, 195) or found differences in only the PNLGs (196). In studies of subtype A and D viruses, recipient viruses have been reported to have shorter variable loops (191, 192), but either the same (191) or fewer PNLGs (192). Comparisons of Env sequences from acutely infected individuals to all sequences in the Los Alamos National Laboratory HIV sequence database yielded shorter variable loops and fewer PNLGs for subtype A but not subtype B (192). Thus, while these Env genotypic features appear to be selected in certain cohorts and subtypes, they are not easily generalizable. Additionally, some of these differences can be attributed to differences in the populations and cohorts studied.

TF viruses have been shown to be more closely related to minor variants in the donor quasispecies (121). A large signature analysis study of subtype B Env sequences identified transmitted signatures in the signal peptide and in gp120, the latter involved the loss of a glycan which has been shown to be associated with immune escape (197). A recent study comparing

sequences from 137 transmission pair donors and recipients and found that recipient sequences were closer to the subtype consensus sequence than the matched donor sequences (31). The transmission of more 'ancestral' forms has been described previously (198-201) and is interpreted to indicate the transmission of more 'fit' viruses. In fact, in chronically infected individuals, viruses with immune escape mutations often infer a fitness cost (181, 184, 202-207). Upon transmission to a naïve recipient, these mutations often revert to consensus amino acid residues (208). The suggestion that more ancestral genomes were indicative of higher fitness merited an investigation into the biological properties of TF viruses.

The most widely observed, robust finding is that transmitted variants use CCR5 as a coreceptor for entry (52, 53, 118, 188, 189, 193). Multiple studies have interrogated the efficiency of receptor and coreceptor usage, hypothesizing that efficient viral replication might require enhanced receptor binding. However, no differences between TF and chronic Envs have been reported in the efficiency and speed of fusion of viral Envs with CD4 (188, 189), and TF viruses have been reported to require high levels of CD4 to mediate entry (118). Additionally, subtype B and C TF Envs were indistinguishable from chronic Envs in their entry into different primary CD4+ T-cell subsets (188, 189). While some reports have identified the integrin a4b7 as a molecule preferentially bound by TF Envs (209)(Arthos), a subsequent study looked at a larger panel of Envs and failed to see these differences (189). We and others have observed that TF Envs are more sensitive to inhibition by maraviroc, a drug that blocks Env- CCR5 interactions, and this is observed for both subtypes B and C (118, 193).

These previous studies have focused on Env in isolation, and to more thoroughly study TF biology, the interrogation of other viral proteins is important. A comprehensive study of full-length replication competent viruses from subtypes B and C found TF viruses have more Env per particle, were more infectious, interacted more efficiently with DC, were transferred more efficiently from DC to CD4+ T-cells and were more resistant to IFN-α (54). The caveat to this study was that the acute and chronic viruses were derived from unmatched individuals. In

agreement with the results from Parrish and colleages, Fenton-May et al., found that transmitted viruses were resistant to IFN-α, and that this resistance declined six months after transmission (210). In this study, the authors compared the IFN-α resistance of the TF from an infected subject to the consensus virus six months later. To determine if these properties were observed in viruses from linked donors and recipients, subsequent studies used known transmission pairs (195, 201). Surprisingly, these studies did not reproduce Parrish and colleagues' findings. They found that recipient viruses were equally infectious and have similar replicative capacity (195, 201). Deymier et al found that recipient viruses were equally resistant to IFN-α, while Oberle and colleagues found recipient viruses to be slightly more sensitive to IFN-α. Limited sampling of the donor and the source of the donor viruses were caveats in these studies that might have resulted in their findings. Thus, while there are hints that transmitted viruses are distinguished by genetic and biological properties, a more thorough investigation, with larger panels of viruses are warranted.

**Section 1.4 HIV-1: accessory proteins and ISG counteraction**

HIV-1 has a positive sense RNA genome that encodes three structural proteins –group-specific antigen (gag), polymerase (pol) an envelope (env) and 2 regulatory proteins – transactivator of transcription (tat) and regulator of expression of virion proteins (rev) (211). In addition, the virus encodes four accessory proteins, so named because they were believed to be dispensible for replication in-vitro (212). They are virion infectivity factor (vif), viral protein r (vpr), viral protein u (vpu) and negative factor (nef). Their organization is shown in Fig 1.4

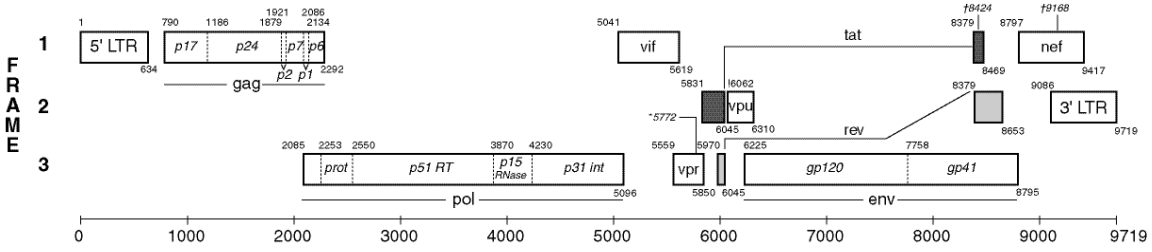


**Fig 1.4** Genomic organization of the coding and non-coding regions of HIV-1. Viral polyproteins and their constituent proteins are indicated. In addition, exons and splice junctions of accessory proteins are indicated. The genes are organized by reading frame and aligned to HXB2 as a reference. Figure from http://www.hiv.lanl.gov/

The four accessory proteins are involved in escape from and manipulation of innate and adaptive immune responses (213). None of these proteins have enzymatic activity; instead they act as molecular adaptors linking their targets to the host degradation pathway (214). Multiple host proteins termed restriction factors act in concert to restrict various stages of the viral life

cycle. These factors are distinguished by being inducible by type I IFNs, encoded by the germline, under positive Darwinian selection and they are frequently counteracted by viral proteins (141). These factors act to restrict virus replication and transmission between individuals of the same species but also can act as barriers to cross-species transmission events (3, 215-217).

A role for Vif was identified by researchers who observed a reduction in viral infectivity of Vif deficient viruses in primary CD4+ T-cells and certain cell lines (218-221). Sheehy and colleagues identified apolipoprotein B mRNA-editing enzyme, catalytic polypeptide like 3G (APOBEC3G) as the host protein that is packaged into viral particles and deaminates nascent reverse trancripts (222). This deamination results in the insertion of alanines in place of guanines during second strand cDNA synthesis, and is referred to as G-A hypermutation. A3G is a member of the A3 family of type I IFN-inducible, cytidine deaminase proteins, and A3 D, E, F, G and H are reported to have anti-HIV activity (223). A3G deamination results in hypermutated viral DNA, which can either be destroyed prior to integration or might integrate, largely encoding defective viruses. Binding of A3G to nascent trancsripts can also inhibit reverse transcription and block the process of integration (223). In the presence of Vif, members of the CUL5-EloB/ C-RBX2-E2 complex are recruited to A3, resulting in their polyubiquitination and proteosomal degradation. Thus, A3G is excluded from the viral particle and cannot influence infectivity in the subsequent round of infection(223).

Vpu was first shown to be important for the efficient release of viral particles from certain cell types (224-227). Based on the requirement for Vpu, cells could be classified as permissive and non-permissive. In the absence of vpu, in non-permissive cells, virions stay associated with the infected cell surface (226), held there by a proteinaceous tether (228, 229), and are thus not

released efficiently (226, 230). The responsible host protein, tetherin/ CD317/ BST2 (229, 231) is IFN-α inducible (228, 229) and retains viral particles which can then be endoctyosed (228, 230). Tetherin is a transmembrane protein with a C-terminal glycosylphophatidylinositol (GPI) anchor. It is expressed on cells as a short and long isoform due to the presence of an internal initiation codon (232), both of which are restrictive, but only the long form retains Vpu sensitivity. Vpu directs the ubiquitination and lysosomal degradation of tetherin via linking it to the adaptor b-TRCP(233). While tetherin can inhibit virus release, its role in the restriction of virus replication is contentious. Some groups have reported the ability of cell-cell spread of HIV-1 to overcome restriction by tetherin (234), while others have demonstrated that tetherin can restrict both cell-free and cell-cell spread of HIV-1(235). Thus, it appears that tetherin at least plays an important role in limiting cell-free virus production, which could influence transmission.

Vpu is not the only lentiviral protein capable of antagonizing tetherin. Certain SIVs and HIV-2 use Nef and Env respectively to counteract tetherin, underscoring the importance of the counteraction of this restriction factor (214). SIVcpz and SIV gor viruses utilize their Nef protein to counteract tetherin. Human tetherin has a 5 amino acid deletion in the N-terminal cytoplasmic region. This deletion renders human tetherin resistant to antagonism by Nef, whose binding site overlaps with this region (215, 236). Upon cross-species transmission of SIVcpz to humans, HIV-1 M adapted to use Vpu to counteract this host protein, switching from interacting with the cytoplasmic domain using Nef to the transmembrane domain using Vpu (215). Indeed this adaptation is believed to be critical for HIV-1's effective spread in the human population (3). Among the groups of HIV-1, M and N use Vpu to antagonize tetherin, although the latter is very inefficient (215, 237). Previous reports indicate that groups O and P Vpu proteins do not counteract tetherin (215, 236, 238-240).

In addition to the counteraction of tetherin, Vpu also downmodulates CD4 and NFkB. The downregulation of CD4 prevents viral superinfection of an infected cell and prevents the interaction of CD4 and Env intracellularly, thus freeing newly synthesized Env for incorporation into particles and viral release (241-244). NFkB can induce antiviral gene expression, and thus especially in late stages of viral replication, its expression can be disadvantageous. Vpu proteins of different SIVs and HIV-1 downregulate NFkB, thus preventing sensing of the virus, and downstream antiviral effector expression (245, 246). Thus, in addition to the counteraction of ISGs, accessory proteins also interfere with viral sensing to avoid the induction of type I IFNs.

Vpr has been reported to induce cell cycle arrest at the G2/M transition (247-249). More recently, Vpr has been demonstrated to interact with the SLX4 scaffold protein and structure-specific endonucleases. The formation of this complex (SLX4com) results in its activation and thus G2/M cell cycle arrest. The activation of SLX4com is beneficial to HIV-1 replication through the impairment of the host cell's ability to sense infection and induce type I IFN production (250, 251). Following activation of the complex, endonucleases degrade viral transcripts enabling the virus to escape detection by the host innate immune mechanism (250).

Nef is an early viral protein, whose expression while dispensable in vitro, is crucial for the maintenance of high viral loads in HIV-1 infected humans and SIV infected macaques in vivo (252, 253) (254). Nef, similar to Vpu, downregulates CD4 from the cell surface (255), and in doing so prevents cell death by the possible recognition of Env-CD4 complexes by antibody-dependent cell-mediated cytotoxicity (ADCC) (256, 257). While Vpu targets newly synthesized CD4 in the endoplasmic reticulum (ER), Nef, by virtue of being an early protein, targets CD4 expressed on the cell surface. Nef recruits adaptor protein 2 (AP-2) and induces the clathrin-dependent endocytosis of CD4 and ultimately its degradation (258). In addition to CD4, Nef also

downmodulates major histocompatibility complex 1 (MCH I) from the cell surface. This reduces the presentation of viral antigens to CD8+ T cells and thus prevents infected cell death (ref). Nef also regulates NFkB expression (259). NFkB has a dual role during viral infection, early in infection it binds to the long terminal repeat (LTR) in the integrated provirus and stimulates transcription (ref), while late in the life cycle its expression can enhance viral sensing. Nef, induces the levels of NFkB early in the viral life cycle, thereby inducing transcription and viral replication (260).

Nef has additionally been shown to be crucial to promote viral infectivity (ref). It was thus hypothesized that Nef altered the cell surface expression and potential incorporation of a cellular factor that limited virion infectivity (261). Two groups recently identified this protein as Serine incorporator (SERINC) 3 and 5 (262, 263). SERINC proteins do not inhibit Env fusion with the target cell, instead they block infection at the expansion of the fusion pore(264). However, SERINC is not IFN-α inducible, and is thus not considered a restriction factor, but a cell intrinsic viral factor. Lastly, as mentioned above many SIVs use their Nef proteins to counteract tetherin. While groups M and N use their Vpu protein to antagonize this host protein, groups O and P do not, and in fact previous studies suggest that neither has evolved an efficient protein capable of counteracting tetherin(215, 238, 239). While consistent with the limited spread of group P viruses in the population, it is puzzling how group O viruses have infected nearly 100,000 people in the absence of an efficient tetherin antagonist.

**Conclusions**

In sum, mucosal transmission of HIV-1 is characterized by a stringent bottleneck. While genetic and phenotypic signatures of transmission have been described for Env proteins, these have not been extended to other viral proteins. Determining the biological phenotypes of full-length, mucosally transmitted HIV-1 will enable a more precise understanding of the processes that limit transmission. I hypothesize that transmitted viruses are characterized by unique biological properties that distinguish them from non-transmitted donor viruses. In addition, these properties will enable the successful transmission across mucosal surfaces, and enable efficient viral replication in a newly infected recipient. In Chapter 2, I investigate the properties of transmitted viruses by comparing them to non-transmitted variants in the context of established transmission pairs. Evaluating the role of restriction factors and their contribution to the mucosal bottleneck could reveal interesting and novel therapeutic angles. Specifically, in chapter 3, I address the contribution of tetherin to the antiviral state established by IFN-α. Complimentary to this, I determine the importance of Vpu in overcoming IFN-mediated HIV-1 restriction.

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# CHAPTER 2

## RESISTANCE TO TYPE 1 INTERFERONS IS A MAJOR DETERMINANT OF HIV-1 TRANSMISSION FITNESS

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S.S.I., F.B.-R., H.J.B., R.M.R., M.V.G., and Y.L. generated isolates and characterized their phenotype; A.G.S., C.Y.B., T.D., and C.M.S. performed single genome amplification and sequence analyses; G.H.L., S.S.-M., L.J.P., and P.M.S, performed statistical and phylogenetic analyses; B.F.H., G.M.S., P.M.S., and P.B. contributed reagents and analytic tools; S.S.I., F.B.-R., S.S.-M., G.M.S., P.M.S., P.B. and B.H.H. conceived the study and wrote the paper.

**Section 2.1 – Abstract**

Sexual transmission of HIV-1 is an inefficient process, with only one or few variants of the donor quasispecies establishing the new infection. A critical, and as yet unresolved, question is whether the mucosal bottleneck selects for viruses with increased transmission fitness. Here, we characterized 300 limiting dilution-derived virus isolates from the plasma, and in some instances genital secretions, of eight HIV-1 donor and recipient pairs. Although there were no differences in the amount of virion-associated envelope glycoprotein, recipient isolates were on average 3-fold more infectious ($P$ = 0.0001), replicated to 1.4-fold higher titers ($P$ = 0.004), were released from infected cells 4.2-fold more efficiently ($P$ < 0.00001), and were significantly more resistant to type I interferons (IFNs) than the corresponding donor isolates. Remarkably, transmitted viruses exhibited 7.8-fold higher IFNα2 ($P$ < 0.00001) and 39-fold higher IFNβ ($P$ < 0.00001) half-maximal inhibitory concentrations ($IC_{50}$) than did donor isolates, and their odds of replicating in CD4+ T cells at the highest IFNα2 and IFNβ doses were 35-fold ($P$ < 0.00001) and 250-fold ($P$ < 0.00001) greater, respectively. Interestingly, pretreatment of CD4+ T cells with IFNβ, but not IFNα2, selected donor plasma isolates that exhibited a transmitted virus-like phenotype, and such viruses were also detected in the donor genital tract. These data indicate that transmitted viruses are phenotypically distinct, and that increased IFN resistance represents their most distinguishing property. Thus, the mucosal bottleneck selects for viruses that are able to replicate and spread efficiently in the face of a potent innate immune response.

**Section 2.2 – Significance**

Effective prevention strategies are urgently needed to control the spread of HIV-1. A critical barrier to developing such strategies is the lack of understanding of the host antiviral defenses that control HIV-1 replication in the mucosa at the site of entry. Here, we characterized viruses from matched donor and recipient pairs to determine whether transmitted HIV-1 strains exhibit traits that increase their transmission fitness. Characterizing 300 limiting dilution-derived isolates, we identified several properties that enhance virus replication in the face of a vigorous innate immune response, of which resistance to type 1 IFNs is the most important. These results provide new insights into the HIV-1 transmission process and define possible new targets for AIDS prevention and therapy.

**Section 2.3 – Introduction**

Understanding the host and viral factors that influence HIV-1 transmission may aid the development of an effective AIDS vaccine. In 2015, approximately 2 million individuals were newly infected with HIV-1, the great majority of whom acquired the virus by sexual routes (1). Although a number of factors, such as high donor viral loads, genital inflammation, altered mucosal microbiota, and recipient gender, are known to increase the infection risk (2-4), virus transmission across intact mucosal surfaces is inherently inefficient, with only a small fraction (less than 1%) of unprotected sexual exposures leading to productive infection (5-8). This inefficiency is exemplified by a stringent population bottleneck, in which only one or a limited number of variants from the diverse quasispecies of the transmitting donor establish the new infection (9). Transmitted viruses are not usually the most abundant strains in the genital secretions of infected donors (10), and analyses of viral sequences from 137 matched donor and recipient pairs indicated that viruses with a more ancestral genotype are preferentially transmitted (11). These data suggested that mucosal transmission selects for variants with enhanced transmission fitness (11). However, the viral properties that contribute to this transmission fitness have not been defined.

For obvious reasons, viruses cannot be collected from, or studied in, humans at the time of transmission. However, by sequencing plasma virion RNA (vRNA) in the first few weeks following transmission, it is possible to enumerate and infer the genome(s) of the virus(es) that established the infection (9, 12-14). In the absence of adaptive immune pressures, HIV-1 diversifies in a random fashion, with viral sequences exhibiting a Poisson distribution of mutations and a star-like phylogeny that coalesces to an inferred consensus sequence. This consensus sequence represents the genome of the virus that initiated the infection, termed the transmitted founder (TF) virus (9). Single genome amplification (SGA) of plasma vRNA, which precludes PCR

artifacts such as Taq polymerase mediated recombination (15-18), revealed that in the great majority (~80%) of sexual transmission cases, a single TF virus establishes the new infection (9, 12, 13, 19-21).

The ability to infer and molecularly clone the genomes of TF viruses has permitted their biological characterization. Initial studies showed that TF viruses use CD4 and CCR5 as their receptor and co-receptor, and replicate efficiently in activated CD4+ T cells but not macrophages (14, 22-25). Moreover, analysis of a comprehensive panel of infectious molecular clones (IMCs) showed that TF viruses packaged more envelope glycoprotein (Env), exhibited greater infectivity, bound to monocyte-derived dendritic cells more efficiently, and replicated to higher titers in CD4+ T cells in the presence of the type 1 interferon IFNα2 than chronic control (CC) viruses (26). However, a potential confounder of these studies was the fact that TF and CC viruses were not derived from epidemiologically linked transmission pairs. To compare transmitted and non-transmitted viruses close to the time of transmission, two recent studies characterized the phenotype of viruses from matched donor and recipient pairs (27, 28). Examining various biological properties, including the sensitivity of donor and recipient viruses to IFNα2, both studies failed to identify viral traits that were indicative of enhanced transmission fitness (27, 28).

Innate immune responses, in particular type 1 IFNs, represent a potent first-line defense against many pathogens, including primate lentiviruses (29-33). Consistent with this, treatment of rhesus macaques with pegylated IFNα2 increased the number of intrarectal challenges required to achieve systemic SIVmac infection and decreased the number of transmitted founder viruses (34). Similarly, mucosal application of IFNβ protected macaques from repeated intrarectal and intravaginal challenges with a simian-human immunodeficiency virus (SHIV) (35). Since type 1 IFNs are rapidly upregulated at mucosal sites of virus replication in SIVmac infected macaques (36), and bioactive IFN levels are highly elevated during acute HIV-1 infection (37), we hypothesized that IFN-mediated antiviral activity contributes to the HIV-1 transmission bottleneck. To test this, we generated a large panel of limiting dilution derived isolates from the plasma and

genital secretions of chronically infected donors and their matched recipients. Analyzing 300 such isolates, we identified a number of biological properties that are associated with increased transmission fitness, all of which serve to enhance HIV-1 replication and spread in the face of a vigorous innate immune response.

**Section 2.4 – Results**

**Generation of limiting dilution HIV-1 isolates from sexual transmission pairs.** Molecular cloning of HIV-1 genomes is labor intensive and thus limits the number of IMCs that can reasonably be characterized. Moreover, predicting which viral genomes are functional in chronically infected individuals is challenging, because immune escape mutations frequently incur fitness costs (38-45). Virus isolation represents an alternative to cloning, but bulk cultures cannot account for the biological variation of individual quasispecies members. Here, we used limiting dilution virus isolation to generate single virion-derived HIV-1 strains from eight sexual transmission pairs. These included four female-to-male (FTM) transmissions (subtype C) from southern Africa as well as from one male-to-female (MTF) and three men-who-have-sex-with-men (MSM) transmissions (subtype B) from the US (*SI Appendix*, Table S1). In all but one case, the newly infected recipient was identified first as part of an acute infection cohort, while the transmitting partner was identified retrospectively. Phylogenetic analysis of SGA-derived plasma viral sequences confirmed that all transmission pairs were epidemiologically linked (*SI Appendix*, Fig. S2.1) and showed that two recipients (CH378, CH831) had acquired their infection from the same donor (CH742). Seven of the eight recipients were infected with a single TF virus, while the remaining subject (CH378) acquired at least two TF viruses (*SI Appendix*, Fig. S2.2). All subjects remained treatment naïve throughout the study.

To generate limiting dilution-derived viral isolates, plasma as well as cell-free fractions of cervicovaginal lavage (CVL) and semen (SEM) samples were end-point diluted and used to infect activated normal donor CD4+ T-cells in 24 well plates. According to a Poisson distribution, a dilution that yields positive cultures in no more than 30% of wells should contain a single infectious unit more than 80% of the time. Cultures were maintained for 20 days, tested for p24

antigen production, and virus positive wells were expanded further in normal donor CD4+ T cells for an additional 10 days. The resulting viral stocks were used for all subsequent genetic and biological analyses.

To ensure that the limiting dilution isolates were indeed single virion-derived, we sequenced all stocks prior to biological characterization. Briefly, 5' and 3' half genomes were PCR amplified, MiSeq sequenced, and the resulting reads assembled to generate an isolate specific consensus sequence. Viral reads were then mapped to this consensus sequence and the extent of genetic diversity was examined for each position along the genome. Isolates that exhibited more than 15% diversity at any one position in the alignment were considered to contain more than one variant and thus removed from further analysis. To control for the emergence of phenotypically distinct variants in the culture, we generated limiting dilution isolates from all acutely infected subjects even though TF IMCs were available for two recipients (14, 26). Using plasma samples collected closest to the time of transmission, we generated 95 donor and 61 recipient isolates (*SI Appendix*, Table S2.1). Virus isolation from CVL and SEM samples was more challenging, because of lower viral loads, frequent bacterial and yeast contaminations, and the fact that many genital secretions were inherently cytotoxic for CD4+ T cells (46). Nonetheless, we were able to generate limiting dilution isolates from the CVL or semen samples of three transmitting donors (*SI Appendix*, Table S1).

**Limiting dilution HIV-1 isolates are representative of the donor quasispecies.** To determine whether the limiting dilution isolates were representative of the viral quasispecies present in both donors and recipients, we compared all isolate-derived sequences to SGA derived vRNA sequences amplified directly from the blood of the same individual. In phylogenetic trees of 3' half genome sequences, isolate and plasma vRNA sequences were completely interspersed (Figs. 2.1*A* and *SI Appendix*, Fig. S2.3). To assess whether isolate and plasma viral sequences from

chronically infected donors were segregated, we calculated their genealogical sorting index (gsi) (47). Two donor samples yielded gsi values that were higher than expected from random segregation (*SI Appendix*, Table S2.2*A*). In one case (CH212), available isolates represented only two of three diverse viral lineages present in this donor's quasispecies, indicating limited sampling (*SI Appendix*, Fig. S2.3*F*). In the other case (CH728), two pairs of near identical isolate sequences indicated repeat culture of the same virus (*SI Appendix*, Fig. S2.3*C*). Collapsing one of these to a single sequence rendered the gsi value non-significant. For all other subjects, there was no evidence for segregation (*SI Appendix*, Table S2.2*A*), indicating that the isolates were fully representative of the viral diversity present in the plasma. As expected, plasma isolates from single TF infections were very closely related, differing from each other by fewer than 8 (range 2-7) and from the inferred TF genome by fewer than 12 (range 2-11) nucleotides across the entire genome (*SI Appendix*, Fig. S2.2). Plasma isolates from subject CH378 exhibited greater diversity, because they represented the progeny of two TF viruses as well as their recombinants (*SI Appendix*, Fig. S2.2*A*). Unlike in some previous studies (8), there was no evidence of compartmentalization of plasma and genital secretion isolates from donors CH492 and CH742 (*SI Appendix*, Fig. S2.3 and Table S2.2*B*).

**Increased Env content is not a characteristic feature of transmitted viruses.** Comparing viruses from unrelated subjects, we previously reported that TF IMCs package on average 1.9-fold more envelope glycoprotein (Env) than viruses circulating in the plasma of chronically infected individuals (26). To examine the Env content of matched donor and recipient isolates, we generated viral stocks in normal donor CD4+ T cells, depleted these of microvesicles, purified virions using antibody coated magnetic beads, and quantified Env by enzyme-linked immunosorbent assay (ELISA) per unit of RT activity. We found that plasma isolates varied widely in the amounts of Env that they packaged, but failed to identify consistent differences between donor and recipient isolates. Recipient isolates packaged either significantly more, less,

or similar amounts of Env compared to their corresponding donor viruses (Figs. 2.1*B* and *SI Appendix*, Fig. S2.4*A*). For one donor (CH492), genital tract isolates had a 2.4-fold higher mean Env content than the corresponding plasma isolates, but this was not the case for the other two donors (Figs. 2.1*B* and *SI Appendix*, Fig. S2.4*A*). When data from all pairs were combined, no significant differences in Env content were observed between donor and recipient isolates, plasma and genital secretion isolates, and subtype B and C recipient isolates (Fig. 2.1*C*). These data indicate that mucosal transmission does not select for viruses with an increased Env content.

**Transmitted viruses exhibit increased particle infectivity.** We previously reported that TF viruses were 2-fold more infectious than chronic viruses from unrelated subjects (26), but two subsequent studies failed to identify virus infectivity as a determinant of transmission fitness (27, 28). Here, we used TZM-bl cells, which express luciferase under the control of an HIV-1 promoter (48, 49), to determine the per-particle infectivity of CD4+ T cell-derived viral stocks. To limit virus infection to a single round, we added the fusion inhibitor T1249 (50) to all cultures 12-15 hours following infection. Plotting relative light units (RLUs) per amount of input virus (pg of RT), we found that donor plasma isolates exhibited a wide range of particle infectivity both within and between individuals, while the infectivity of recipient isolates was much less variable. Moreover, for seven transmission pairs, recipient viruses were significantly (2 to 8-fold) more infectious than the corresponding donor viruses, with a trend observed for the eighth pair (Figs. 2.1*D* and *SI Appendix*, Fig. S2.4*B*). Higher particle infectivity relative to plasma viruses was also observed for CVL and SEM isolates from two donors (1.8- and 3.2-fold, respectively), but not for the third donor, although in the latter case only two CVL isolates were available for comparison (Fig. 2.1*D* and *SI Appendix*, Fig. S2.4*B*). When data from all transmission pairs were combined, recipient isolates were on average 3-fold more infectious ($P$ = 0.0001) than the corresponding donor isolates irrespective of their subtype (Fig. 2.1*E*). Donor genital secretion isolates tended to be

more infectious than the corresponding plasma isolates, but this did not reach statistical significance. Thus, mucosal transmission selects for viruses with increased particle infectivity, some of which are present in genital secretions.

**Transmitted viruses replicate to higher titers**. The replicative capacity of viruses can influence their reproductive ratio ($R_0$) and thus their ability to expand an initial infection (51). Comparing IMCs from unrelated subjects, we previously failed to detect differences in the growth potential of TF and chronic HIV-1 strains (26), and similar results were reported for donor and recipient viruses from transmission pairs (27, 28). Here, we compared the replicative capacity of limiting dilution-derived isolates in normal donor CD4+ T cells. Using equal numbers of particles for viral input (1 ng of RT activity), we monitored the growth kinetics of a subset of isolates ($n$ = 25) for 9 days by measuring p24 antigen in culture supernatants every 48 hours. We then determined the area under the curve (AUC) and compared it with p24 values measured at individual time points. This analysis revealed a strong correlation between the AUC and p24 production at day 7 ($r$ = 0.99, $P$ < 0.0001). We thus used the latter as a measure of viral replicative capacity for all remaining isolates.

Transmitting donor isolates varied widely in their replicative capacity, and this was also true for some recipient isolates. However, recipient isolates replicated on average between 1.2 and 1.7-fold more efficiently than viruses isolated from the corresponding donors (Figs. 2.1*F* and *SI Appendix*, Fig. S2.4*C*). These differences were significant for seven transmission pairs, with a trend observed for the eighth pair (Figs. 2.1*F* and *SI Appendix*, Fig. S2.4*C*). In contrast, genital secretion isolates did not exhibit an increased replicative capacity. Combining results from all transmission pairs, we found that on average recipient isolates grew to 1.4-fold higher titers than their corresponding donor isolates ($P$ = 0.004), while no significant differences were observed between plasma and genital secretion isolates, or between recipient isolates representing

subtype B and C infections (Fig. 2.1*G*). These data indicate that mucosal transmission selects for viruses with enhanced replicative capacity.

**Transmitted viruses are uniformly resistant to type I interferons.** We previously reported that TF viruses are more resistant to IFNα2 than viruses from chronically infected individuals (26, 52). However, two subsequent studies of linked transmission pairs failed to confirm this phenotype, with one study finding no differences in IFNα2 resistance between transmitted and non-transmitted viruses (27), and the other reporting transmitted viruses being more IFNα2 sensitive (28). To resolve these differences, we tested the IFN sensitivity of the limiting dilution-derived isolates, but with some experimental modifications. First, instead of testing only IFNα2, we measured the antiviral effect of a second potent inhibitor of HIV-1, IFNβ (35, 52) Second, rather than examining the effect of only a single IFN inhibitory dose (26-28, 53), we determined the half-maximal inhibitory concentration ($IC_{50}$) of both IFNα2 and IFNβ for every single isolate. This was done by treating normal donor CD4+ T cells with increasing quantities of IFN, infecting them with equal amounts of virus, and culturing the cells for 7 days while replenishing IFN-containing medium. Virus replication was then measured for each IFN concentration as the amount of p24 produced at day 7 and plotted as the percentage of viral growth in the absence of IFN, which was set to 100% (Fig. 2.2*A* and *B*). As an independent measure of IFN resistance, we also measured viral replication at the highest IFN dose and expressed this residual replication capacity (Vres) as the percentage of viral growth in the absence of IFN (Fig. 2.2*A* and *B*, *SI Appendix*, Fig. S2.5*B* and *D*).

For each transmission pair, plasma isolates from donors exhibited a wide range of sensitivities to both IFNα2 and IFNβ, while recipient isolates were much less variable as well as uniformly more resistant to both IFNα2 and IFNβ (Fig. 2.2*C* and *E*). Compared to the respective donor isolates, recipient isolates exhibited on average 6- to 11-fold higher IFNα2, and 15- to 71-

fold higher IFNβ $IC_{50}$ values (*SI Appendix*, Fig. S2.5*A* and *C*). Analysis of the residual replicative capacity, Vres, yielded similar results, although the differences between donor and recipient isolates were much more pronounced. At the highest IFNα2 dose (5.5 pg/ml), recipient isolates retained on average 15% to 26% of their replicative capacity, while the corresponding donor viruses reached only 0.8% to 2% (*SI Appendix*, Fig. S2.5*B*). At the highest IFNβ dose (0.44 pg/ml), recipient viruses retained on average 4% to 13% of their replicative capacity, while the corresponding donor isolates were either suppressed below the limits of p24 detection or reached Vres values of 0.01% to 0.1% (*SI Appendix*, Fig. S2.5*D*). Thus, the ability of recipient isolates to replicate at the highest IFN dose was 13- to 51-fold higher for IFNα2, and 123- to 541-fold higher for IFNβ, compared to the corresponding donor viruses (*SI Appendix*, Fig. S2.5*B* and *D*).

Examining genital tract isolates, we found that they also exhibited higher IFNα2 and IFNβ $IC_{50}$ values than the corresponding plasma isolates, although significance was reached only for IFNβ ($P = 0.04$) (Fig. 2.2*F*). In addition, genital tract isolates exhibited higher Vres values, but in this case significance was reached only for IFNα2 ($P = 0.008$) (*SI Appendix*, Fig. S2.6*B*). Comparing $IC_{50}$ and Vres, we found that these values correlated strongly for donor plasma isolates (IFNα2: $r = 0.89$, $P < 0.0001$; IFNβ: $r = 0.57$, $P < 0.0001$), but only weakly for donor genital secretion isolates (IFNα2: $r = 0.34$, $P < 0.05$; IFNβ: $r = 0.40$, $P < 0.01$), indicating that $IC_{50}$ and Vres provide different measures of the antiviral effect of IFNs in these compartments. Similarly, $IC_{50}$ values for IFNα2 and IFNβ correlated only weakly ($r = 0.33$, $P = 0.048$), indicating only a partial overlap in the effects of the two IFN subtypes on the activation state, survival, and ISG expression levels of CD4+ T cells.

Combining data from all transmission pairs, we found that recipient isolates were on average significantly more resistant to both IFNα2 and IFNβ than the corresponding donor isolates, exhibiting 7.8-fold ($P < 0.00001$) and 39-fold ($P < 0.00001$) higher $IC_{50}$ values, respectively (Fig. 2.2*D* and *F*). Moreover, recipient isolates had 35-fold ($P < 0.00001$) and 250-fold ($P < 0.00001$) greater odds of replicating at the highest IFNα2 (*SI Appendix*, Fig. S2.6*B*) and

IFNβ (*SI Appendix*, Fig. S2.6*D*) doses than the great majority of donor viruses, respectively. These differences were not dependent on the viral subtype (Fig. 2.2*D* and *F*). Thus, resistance to type 1 IFNs is a characteristic feature of transmitted viruses.

**Selection with IFNβ, but not IFNα2, yields donor isolates with a transmitted virus-like phenotype.** To search for IFN resistant viruses in donor plasma, we treated CD4+ T-cells with high doses of IFNα2 (4.0 pg/ml) or IFNβ (0.44 ng/ml) 24 hours prior to virus isolation. The rationale was to maximally upregulate antiviral ISGs in these target cells (without causing cell toxicity), thereby simulating, at least in part, conditions during the earliest stages of HIV-1 infection. As a control, the same approach was used to isolate viruses from recipient plasma. As expected, the number of viral isolates recovered from pretreated CD4+ T cells was lower than from untreated CD4+ T cells, especially when IFNβ was used for selection (*SI Appendix*, Table S1). Thus, while IFNα2 pretreatment yielded plasma isolates for all donors and recipients, only three donors and two recipients yielded IFNβ preselected plasma isolates. This was as expected since the selection dose of IFNβ was six orders of magnitude higher than the average IFNβ $IC_{50}$ value of all isolates (IFNα2 doses higher than 5.5 pg/ml caused cell toxicity). Phylogenetic analyses of full-length genome sequences revealed no evidence of compartmentalization of selected and non-selected isolates (*SI Appendix*, Fig. S2.7, Table S2.2*C*).

$IC_{50}$ determinations confirmed that donor isolates from IFN pretreated cells were indeed more IFN resistant than those derived from untreated CD4+ T cells, while no changes were observed for recipient isolates (Fig. 2.3). For example, IFNα2- and IFNβ-selected plasma isolates from donor CH492 had mean IFNα2 and IFNβ $IC_{50}$ values than were 7.6-fold and 31-fold higher than those of untreated plasma isolates (Fig. 2.3*A* and *C*). However, resistance to one IFN subtype did not always predict resistance to the other. For donor CH492, IFNβ pretreatment generated isolates that were also highly resistant to IFNα2 (Fig. 2.3*A*), while IFNα2 pretreatment

generated isolates with a wide range of IFNβ $IC_{50}$ values, including some as low as untreated isolates (Fig. 2.3*C*). When results from all donors were combined, both IFNα2- and IFNβ-selected isolates were as resistant to IFNα2 as were untreated recipient isolates (Fig. 2.3*B*). In contrast, IFNα2-selected isolates were 7-fold less resistant to IFNβ than IFNβ-selected isolates, and these in turn were 2-fold less resistant than untreated recipient isolates (Fig. 2.3*D*). Similar results were obtained for Vres, which showed that IFNα2 selection did not generally yield IFNβ resistant isolates, and that IFNβ selected isolates were less resistant to IFNβ than untreated recipient isolates (*SI Appendix*, Fig. S2.6). Interestingly, IFN selection did not increase the $IC_{50}$ or Vres values of recipient isolates, suggesting that transmitted viruses are already maximally resistant to both of these IFN subtypes (Fig. 2.3*B* and *D; SI Appendix*, Fig. S2.6*B* and *D*).

Having generated IFNα2 or IFNβ preselected isolates, we next examined their biological properties. For donor CH492, IFNα2 and IFNβ pretreatment resulted in isolates that packaged 2.0- and 3.3-fold more Env than untreated isolates, respectively (Fig. 2.3*E*). However, no significant differences in Env content were detected between treated and untreated isolates when data from all subjects were combined (Fig. 2.3*F*). However, pretreatment with IFNα2 and IFNβ resulted in donor isolates that exhibited increased infectivity. This was observed for donor CH492 (Fig. 2.3*G*) as well as all donor isolates combined (Fig. 3*H*). IFNα2 and IFNβ pretreatment yielded plasma isolates that were on average 2- and 2.2-fold more infectious, respectively, than isolates obtained without selection, although neither pretreated group was as infectious as the recipient isolates. Interestingly, IFN pretreatment had no effect on the infectivity of recipient isolates (Fig. 2.3*H*).

Reasoning that IFN pretreatment may favor the outgrowth of viruses that replicated to higher titers, we compared the replicative capacity of IFN-selected and unselected donor and recipient isolates. Indeed, pretreatment of CD4+ target cells with IFNβ resulted in donor isolates that replicated more efficiently than untreated viruses, both for CH492 (1.3-fold; Fig. 2.3*I*) and all donor isolates combined (1.3-fold; Fig. 2.3*J*). However, this was not observed when CD4+ T cells

were pretreated with IFNα2. Surprisingly, IFNα2-selected isolates replicated significantly less well, both for donor CH492 (1.7-fold; Fig. 2.3I) and all donor isolates combined (2.1-fold; Fig. 2.3*J*). For each of the seven donors, IFNα2 treatment selected isolates whose replicative capacity was much reduced compared to untreated isolates despite higher infectivity and in some cases greater amounts of packaged Env (e.g., CH492). These data indicate that IFNα2 and IFNβ selection can have opposing effects on some viral properties, and that in contrast to previous suggestions (27), IFN resistance is not simply a consequence of a higher replicative fitness. As expected, IFNα2 and IFNβ selection did not increase the growth potential of recipient isolates (Fig. 2.3*J*). Taken together, these results indicate that both IFNα2 and IFNβ resistant viruses are present, albeit at low levels, in the plasma of chronically infected individuals, and that *in vitro* treatment of CD4+ T cells with IFNβ, but not IFNα2, selects isolates that approach the phenotype of transmitted viruses (Figs. 2.3 and *SI Appendix*, Fig. S2.6).

**Transmitted viruses are more efficiently released from infected cells.** We previously reported that CD4+ T cells infected with TF viruses released larger quantities of cell-free virions than cultures infected with CC viruses (54). However, since only two TF and two CC IMCs were studied, we examined this property in a much larger number ($n$ = 127) of matched donor and recipient isolates. To quantify particle release from infected CD4+ T cells, we measured the amounts of cell-free and cell-associated p24 antigen seven days post-infection, and used these values to calculate the percentage of p24 that was released into the supernatant. Consistent with our previous observations (54), we found that donor isolates produced on average much less cell-free virus than recipient isolates (Fig. 2.4), although the total amount of p24 in these cultures was comparable. Plasma and genital secretion isolates from chronically infected donors released on average 31% and 38% of their total p24, respectively, while recipient isolates released 65%. In addition, IFN selected isolates released more p24 than unselected donor isolates, although this effect was less pronounced for IFNα2 (42%) than for IFNβ (64%). Combining results from all

isolates, the odds of p24 antigen being released from CD4+ T cell cultures infected with IFNα2- and IFNβ-selected donor isolates were 1.6-fold and 3.8-fold higher, respectively, than from cultures infected with untreated donor isolates, and the odds of release were even higher (4.2-fold) for untreated recipient isolates (Fig. 2.4*B*). In contrast, no differences were observed for donor genital secretion isolates as well as for IFN-treated and untreated recipient isolates (Fig. 2.4*B*). Thus, mucosal transmission selects for viruses with a significantly enhanced particle release capacity, suggesting that the production of cell-free virions is important in the transmission process.

**Section 2.5 – Discussion**

An effective AIDS vaccine will need to prevent acquisition of HIV-1 at mucosal surfaces (5). In this context, it is critical to know whether transmitted viruses possess unique biological properties that predispose them to establish new infections more efficiently. This is a controversial topic, since some studies have reported TF-specific traits (22, 24, 26, 52, 55-57), while others have failed to confirm these results (27, 28, 53, 58, 59). Some of these discrepancies are likely due to the fact that most previous analyses did not compare HIV-1 strains from transmission pairs. Using a more rigorous approach, two recent studies characterized viruses from matched donors and recipients, but failed to identify viral properties that were indicative of enhanced transmission fitness (27, 28). These findings led to the prevailing view that HIV-1 transmission is a stochastic process in which any reasonably fit virus has the potential of crossing the mucosa.

Both transmission pair studies characterized only very few donor and recipient viruses, using either infectious molecular clones (27) or PBMC-derived bulk cultures (28). Reasoning that this approach had likely led to erroneous conclusions, we used limiting dilution isolation to generate a much larger number of donor and recipient viruses for phenotypic comparisons. We also used plasma rather than PBMCs for virus isolation to preclude the characterization of archived HIV-1 strains, generated genital secretion isolates for a subset of donors, and examined viral properties, such as virion release and resistance to IFNβ, which have not previously been characterized. Finally, we rendered the CD4+ T cells used for virus isolation more resistant to infection by treating them with high doses of type 1 IFNs to simulate host innate defenses that may be operative during the earliest stages of infection. We found that both recipient and *in vitro* IFN-selected donor isolates were more infectious, replicated to higher titers, were released from infected cells more efficiently, and were much more resistant to both IFNα2 and IFNβ than the

great majority of unselected donor isolates (Figs. 2.1-4). Thus, it seems clear that these viral properties collectively contribute to transmission fitness.

To visualize the biological properties examined for all virus isolates (particle Env content, infectivity, replicative capacity, IFN $IC_{50}$ and Vres values) in combination, we conducted a principal component analysis (Fig. 2.5*A* and *B*). This approach revealed two major groups, one that contained all plasma and genital secretion isolates from chronically infected donors, and another that included all plasma isolates from acutely infected recipients (Fig. 2.5*A*). The fact that there was no overlap between these groups indicates that transmitted viruses are phenotypically distinct. This conclusion was confirmed when IFN-treated isolates were plotted on the same principal components (Fig. 2.5*B*). While most IFNα2 selected donor isolates grouped within the untreated donor cluster, most IFNβ selected donor isolates overlapped the cluster of recipient viruses (Fig. 2.5*B*).

To quantify these relationships, we calculated the distance between each virus and its pair-matched recipient average of the first two principal components (Fig. 2.5*C*). As expected, untreated and IFN treated recipient isolates were the closest to the recipient average, exhibiting only minimal variation. In contrast, untreated donor plasma and genital secretion isolates as well as IFNα2-selected donor isolates were most distant from the average position of their respective recipient isolates and exhibited a wide distribution of distances. Interestingly, IFNβ-selected donor isolates were much closer to their recipient isolate average, consistent with IFNβ selection yielding a transmitted virus-like phenotype. We also examined the accuracy with which an isolate could be predicted to be derived from either a donor or a recipient on the basis of the seven biological properties examined (Fig. 2.5*D*). This analysis showed that IFN $IC_{50}$ and Vres values predicted donor and recipient isolates with near 100% accuracy, indicating that resistance to type 1 IFNs is the most distinguishing characteristic of transmitted viruses.

If IFN resistance represents such a discerning feature, why did previous transmission pair studies miss this property? As shown in Fig. 2.2, chronic viruses exhibit a wide range of IFN $IC_{50}$ values, indicating that random selection of just two such viruses per transmitting donor as reported by Deymier *et al* (27) may not reveal donor/recipient differences. Moreover, measuring viral inhibition in response to a single IFN dose (26-28, 53) is likely less accurate than a formal $IC_{50}$ determination. It should also be noted that the resistance of HIV-1 to IFNs is not constant during the course of infection. IFN resistance declines rapidly within the first six months (52, 57), but then increases again when subjects progress toward AIDS (52, 60, 61). Thus, depending when during the course of infection a virus is transmitted to another person, donor viruses may be more or less IFN resistant. For example, viruses from donors who transmit during acute HIV-1 infection or immediately following treatment interruption as described by Oberle *et al* (28) would be expected to exhibit much higher levels of IFN resistance than viruses from subjects who transmit during asymptomatic chronic infection. In addition, PBMC cultures may reactivate latent viruses, which would be expected to exhibit IFN resistance levels consistent with their entry into the latent pool.

None of the previous transmission pair studies analyzed viral resistance to IFNβ, which produced the most pronounced donor/recipient differences. Indeed, the 39-fold higher IFNβ $IC_{50}$ values of recipient isolates (Fig. 2.2*F*) is likely a gross underestimate, since many donor viruses were already more than 50% inhibited at the lowest IFNβ dose (Fig. 2.2*B*). This explains why the donor/recipient differences for IFNβ Vres values are so much higher than the corresponding $IC_{50}$ values, and why this is not observed for IFNα2 (Figs. 2.2*F* and *SI Appendix*, Fig. S2.5*D*). While both $IC_{50}$ and Vres values provide an indicator of IFN resistance, they seem to describe only partially overlapping biological effects. For example, the strong correlation of both IFNα2 and IFNβ $IC_{50}$ and Vres values for donor plasma isolates likely indicates restriction by an IFN dose-driven increase in interferon stimulated gene (ISG) activity. In contrast, the lack of a similarly strong correlation for donor genital secretion isolates suggests that some of these viruses are

restricted by ISGs whose inhibitory activity is not IFN dose dependent. In addition, Vres may be a more relevant indicator of IFN resistance during the acute phase of infection when IFN levels are particularly high in the mucosa, while $IC_{50}$ may be a more appropriate measure of systemic immune activation during later stages of infection. Future studies will need to determine the full range of IFNα2 and IFNβ $IC_{50}$ and Vres values in HIV-1 infected subjects over time.

Not all viral properties studied contributed, or contributed equally, to HIV-1 transmission fitness. For example, virion associated Env content, although previously identified as a characteristic feature of TF viruses (26), did not differentiate donor and recipient isolates (Figs. 2.1*C* and 2.5*D*). Nonetheless, in half of the transmission pairs studied, recipient isolates packaged significantly more Env than the respective donor viruses (Fig. 2.1*B*), suggesting that increased Env content may increase transmission fitness under certain circumstances. Similarly, particle infectivity and replicative capacity were significantly increased in most, but not all, recipient isolates. The successful transmission of viruses lacking these properties suggests that they are not absolutely required and/or that their absence can be compensated by other factors. In contrast, enhanced resistance to type I IFNs was observed for every single recipient isolate, indicating that the ability to counteract these innate immune responses is essential for successful mucosal transmission.

The need to overcome innate defenses is also exemplified by the fact that recipient and IFNβ-selected donor isolates produced much higher levels of cell-free virus than the corresponding untreated donor isolates (Fig. 2.4). Type 1 IFNs induce tetherin, which prevents the release of virus particles from the plasma membrane of infected cells. HIV-1 counteracts tetherin using its Vpu protein, which binds tetherin and prevents its expression on the cell surface (62-64). However, TF Vpu proteins do not seem to counteract tetherin more effectively than the Vpu protein of chronic viruses (65). Moreover, TF infected CD4 T cells were shown to produce more cell free virions even in the absence of Vpu (54). Although we have not mapped the genomic region(s) responsible for the significantly enhanced virion release capacity of recipient

isolates, it is unlikely that Vpu alone is responsible. In fact, several isolates that differed significantly in their particle release function encoded identical *vpu* genes (*SI Appendix*, Fig. S2.7). Thus, it seems clear that other as-yet-unknown factors must be responsible for the increased particle release function of recipient (and IFNβ-selected donor) isolates, which may be critical to enhance virus spread in the mucosa during the first rounds of replication when extracellular IFN levels are particularly high.

In summary, we have identified resistance to type 1 IFNs, in particular IFNβ, as a key determinant of HIV-1 transmission fitness. This observation is consistent with previous studies showing that innate responses in the mucosa immediately following infection are inducing a potent antiviral state through the upregulation of ISGs, many of which have anti-HIV-1 activity (63, 64, 66-73). All IFN subtypes signal through the same heterodimeric receptor (30), but differences in receptor binding and/or downstream signal transduction pathways are thought to be responsible for IFN subtype-specific biological effects (74-77). IFNβ has been reported to bind the IFN receptor (IFNAR) with the highest affinity (76) and ligates the IFNAR1 chain in an IFNAR2-independent manner, resulting in the expression of a distinct set of genes (78). Either of these properties could explain its greater potency in placing selection on the transmitted virus pool. Nonetheless, IFNβ selection did not fully recapitulate the biological properties of recipient isolates despite the extremely high dose that was used to treat the target cells (Figs. 2.3*D,* 5*B,* 5*C* and *SI Appendix*, Fig. S2.6*D*). These results indicate that additional factors, possibly including IFNα2 and/or other IFN subtypes, shape the transmitted founder phenotype. Since there are a total of 13 IFNα subtypes as well as other type 1 IFNs such as IFNω, some of which inhibit HIV-1 even more potently *in vitro* and in animal models (79-81), it will be critical to evaluate to what extent they contribute alone, or in combination, to the HIV-1 transmission bottleneck.

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**Section 2.8 – Chapter Figures**

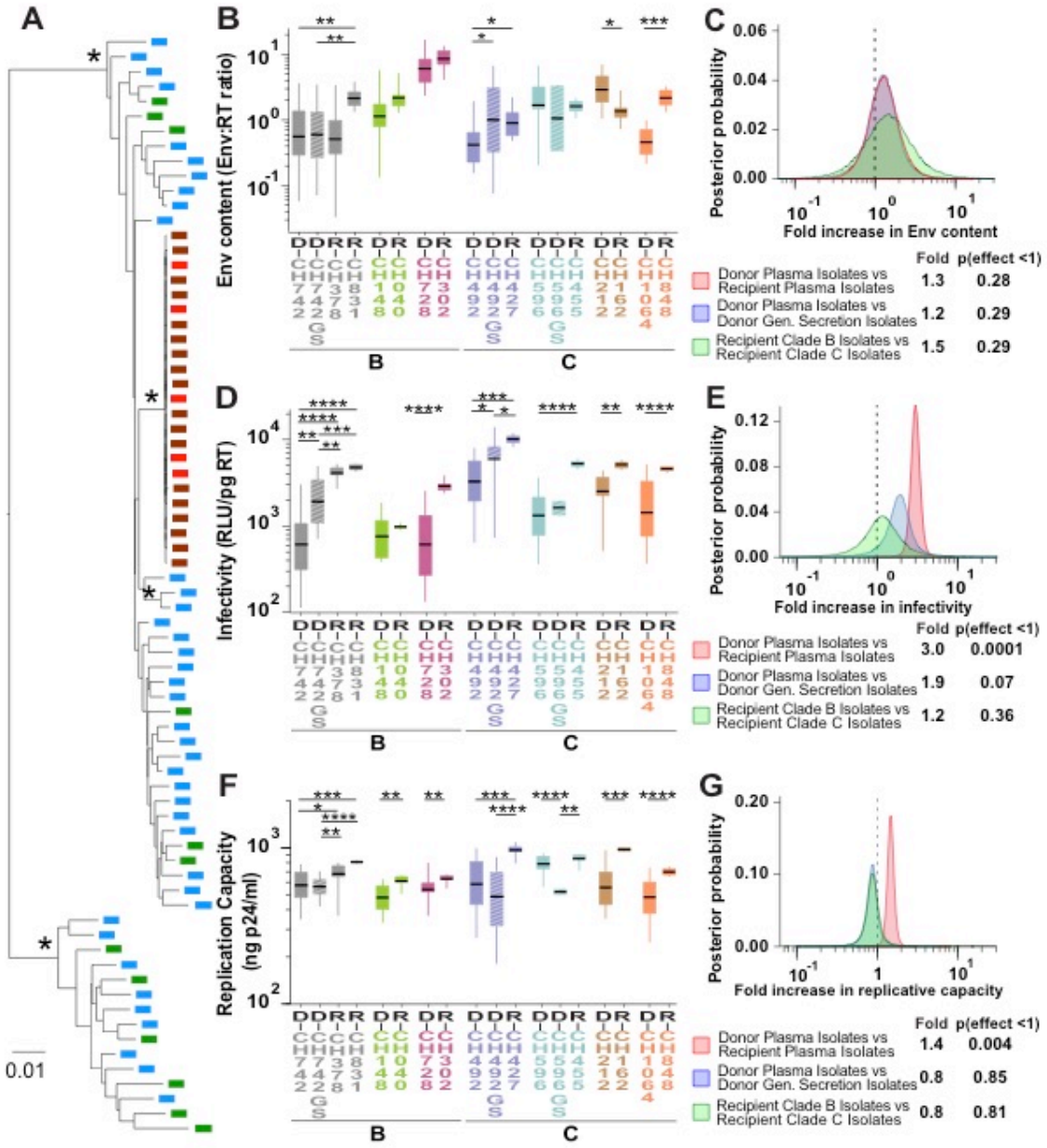

**Fig. 2.1. Genetic and biological characterization of matched donor and recipient limiting dilution-derived isolates.** (*A*) The phylogenetic relationships of donor (green) and recipient (brown) isolate sequences to donor (blue) and recipient (red) SGA-derived plasma viral sequences are shown for the CH596-CH455 transmission pair (maximum likelihood trees for all other transmission pairs are shown in *SI Appendix*, Fig. S2.3). Asterisks denote nodes with 100% bootstrap support (the scale bar indicates 0.01 substitutions per site). (*B, D, F*) Viral Env content (mass ratio of gp120 and RT), particle infectivity (relative light units [RLU] in TZM-bl cells per picogram of RT), and replicative capacity (p24 antigen levels in CD4+ T cell culture supernatants seven days post-infection) of plasma isolates from matched donor (D) and recipient (R) pairs (color-coded) are shown, with HIV-1 subtype classification indicated below. Data are grouped for each transmission pair, with genital secretion isolates (GS) shown as hashed boxes. Donor D-CH472 transmitted to two recipients R-CH378 and R-CH831. Boxes show the interquartile range, a black bar within each box indicates the geometric mean, and whiskers span the range of the data, respectively. Asterisks indicate significant differences (determined by unpaired t-test) between groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). (*C, E, G*) A hierarchical Bayesian regression model was used to estimate the population-wide fold change of Env content (*C*), particle infectivity (*E*), and replicative capacity (*G*) across all transmission pairs between donor and recipient plasma isolates (red), donor plasma and genital (Gen.) secretion isolates (blue), and clade B and C recipient isolates (green). A dashed line indicates a fold change of 1, indicating no effect. The estimated posterior probability distribution for each parameter is shown along with a table summarizing the expected fold change and the probability that the effect is less than 1 (analogous to a one-sided p-value).

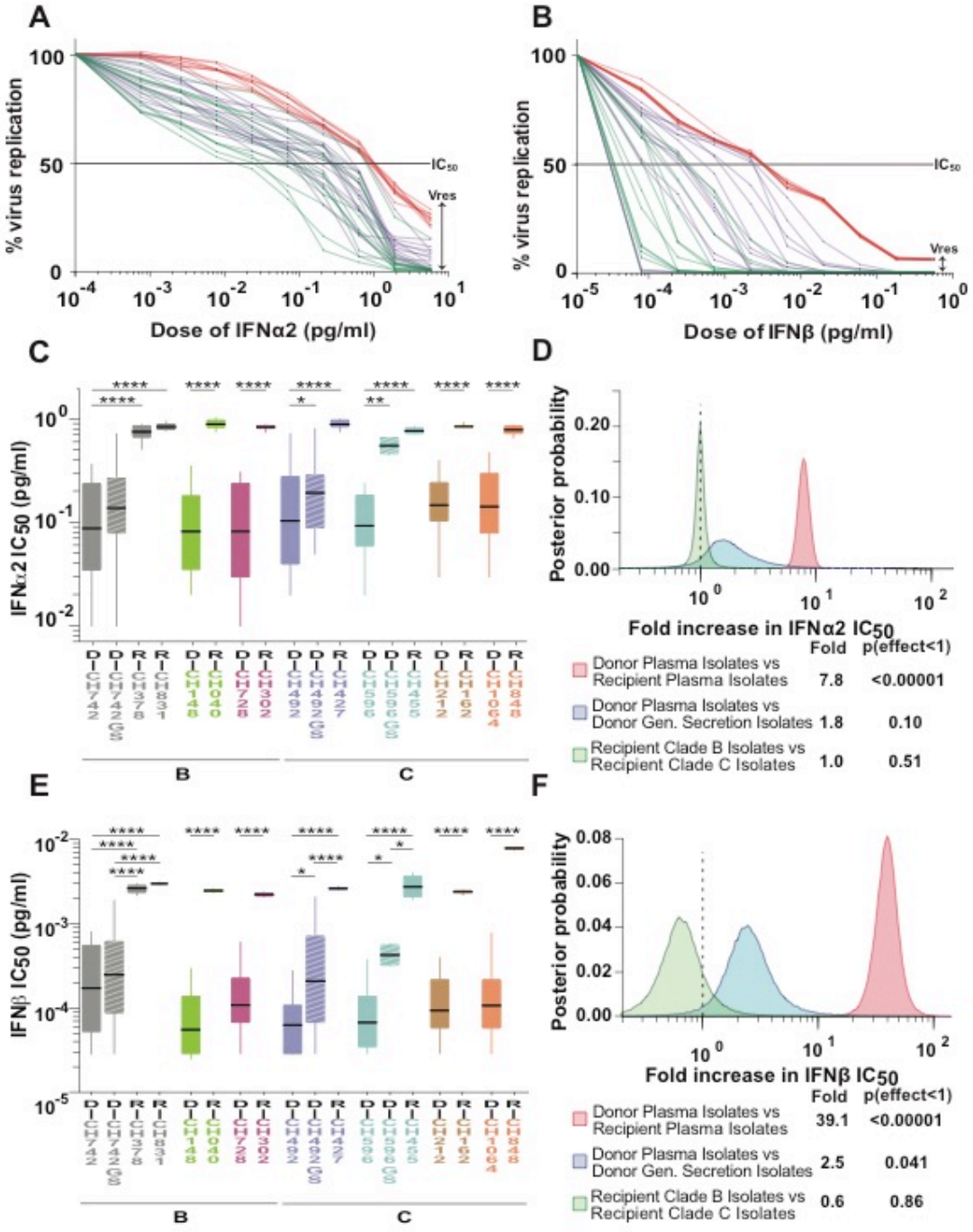
A
% virus replication
IC50
Vres
Dose of IFNα2 (pg/ml)
B
% virus replication
IC50
Vres
Dose of IFNβ (pg/ml)
C
IFNα2 IC50 (pg/ml)
B
C
D
Posterior probability
Fold increase in IFNα2 IC50
Fold
p(effect<1)
Donor Plasma Isolates vs Recipient Plasma Isolates 7.8 <0.00001
Donor Plasma Isolates vs Donor Gen. Secretion Isolates 1.8 0.10
Recipient Clade B Isolates vs Recipient Clade C Isolates 1.0 0.51
E
IFNβ IC50 (pg/ml)
B
C
F
Posterior probability
Fold increase in IFNβ IC50
Fold
p(effect<1)
Donor Plasma Isolates vs Recipient Plasma Isolates 39.1 <0.00001
Donor Plasma Isolates vs Donor Gen. Secretion Isolates 2.5 0.041
Recipient Clade B Isolates vs Recipient Clade C Isolates 0.6 0.86

**Fig. 2.2. IFN resistance of matched donor and recipient isolates.** (*A, B*) Dose response curves for IFNα2 (*A*) and IFNβ (*B*) are shown for plasma (green) and genital secretion (magenta) isolates of one chronically infected donor as well as plasma isolates of the corresponding acutely infected recipient (red) of a representative transmission pair (CH492-CH427). A black line indicates the half-maximal inhibitory concentration ($IC_{50}$), and a double arrow the residual viral replication (Vres) capacity at the highest IFN dose. (*C, E*) IFNα2 (*C*) and IFNβ (*E*) concentrations (picograms per ml), which resulted in 50% viral inhibition, are shown for plasma isolates from matched donor (D) and recipient (R) pairs (color-coded as in Fig. 2.1), with HIV-1 subtype classification indicated below. Data are grouped for each transmission pair, with genital secretion isolates (GS) shown as hashed boxes. Donor D-CH472 transmitted to two recipients R-CH378 and R-CH831. Boxes show the interquartile range, a black bar within each box indicates the geometric mean, and whiskers span the range of the data, respectively. Asterisks indicate significant differences (determined by unpaired t-test) between groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). IFN $IC_{50}$ values were determined in pooled CD4+ T-cells from multiple donors. (*D, F*) A hierarchical Bayesian regression model was used to estimate the population-wide fold change of IFNα2 (*D*) and IFNβ (*F*) $IC_{50}$ values across all transmission pairs between donor and recipient plasma isolates (red), donor plasma and genital (Gen.) secretion isolates (blue), and clade B and C recipient isolates (green). A dashed vertical line marks a fold change of 1 indicating no effect. The estimated posterior probability distribution for each parameter is shown along with a table summarizing the expected fold change and the probability that the effect is less than 1 (analogous to a one-sided p-value).

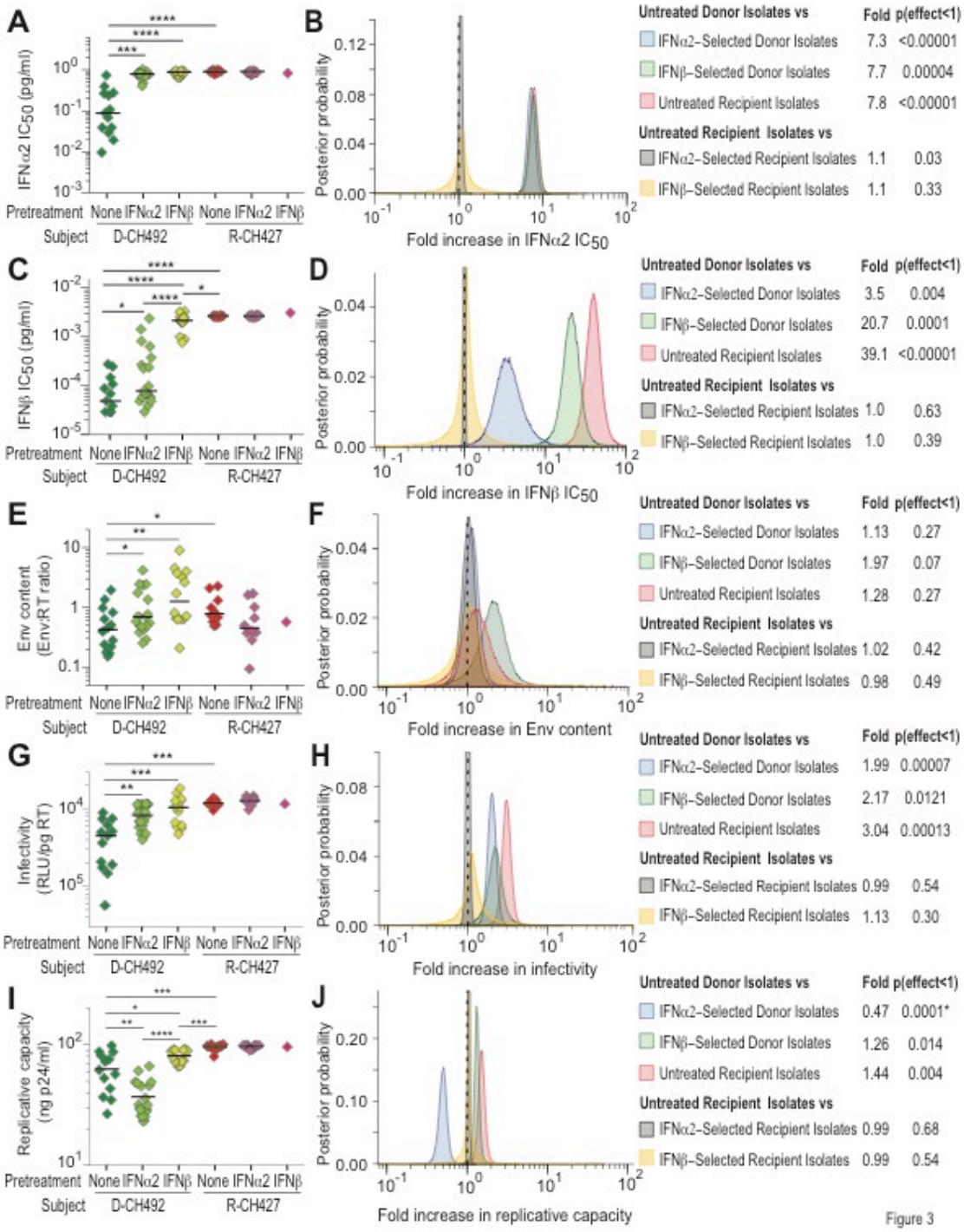
A
IFNα2 IC50 (pg/ml)
Pretreatment None IFNα2 IFNβ None IFNα2 IFNβ
Subject D-CH492 R-CH427
B
Posterior probability
Fold increase in IFNα2 IC50
Untreated Donor Isolates vs Fold p(effect<1)
IFNα2-Selected Donor Isolates 7.3 <0.00001
IFNβ-Selected Donor Isolates 7.7 0.00004
Untreated Recipient Isolates 7.8 <0.00001
Untreated Recipient Isolates vs
IFNα2-Selected Recipient Isolates 1.1 0.03
IFNβ-Selected Recipient Isolates 1.1 0.33
C
IFNβ IC50 (pg/ml)
D
Fold increase in IFNβ IC50
Untreated Donor Isolates vs Fold p(effect<1)
IFNα2-Selected Donor Isolates 3.5 0.004
IFNβ-Selected Donor Isolates 20.7 0.0001
Untreated Recipient Isolates 39.1 <0.00001
Untreated Recipient Isolates vs
IFNα2-Selected Recipient Isolates 1.0 0.63
IFNβ-Selected Recipient Isolates 1.0 0.39
E
Env content (Env:RT ratio)
F
Fold increase in Env content
Untreated Donor Isolates vs Fold p(effect<1)
IFNα2-Selected Donor Isolates 1.13 0.27
IFNβ-Selected Donor Isolates 1.97 0.07
Untreated Recipient Isolates 1.28 0.27
Untreated Recipient Isolates vs
IFNα2-Selected Recipient Isolates 1.02 0.42
IFNβ-Selected Recipient Isolates 0.98 0.49
G
Infectivity (RLU/pg RT)
H
Fold increase in infectivity
Untreated Donor Isolates vs Fold p(effect<1)
IFNα2-Selected Donor Isolates 1.99 0.00007
IFNβ-Selected Donor Isolates 2.17 0.0121
Untreated Recipient Isolates 3.04 0.00013
Untreated Recipient Isolates vs
IFNα2-Selected Recipient Isolates 0.99 0.54
IFNβ-Selected Recipient Isolates 1.13 0.30
I
Replicative capacity (ng p24/ml)
J
Fold increase in replicative capacity
Untreated Donor Isolates vs Fold p(effect<1)
IFNα2-Selected Donor Isolates 0.47 0.0001*
IFNβ-Selected Donor Isolates 1.26 0.014
Untreated Recipient Isolates 1.44 0.004
Untreated Recipient Isolates vs
IFNα2-Selected Recipient Isolates 0.99 0.68
IFNβ-Selected Recipient Isolates 0.99 0.54

Figure 3

**Fig. 2.3. Biological characterization of IFNα2- and IFNβ-selected donor and recipient isolates.** (*A, C, E, G, I*) IFNα2 $IC_{50}$ (picogram per ml) (*A*), IFNβ $IC_{50}$ (picogram per ml) (*C*), viral Env content (mass ratio of gp120 and RT) (*E*), particle infectivity (RLU per picogram of RT) (*G*), and replicative capacity in CD4+ T cells (ng of p24 antigen per ml) (*I*) values are shown for limiting dilution derived viral isolates from one representative matched donor (D-CH492) and recipient (R-CH427) pair. In each panel, untreated (dark green), IFNα2-selected (light green), and IFNβ–selected (yellow) isolates from the donor (D-492) are compared to untreated (red), IFNα2-selected (dark pink) and IFNβ-selected (light pink) isolates from the corresponding recipient R-CH427. Boxes show the interquartile range, a black bar within each box indicates the geometric mean and whiskers span the range of the data, respectively. Asterisks indicate significant differences (determined by unpaired t-test) between groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Since IFN selection did not alter the phenotype of recipient isolates, only statistical comparisons of donor isolates to untreated recipient isolates are shown. (*B, D, F, H, J*) A hierarchical Bayesian regression model was used to estimate the population-wide fold change of IFNα2 $IC_{50}$ (*B*), IFNβ $IC_{50}$ (*D*), Env content (*F*), particle infectivity (*H*), and replicative capacity in CD4+ T cells (*J*) across all transmission pairs between untreated and IFNα2-selected donor isolates (blue), untreated and IFNβ-selected donor isolates (green), untreated and IFNα2-selected recipient isolates (grey), and untreated and IFNβ-selected recipient isolates (yellow). The fold change between untreated donor and recipient plasma isolates (red), as in Figs. 2.1 and 2.2, is also shown for comparison. A dashed vertical line marks a fold change of 1 indicating no effect. The estimated posterior probability distribution for each parameter is shown along with a table summarizing the expected fold change and the probability that the effect is less than 1 (or where indicated by an asterisk (*) the probability that the effect is greater than 1).

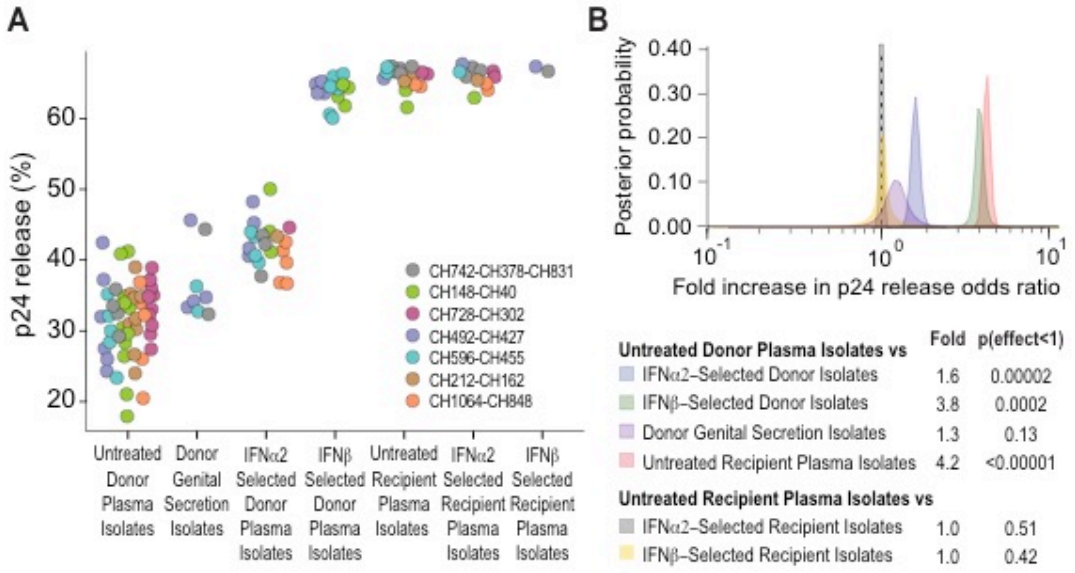


**Fig. 2.4. Particle release capacity of matched donor and recipient isolates.** (*A*) Donor and recipient isolates were tested for their ability to be released from infected CD4+ T cells. The percent of viral release was determined as the ratio of cell-free p24 divided by the total amount (cell-associated plus cell-free) of p24 seven days post-infection. Only a subset of isolates (*n* = 132) was tested. Values are color coded by transmission pair. (*B*) A hierarchical Bayesian regression model was used to estimate the population-wide fold change in the odds of release (the probability of release divided by the probability of retention) of p24 between untreated and IFNα2-selected donor plasma isolates (blue), untreated and IFNβ-selected donor plasma isolates (green), untreated donor plasma and genital secretion isolates (purple), untreated donor and recipient plasma isolates (red), untreated and IFNα2 selected recipient isolates (grey), and untreated and IFNβ-selected recipient isolates (yellow). A dashed vertical line marks a fold change of 1 indicating no effect. The estimated posterior probability distribution for each parameter is shown along with a table summarizing the expected fold change and the probability that the effect is less than 1 (analogous to a one-sided p-value).

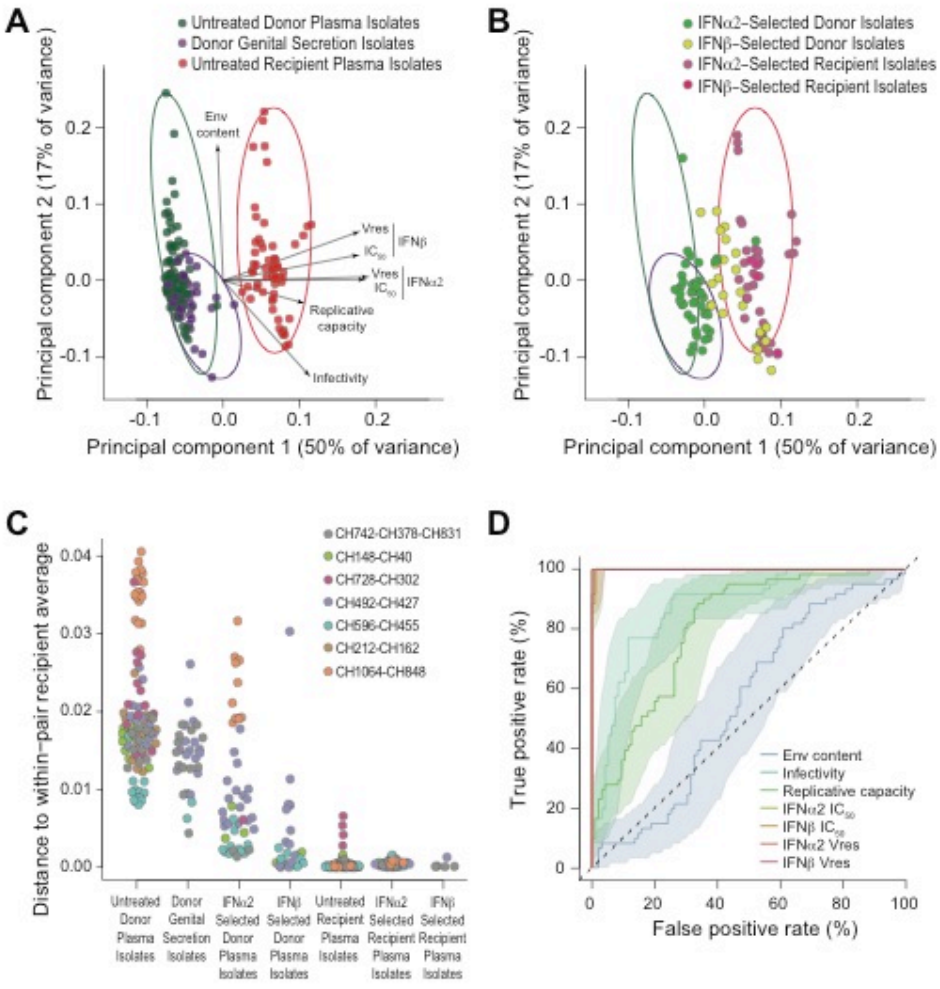


**Fig. 2.5. Phenotypic properties distinguishing donor and recipient isolates.** (*A*) A principal component analysis was used to visualize properties that were determined for all viral isolates (Env content, particle infectivity, replicative capacity, IFNα2 $IC_{50}$, IFNβ $IC_{50,}$ IFNα2 Vres and IFNβ Vres) in combination. The positions of untreated donor plasma (green), donor genital secretion (purple) and recipient plasma (red) isolates are shown on the first two components. Length and direction of arrows show how each variable contributes to the two axes. The minimum spanning ellipses that contain all data points for each group are shown in corresponding colors. (*B*) To visualize the effect of IFN-selection, IFNα2-selected (green) and IFNβ-selected (yellow) donor

isolates are plotted together with IFNα2-selected (dark pink) and IFNβ-selected (light pink) recipient isolates on the same principal components as in *A*. Minimum spanning ellipses encompassing the untreated donor plasma isolates (green), donor genital secretion isolates (purple) and untreated recipient plasma isolates (red) as shown in *A* were retained. (*C*) To quantify the groupings apparent in *A* and *B*, we calculated the distance of the first two principal components for each isolate to the average position of the corresponding untreated recipient isolates for that transmission pair. Isolates are color-coded by transmission pairs and grouped as in *A* and *B*. (*D*) The accuracy with which the seven viral properties predicted whether an isolate came from a donor or recipient was measured using receiver operating characteristic curves. Each line indicates the trade-off between true and false positive rate as a threshold is moved through the range of the data. Shading indicates the 95% confidence interval of the true positive rate. The dashed line indicates the expected performance of a predictor with no relationship to donor-recipient status. A line that reaches a true positive rate of 100% with a 0% false positive rate indicates that there is perfect separation between donor and recipient isolates.

**Section 2. 9 – Supplemental Information and Materials Methods**

**Study subjects.** Plasma samples were obtained for seven chronically HIV-1 infected transmitting donors and eight matched acutely infected recipients enrolled in the CHAVI-001 acute and established HIV-1 infection cohorts (1). A summary of available epidemiological, clinical and infection status data is shown in Table S1. In all but one case, acutely infected individuals were identified first and staged using the Fiebig classification (2), while the transmitting partners were identified retrospectively. Epidemiological linkage was confirmed through viral sequence analysis, which also indicated considerable quasispecies diversity in all transmitting donors except CH1064, who may have transmitted during earlier stages of infection (Fig. S2.1). Transmission pairs were selected based on the following criteria: (i) high transmitting donor plasma viral loads (generally >100,000 vRNA copies/ml) to increase the likelihood of obtaining virus isolates, (ii) availability of donor genital secretions within a year following transmission, (iii) single transmitted founder infections of the recipients to ensure a stringent mucosal bottleneck (one recipient was subsequently found to harbor two transmitted founder viruses), and (iv) absence of antiretroviral treatment. Whole blood was collected in acid citrate dextrose, and plasma was separated and stored at −80°C. In addition, cell-free fractions of genital secretions (GS) were obtained from five of the seven donors. Ectocervicovaginal lavage (CVL) was performed on non-menstruating women using 10 ml of phosphate buffered saline (PBS). Semen ejaculate was collected in 2.5ml of transport medium (RPMI 1640, 1,000 U/ml penicillin, 1 mg/ml streptomycin, 200U/ml nystatin). Genital secretion samples were centrifuged for 10 min at 800g to pellet cells; supernatants were harvested, aliquoted and stored at -80˚C. Written informed consent was obtained from each subject and the study was approved by the Institutional Review Boards of the University of Pennsylvania and Duke University.

**Human CD4+ T-cell isolation and activation.** CD4+ T-cells were positively selected from buffy coats of normal subjects (Research Blood Component, Boston, MA or ZenBio Inc., Research Triangle Park, NC) using Human CD4 Microbeads (Miltenyi Biotec Inc., San Diego, CA), viably frozen in CryoStor® CS5 medium (Sigma-Aldrich, St Louis, MO), and stored in liquid nitrogen. Cell aliquots were thawed quickly in a 37°C water bath, resuspended at a density of $2\times10^6$ cells/ml, and allowed to recover overnight in RPMI 1640 medium containing 15% (vol/vol) fetal bovine serum (FBS) and 30 IU/mL interleukin-2 (IL-2) (CD4+ T-cell medium) in a 37°C incubator with 5% (vol/vol) $CO_2$. Cells were stimulated using the Human T Cell Activation/Expansion Kit (Miltenyi Biotec Inc., San Diego, CA) and expanded for 4-5 days in CD4+ T-cell medium following the manufacturer's protocol.

**Interferons and cytokines.** IFN$\alpha$2 was purchased from PBL Assay Science (Piscataway, NJ). IFN$\beta$ was purchased from PBL Assay Science and EMD Serono USA (Rebif®, EMD Serono Inc., Rockland, MD). Interleukin-2 was purchased from the Hospital of the University of Pennsylvania pharmacy (Aldesleukin).

**Virus quantification.** Viral stocks were characterized by determining their reverse transcriptase (RT) activity using the colorimetric Reverse Transcriptase Assay (Sigma-Aldrich, St Louis, MO), and their p24 antigen content was determined using the HIV p24 (high sensitivity) AlphaLISA Detection Kit (Perkin Elmer Inc., Boston, MA).

**Generation of limiting dilution-derived HIV-1 isolates.** To generate limiting dilution-derived viral isolates, plasma samples were end-point diluted and used to infect activated normal donor CD4+ T-cells in 24 well plates such that no more than 30% of wells became p24 antigen positive. Assuming that approximately one virus per 1,000 particles is infectious, we started with ~500 vRNA copies/well. Plasma aliquots containing ~12,500 vRNA copies were diluted in 1ml of CD4+ T-cell medium containing 50µl of HIV Infectivity Enhancement Reagent (Miltenyi Biotec Inc., San Diego, CA). To allow the formation of HIV-1 enhancement complexes, tubes were placed on a MACSmix Tubes Rotator (Miltenyi Biotec Inc., San Diego, CA) and incubated at 4˚C under constant rotation at 4 rpm for 30 min. Activated CD4+ T-cells were seeded ($1x10^6$ per well) in a 24-well plate in 500µl of fresh CD4+ T-cell medium; 40µl of the complex-containing solution were added to each well (500 vRNA copies/well), incubated for 12-16 hours in a 37°C incubator with 5% (vol/vol) $CO_2$, and then supplemented with an additional 1ml of T-cell medium. At days 5, 10 and 15, activated CD4+ T-cells ($1x10^6$ in 500µl of T-cell medium) were added to each well to provide new target cells for virus replication. At day 20, p24 positive wells were identified using the HIV p24 (high sensitivity) AlphaLISA detection kit (Perkin Elmer Inc., Boston, MA). For some plasma samples the number of vRNA copies per well had to be adjusted to reach limiting-dilution conditions. These values ranged between 3 vRNA copies/well (CH831) to 2,000 vRNA copies/well (CH040), indicating a wide range of per particle infectivity, including in acute infection plasmas.

Low viral loads in plasma samples from subjects CH162, CH728 and CH302 (Table S1) required larger volumes of plasma to reach the target dose of 500 vRNA copies per well. These larger volumes decreased cell viability during the first incubation step of the isolation procedure. We thus purified the same amount of virus particles from these samples prior to infection using the µMACS VitalVirus HIV Isolation Kit (Miltenyi Biotec Inc., San Diego, CA). Virus-microbead complexes were eluted from µ-Columns in 1ml of T-cell medium and 40µl were added to each well (500 vRNA copies/well) of activated target T-cells as described above.

To generate IFN resistant virus isolates, activated CD4+ T-cells were pre-treated with 4.0 pg/ml of IFN$\alpha$2 or 44 ng/ml of IFN$\beta$ for 24 hours prior to isolation with no further addition of IFN. The rationale was to maximally upregulate antiviral ISGs in these target cells, but to then isolate virus in the absence of interferon. For IFN$\beta$, we were able to use a selection dose that was six orders of magnitude higher than the average IFN$\beta$ $IC_{50}$ value of all isolates. However, this was not possible for IFNα2 since doses higher than 5.5 pg/ml caused toxicity in the culture. Thus, IFNα2 pretreatment was kept at 4.0 pg/ml. Following treatment, cells were washed once in T-cell medium before being plated in 24-well plates as described above.

For virus isolation from CVL and semen samples, aliquots were thawed at room temperature, fetal bovine serum was added to a final concentration of 2%, and virus isolation was performed as described above. Since virus load information was not available, 1ml aliquots were used per well for one 24-well isolation plate. To inhibit the growth of bacteria, yeast and fungi, T-cell medium was supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and Amphotericin B (0.25 µg/ml) (Gibco® Antibiotic-Antimycotic, ThermoFisher Scientific, Waltham, MA). While isolation attempts from all plasma samples were successful, the efficiency of isolation from genital secretion samples was variable: only 1 of 4 CVL samples from donor CH492, 1 of 2 CVL samples from donor CH596, and 4 of 5 semen samples from donor CH742 yielded isolates (Table S1). In contrast, none of 4 CVL and semen samples from donors CH1064 and CH728, respectively, yielded isolates (Table S1).

**Virus stock preparation and genotyping**. Cells and supernatants from p24 positive wells were transferred to T25 flasks containing $10x10^6$ activated T-cells in 10ml of fresh T-cell medium. After 5 days of culture, an additional $10x10^6$ activated T-cells in 10ml of fresh T-cell medium were added to each flask. At day 10 post-infection, virus-containing supernatant was passed through a 0.45µm nylon membrane syringe filter (Corning, NY) and stored in aliquots at -80˚C.

To sequence each expanded virus isolate prior to its biological characterization, viral RNA was extracted from 100µl of culture supernatant, reverse-transcribed using SuperScript III Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA) and previously published primers (3, 4), and the resulting cDNA was used to amplify overlapping 5' and 3' genome halves in separate triplicate PCR reactions as described (3-5). Ten nanograms (1µl of a 1/25 dilution of the PCR reaction) of each of the 5' and 3' amplicons were then pooled and sequenced using an Illumina NGS platform. DNA libraries were prepared using the Nextera DNA Library Preparation Kit (Illumina Inc, San Diego, CA), as previously described (6, 7) with minor modifications. Briefly, amplicons were fragmented using Nextera tagmentation buffers TD and TDE1 in a final volume of 2.5µl. The tagmentation reaction was subjected to two rounds of PCR amplification using the KAPA Library Amplification Kit (Kapa Biosystems Inc., Wilmington, MA). The first round of PCR incorporated Index 1 (N7xx) and Index 2 (S5xx) adapters (final volume 7.5µl; 98°C for 3 min followed by 8 cycles, 98°C for 15 sec, 62°C for 30 sec, 72°C for 1.5 min); the second round of PCR (final volume 17µl; 95°C for 5 min followed by 7 cycles, 98°C for 20 sec, 62°C for 20 sec, 72°C for 30 sec) was performed with the Nextera adapter primers P1 (5'-AATGATACGGCGACCACCGA-3') and P2 (5'-CAAGCAGAAGACGGCATACGA-3') to enrich the library for tagmented fragments containing the Index 1-Index 2 adapter combination at their ends. DNA libraries were prepared in 96-well plates. Agencourt AMPure XP beads (Beckman Coulter Inc., Indianapolis, IN) were used to purify PCR amplicons and to size select ~300bp fragments; eight PCR reactions from each column were combined into a single tube and incubated with 136µl of AMPure XP beads for 5 min at room temperature. Beads were washed thrice with 70% ethanol, air-dried for 5 min, and the bound DNA was eluted in 100µl of 10mM Tris-HCl (pH 8.0). Eluted DNA from all 96 wells were pooled and quantified using Qubit (ThermoFisher Scientific, Waltham, MA) and the Agilent DNA 1000 Kit on a 2200 TapeStation instrument (Agilent Technologies, Santa Clara, CA) to determine the molar concentration of the ~300bp fragments. The pooled library was then diluted to a concentration of 4nM in 10mM Tris-HCl (pH 8.0) and stored at -20˚C until sequencing. Sequencing was performed using Illumina MiSeq or MiniSeq

instruments (Illumina Inc, San Diego, CA). Libraries containing 96 samples were run using Illumina MiSeq Nano Kit v2 300. Libraries containing 192 samples were run using Illumina MiSeq Micro Kit v2 300 or Illumina MiniSeq Mid Output Kit 300 (Illumina Inc., San Diego, CA).

All reads were trimmed of adapter sequences, and paired-end reads were combined into a single file, binned for each sample based on index sequences, and assembled to a subtype-matched HIV-1 reference genome using the Cutadapt and SPAdes genome assembler utilities (8, 9). Contigs that aligned to the HIV-1 reference sequence were then used to generate a new sample-specific consensus sequence using reads from triplicate amplifications. Reads were then re-aligned to this sample-matched reference to generate a final full-length isolate consensus sequence using Geneious 9.0 (10). Each nucleotide position of this consensus sequence was inspected for the presence of mixed bases, and isolates that exhibited more than 15% diversity at any one position in the alignment were considered to contain more than one variant and removed from further analysis.

**Particle Env content.** 250µl aliquots of viral stocks were depleted of microvesicles using magnetic beads coated with anti-CD45 antibody (ThermoFisher Scientific, Waltham, MA). Virions were then isolated from the microvesicle-depleted supernatants using the VitalVirus HIV Isolation kit (Miltenyi Biotec Inc., San Diego, CA) and lysed to release viral proteins. The amount of particle-associated reverse transcriptase activity was determined using a colorimetric Reverse Transcriptase Assay (Sigma-Aldrich, St Louis, MO). Particle-associated Env content was determined using an in-house quantitative ELISA. 96-well plates were coated with 200ng of eCD4-Ig (11) in 100µl of 0.2M sodium carbonate/bicarbonate buffer overnight at 4°C. Wells were washed twice with 200µl of PBS containing 0.2% Tween 20 (PBS-T), blocked at room temperature for two hours with 200µl of 5% milk in PBS-T, and washed three times with PBS-T. 100µl of virus lysate was added per well and incubated at 37°C for two hours. After washing wells

five times with PBS-T, 100µl of polyclonal human anti-gp120 conjugated to horseradish peroxidase (ABL Inc., Rockville, MD) was added for one hour at 37°C. Wells were again washed five times with PBS-T, incubated with 100µl o-phenylenediamine dihydrochloride substrate (ThermoFisher Scientific, Waltham, MA) at room temperature for 30 minutes, and then absorbance was read at 450nm. Env content was calculated using a standard curve of recombinant gp120 (10ng to 125pg in 2-fold dilutions), which was then normalized by reverse transcriptase activity. Stocks previously characterized by a similar protocol (12), as well as viral stocks independently quantified for Env content (13), were used to validate the Env ELISA ($r$ = 0.82, $P$ < 0.0001).

**Analysis of per-particle infectivity.** Individual wells of a 96-well plate were seeded with 8.3 x $10^3$ TZM-bl cells in 100µl of 10% FBS-containing DMEM medium to achieve ~30% confluence. 24 hours later, cells were infected with 100µl of virus, diluted serially in 10% DMEM with 80µg/ml DEAE dextran. 12-15 hours post-infection, the T-1249 fusion inhibitor was added (0.01 mg/ml) to prevent multiple rounds of infection. Infections were terminated at 48 hours and cells were lysed in 75µl lysis buffer (Promega Life Sciences, Madison, WI). Relative light units (RLU) generated per volume of each viral stock were calculated by averaging all virus dilutions in the linear range of the assay ($1.5x10^3$ - $7x10^4$ RLUs). The infectivity per particle was then calculated as the RLU generated per pg of RT activity present in each virus stock.

**Replicative capacity and type I IFN resistance.** Activated normal donor CD4+ T cells were left untreated or cultured in the presence increasing amounts of IFNα2 (0.00074 pg/ml - 5.5 pg/ml) or IFNβ (0.000067 pg/ml - 0.44 pg/ml) for 24 hours. Cells were washed, and $1x10^6$ cells were infected overnight with an equivalent amount of each virus (1ng RT activity). Supernatants were sampled every 48 hours, and cultures were maintained for 7 days while replenishing IFN-

containing medium. Virus replication was measured for each IFN concentration as the amount of p24 produced at day 7 and plotted as the percentage of viral growth in the absence of IFN, which was set to 100%. This allowed us to determine the IFN$\alpha$2 and IFN$\beta$ concentrations required to inhibit virus replication by 50% ($IC_{50}$) as well as the residual virus replicative capacity (Vres) in the presence of maximal IFN$\alpha$2 and IFN$\beta$ concentrations. The replicative capacity of each virus isolate was calculated using p24 antigen levels in untreated cells. Some viruses replicated to titers below the limit of detection in the presence of maximal IFN$\beta$ concentrations. For these viruses, the limit of detection (0.1 ng of p24/ml) was used as the numerator to calculate Vres. IFN$\alpha$2 $IC_{50}$ values were determined using pooled CD4+ T-cells from 4 donors, while IFN$\beta$ $IC_{50}$ values were determined using pooled CD4+ T-cells from 3 donors (Fig. 2.2A and B). All viruses were tested in duplicate.

**Quantification of virus release.** CD4+ T-cells were infected as described above. To quantify cell-associated p24, cells and supernatant were harvested 7 days post-infection after centrifugation at 1,200 rpm for 5 min. Cells were lysed, and cell-free and cell-associated p24 antigen levels were quantified using the HIV p24 (high sensitivity) AlphaLISA Detection Kit (Perkin Elmer Inc., Boston, MA). For each isolate, total p24 production was calculated by adding cell-free and cell-associated p24 levels. The percent of released p24 was determined by dividing the cell-free amount of p24 by the total amount of p24 as previously described (14, 15).

**Phylogenetic analyses.** Nucleotide sequences were aligned using CLUSTALW v. 2 (16). Regions that could not be unambiguously aligned were removed. Maximum likelihood trees with bootstrap support (1,000 replicates) were constructed using PhyML v. 3.1 (17) with evolutionary models selected using jModelTest v. 2.1.4 (18, 19), or for larger datasets, RAxML using a GTRGAMMA model (20). Transmitted founder viral genomes were inferred as described

previously (5). Highlighter plots were generated using the Los Alamos National Laboratory HIV Sequence Database Highlighter Tool (https://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). The degree of phylogenetic association of sequences was quantified by calculating their genealogical sorting index (gsi) (21). Maximum likelihood phylogenies were inferred using PhyML (17) and multiple bifurcations with intervening zero-length branches were collapsed to polytomies using the di2multi method implemented in the ape package (22) of R (23). These phylogram topologies were used to calculate the gsi values; p-values were determined using 10,000 replicate permutations (21).

**Statistical analyses.** For intra-pair comparisons of viral properties, p-values were determined using Welch's unequal variances t-test. Analyses were performed in R (23), comparing $\log_{10}$-transformed values for matched donor and recipient isolates for each transmission pair. Some isolates had undetectable p24 values after treatment with IFNβ. Since the unmeasurable range of p24 antigen (0 to 0.1 ng/ml) was negligible in comparison to the measurable range (0.1 to 150 ng/ml), a value of 0.1 ng/ml was used as the numerator for calculating IFNβ Vres values. Principal component and receiver operating characteristic analysis (24) were performed using R v3.3.1 (23).

**Bayesian hierarchical regression models of viral properties.** Each viral property, was modeled using a Bayesian hierarchical model (25), which was based on a linear regression estimating the differences between donor plasma and genital secretion isolates, or donor plasma and recipient plasma isolates, along with the effects of HIV-1 subtype, and IFNα2 and IFNβ-selection. Unlike a normal linear regression, this model accounts for (i) nested measurements within transmission pairs, (ii) multiple transmissions from a single donor, (iii) heteroscedasticity among virus populations, and (iv) censored data where exact measurements were not available

but known to be less than a given value. The hierarchical models are based on the assumption that observations of viral properties are independent and identically normally distributed with mean and variance drawn from common population-level distributions. Estimates of the population-level distributions can then be used to infer broader patterns in the data.

Data were first transformed as follows:

| Variable | Transformation |
|---|---|
| Env/RT | log |
| Infectivity | log |
| Replicative capacity | log |
| IFN$\alpha$2 $IC_{50}$ | log |
| IFN$\beta$ $IC_{50}$ | log |
| IFN$\alpha$2 Vres | logit |
| IFN$\beta$ Vres | logit |
| p24 antigen release | logit |

The observation from each viral isolate $i$ was then modeled as a normal distribution $N(\mu_i, \sigma_i^2)$ with mean $\mu_i$:

$$
\begin{aligned}
\mu_i = \; & \text{donor}_{\text{pair}_i} \\
& + \beta_{\text{recipient,pair}_i} \mathbb{1}(\text{recipient}_i) \\
& + \beta_{\text{genital,pair}_i} \mathbb{1}(\text{genital}_i) \\
& + \beta_{\text{clade,pair}_i} \mathbb{1}(\text{cladeB}_i) \mathbb{1}(\text{recipient}_i) \\
& + \beta_{\text{donorAlpha,pair}_i} \mathbb{1}(\text{donor}_i \;\&\; \text{alphaSelect}_i) \\
& + \beta_{\text{donorBeta,pair}_i} \mathbb{1}(\text{donor}_i \;\&\; \text{betaSelect}_i) \\
& + \beta_{\text{recipientAlpha,pair}_i} \mathbb{1}(\text{recipient}_i \;\&\; \text{alphaSelect}_i) \\
& + \beta_{\text{recipientBeta,pair}_i} \mathbb{1}(\text{recipient}_i \;\&\; \text{betaSelect}_i)
\end{aligned}
$$

and variance $\sigma_i^2$:

$$
\sigma_i^2 = \begin{cases}
\sigma^2_{\text{genital,pair}_i} & \text{if genital}_i \\
\sigma^2_{\text{recipient,pair}_i} & \text{if recipient}_i \\
\sigma^2_{\text{donorAlpha,pair}_i} & \text{if donor}_i \;\&\; \text{alphaSelect}_i \\
\sigma^2_{\text{donorBeta,pair}_i} & \text{if donor}_i \;\&\; \text{betaSelect}_i \\
\sigma^2_{\text{donor,pair}_i} & \text{otherwise}
\end{cases}
$$

where pair $_i$ indicates the pair identity of the $i^{\text{th}}$ observation, $\text{donor}_j$ is the estimated mean of untreated donor plasma viral isolates from pair $j$ and $1\,()$ is an indicator function that is 1 if True and 0 if False. The various $\beta$ values are coefficients modeling the change expected for viruses in recipients, in donor genital samples, in recipients infected with HIV-1 clade B, and the effects of IFN$\alpha$2 and IFN$\beta$-selection on donor or recipient viruses. For example, a donor plasma virus $i$ from pair 2 would have mean $\mu_i = \text{donor}_2$ and an IFN$\alpha$2-selected recipient virus from pair 3 (which happened to be clade B) would have mean:

$$
\mu_i = \text{donor}_3 + \beta_{\text{recipient,3}} + \beta_{\text{clade,3}} + \beta_{\text{recipientAlpha,3}}
$$

For two transmission pairs where one donor (CH742) transmitted viruses to two separate recipients (CH378 and CH831), recipient parameters were estimated independently for each recipient.

Vres measurements were calculated as the amount of p24 released in the presence of the highest IFN dose divided by the released p24 without IFN treatment as measured by AlphaLISA. The limit of detection for these measurements was 0.1, so concentrations $\leq 0.1$ were measured as 0.1. To account for this, the probability of these observations was considered to be:

$$p\left(\text{Vres} = \frac{0.1}{\text{Untreated p24}}\right) = \int_{-\infty}^{\text{logit}\left(\frac{0.1}{\text{Untreated p24}}\right)} N(\mu_i, \sigma_i^2)$$

The coefficients $\beta$ for each pair $j$ come from population-level normal hyperpriors:

$$\begin{aligned}
\text{donor}_j &\sim N(\mu_{\text{donor}}, \sigma^2_{\text{donor}}) \\
\beta_{\text{recipient},j} &\sim N(\mu_{\text{recipient}}, \sigma^2_{\text{recipient}}) \\
\beta_{\text{genital},j} &\sim N(\mu_{\text{genital}}, \sigma^2_{\text{genital}}) \\
\beta_{\text{clade},j} &\sim N(\mu_{\text{clade}}, \sigma^2_{\text{clade}}) \\
\beta_{\text{donorAlpha},j} &\sim N(\mu_{\text{donorAlpha}}, \sigma^2_{\text{donorAlpha}}) \\
\beta_{\text{donorBeta},j} &\sim N(\mu_{\text{donorBeta}}, \sigma^2_{\text{donorBeta}}) \\
\beta_{\text{recipientAlpha},j} &\sim N(\mu_{\text{recipientAlpha}}, \sigma^2_{\text{recipientAlpha}}) \\
\beta_{\text{recipientBeta},j} &\sim N(\mu_{\text{recipientBeta}}, \sigma^2_{\text{recipientBeta}})
\end{aligned}$$

and coefficients $\sigma$ from population-level normal hyperpriors:

$$\begin{aligned}
\sigma_{\text{donor},j} &\sim N(\theta_{\text{donor}}, \phi^2_{\text{donor}}) \\
\sigma_{\text{donorAlpha},j} &\sim N(\theta_{\text{donorAlpha}}, \phi^2_{\text{donorAlpha}}) \\
\sigma_{\text{donorBeta},j} &\sim N(\theta_{\text{donorBeta}}, \phi^2_{\text{donorBeta}}) \\
\sigma_{\text{recipient},j} &\sim N(\theta_{\text{recipient}}, \phi^2_{\text{recipient}}) \\
\sigma_{\text{genital},j} &\sim N(\theta_{\text{genital}}, \phi^2_{\text{genital}})
\end{aligned}$$

The effect hyperparameters $\mu_{\text{recipient}}$, $\mu_{\text{genital}}$, $\mu_{\text{clade}}$, $\mu_{\text{donorAlpha}}$, $\mu_{\text{donorBeta}}$, $\mu_{\text{recipientAlpha}}$ and $\mu_{\text{recipientBeta}}$ were all given a flat prior probability. The variance parameters

$\sigma_{\text{donor}}$,$\sigma_{\text{recipient}}$,$\sigma_{\text{genital}}$,$\sigma_{\text{clade}}$,$\sigma_{\text{donorAlpha}}$,$\sigma_{\text{donorBeta}}$, $\sigma_{\text{recipientAlpha}}$, $\sigma_{\text{recipientBeta}}$, $\phi_{\text{donor}}$, $\phi_{\text{donorAlpha}}$, $\phi_{\text{donorBeta}}$, $\phi_{\text{recipient}}$, $\phi_{\text{genital}}$, $\theta_{\text{donor}}$, $\theta_{\text{donorAlpha}}$, $\theta_{\text{donorBeta}}$, $\theta_{\text{recipient}}$ and $\theta_{\text{genital}}$ were given a prior of Gamma (1,2) reflecting prior knowledge that the standard deviation in these assays was unlikely to be greater than several logs.

Plots and statistics are based on the estimated posterior probabilities of the population-level effects $\mu_{\text{recipient}}$ , $\mu_{\text{genital}}$, $\mu_{\text{clade}}$, $\mu_{\text{donorAlpha}}$, $\mu_{\text{donorBeta}}$, $\mu_{\text{recipientAlpha}}$ and $\mu_{\text{recipientBeta}}$. Markov Chain Monte Carlo sampling of the posterior probability distributions of the models was implemented in Stan (26) using the R package rstan (23) and run in 50 chains with each having a 50,000 iteration burn-in and 50,000 iterations of sampling every 25$^{th}$ iteration.

Biological data and analysis code are archived on Zenodo at:

DOI: 10.5281/zenodo.21645

As an example for why these Bayesian estimates are more conservative than simpler analyses, we can look at the estimated change in IFNβ $IC_{50}$ between untreated donor plasma viruses and IFNβ-selected donor plasma viruses (Fig. 2.3). We observed $\log_{10}(IC_{50})$ in both untreated and IFNβ-selected viral isolates for 3 donors with averages:

| Donor | Untreated | IFNβ-selected | Difference |
|---|---|---|---|
| CH148 | -4.269 | -2.831 | 1.438 |
| CH492 | -4.203 | -2.717 | 1.487 |
| CH596 | -4.162 | -2.820 | 1.343 |

The simplest estimate would be to take the average, 1.423, and the standard deviation, 0.0733, of the three differences and estimate the 95% confidence interval on the mean as:

$$1.423 \pm \frac{1.96 \times 0.0733}{\sqrt{3}} = 1.423 \pm 0.0829$$

Or equivalently an estimate that IFNβ-selected donor viruses have an $IC_{50}$ 26.5-fold (95% confidence interval: 21.9–32.0-fold) higher than untreated isolates. In contrast, the Bayesian model yielded estimates of 20.7-fold (95% credible interval: 11.0–36.2-fold) higher $IC_{50}$ values. Thus, the Bayesian model represents a more conservative approach that yields wider intervals in its estimation due to the incorporation of uncertainty in our estimates of untreated and IFNβ-selected $IC_{50}$ values for each donor.

**Section 2.10 Supplemental References**


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## Section 2.11 – Supplemental figures

**Table S2.1. Generation of limiting-dilution isolates from eight epidemiologically linked transmission pairs**

| Subject | Transmission Partner | Risk Factor | Subtype | Country | Gender | VL | Fiebig Stage | Number of TF Viruses | Sample | Date | UT | IFNα2 selected | IFNβ selected | Days[‡] | Accession Codes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | Isolate Sequences[§] | SGA Sequences[¶] |
| CH0742 | Donor | MSM | B | USA | M | 112,531 | | | PL | 09/10/08 | 15 | 3 | 0 | | KY112461-KY112478 | KY112494-KY112521 |
| | | | | | | na | | | SEM | 08/27/08 | 1 | 0 | 0 | | KY112480 | |
| | | | | | | na | | | SEM | 09/10/08 | 11 | 0 | 0 | | KY112481-KY112491 | |
| | | | | | | na | | | SEM | 10/29/08 | 1 | 0 | 0 | | KY112492 | |
| | | | | | | na | | | SEM | 12/10/08 | 1 | 0 | 0 | | KY112493 | |
| | | | | | | na | | | SEM | 03/18/09 | 0 | 0 | 0 | | | |
| CH0378 | Recipient 1 | | | | M | 265,936 | 5 | 2 | PL | 08/05/08 | 17 | 7 | 0 | 36 | KY112136-KY112159 | KY112160-KY112189 |
| CH0831 | Recipient 2 | | | | M | 261,752 | 3 | 1 | PL | 11/14/08 | 8 | 4 | 4 | 65[#] | KY112522-KY112537 | KY112538-KY112576 |
| CH0148 | Donor | MSM | B | USA | M | 246,017 | | | PL | 12/19/06 | 12 | 3 | 4 | | KY112056-KY112074 | KY111920-KY111946 |
| CH0040 | Recipient | | | | M | 298,026 | 1-2 | 1[†] | PL | 07/27/06 | 5 | 2 | 0 | 145 | KY112190-KY112196 | FJ495827-FJ495838 |
| CH0728 | Donor | MTF | B | USA | M | 23,965 | | | PL | 07/28/08 | 14 | 1 | 0 | | KY112429-KY112445 | KY111983-KY111986 KY112446-KY112460 |
| | | | | | | na | | | SEM | 07/27/08 | 0 | 0 | 0 | | | |
| | | | | | | na | | | SEM | 08/27/08 | 0 | 0 | 0 | | | |
| | | | | | | na | | | SEM | 11/04/09 | 0 | 0 | 0 | | | |
| | | | | | | na | | | SEM | 05/13/10 | 0 | 0 | 0 | | | |
| CH0302 | Recipient | | | | F | 16,218 | 5 | 1 | PL | 04/16/08 | 7 | 3 | 0 | 103 | KY112127-KY112135 | KY111947-KY111964, KY364886 |
| CH0492 | Donor | FTM | C | MWI | F | 472,129 | | | PL | 03/11/08 | 13 | 19 | 14 | | KY112276-KY112321 | KY112322-KY112359 |
| | | | | | | 22,753 | | | CVL | 02/06/08 | 17 | 0 | 0 | | KY112259-KY112275 | |
| | | | | | | 7,582 | | | CVL | 02/21/08 | 0 | 0 | 0 | | | |
| | | | | | | na | | | CVL | 03/11/08 | 0 | 0 | 0 | | | |
| | | | | | | 14,596 | | | CVL | 04/28/08 | 0 | 0 | 0 | | | |
| CH0427 | Recipient | | | | M | 1,644,231 | 1-2 | 1 | PL | 01/23/08 | 10 | 10 | 1 | 47 | KY112197-KY112217 | KY112218-KY112250 |
| CH0596 | Donor | FTM | C | MWI | F | 250,981 | | | PL | 04/03/08 | 12 | 10 | 6 | | KY112362-KY112389 | KY112390-KY112428 |
| | | | | | | na | | | CVL | 04/03/08 | 2 | 0 | 0 | | KY112360-KY112361 | |
| | | | | | | na | | | CVL | 04/17/08 | 0 | 0 | 0 | | | |
| CH0455 | Recipient | | | | M | 502,665 | 3 | 1 | PL | 01/29/08 | 5 | 3 | 0 | 65 | KY112251-KY112258 | KY111965-KY111982 |
| CH0212 | Donor | FTM | C | ZAF | F | 111,427 | | | PL | 08/01/07 | 11 | 1 | 0 | | KY112082-KY112093 | KY112094-KY112126 |
| CH0162 | Recipient | | | | M | 18,260 | 3 | 1[†] | PL | 07/13/07 | 5 | 2 | 0 | 19 | KY112075-KY112081 | JX972986-JX972998 |
| CH1064 | Donor | FTM | C | MWI | F | 323,674 | | | PL | 04/08/09 | 18 | 10 | 0 | | KY111987-KY112014 | KY112015-KY112055 |
| | | | | | | bld | | | CVL | 02/11/09 | 0 | 0 | 0 | | | |
| | | | | | | bld | | | CVL | 02/25/09 | 0 | 0 | 0 | | | |
| | | | | | | na | | | CVL | 04/08/09 | 0 | 0 | 0 | | | |
| | | | | | | 990 | | | CVL | 07/07/09 | 0 | 0 | 0 | | | |
| CH0848 | Recipient | | | | M | 361,254 | 4 | 1 | PL | 07/29/08 | 4 | 4 | 0 | 265 | KY112577-KY112584 | KX216883-KX216893 KX216895 |

MSM, men who have sex with men; MTF, male to female; FTM female to male; USA, United States; MWI, Malawi; ZAF, South Africa; M, male; F, female; PL, plasma; SEM, semen; CVL, cervicovaginal lavage; VL, viral load (RNA copies per milliliter of plasma); na, data not available; bld, below limit of detection; UT, untreated. Viral load determination, Fiebig staging, and limiting dilution virus isolation were performed on the same sample from the indicated time point.

[*] defined as previously described (2).

[†] infectious molecular clone (IMC) available

[‡] number of days between recipient and first available donor samples.

[#] in contrast to all other recipients, CH831 was sampled 64 days after donor CH742.

[§] Isolate sequences represent near-complete viral genomes (8,750-9,208 bp).

[¶] SGA sequences span *rev-vpu-env-nef* gene regions (2,922-2,973 bp for KY112446-KY112460) or 3' half genomes (4,112-4,936 bp).

**Table S2.2A.** Genealogical sorting index analysis of sequences from uncultured plasma and limiting dilution isolates

| Donor | Plasma SGA | Plasma Isolates |
|---|---|---|
| CH1064 | 0.039 | 0.120 |
| CH148 | 0.059 | 0.092 |
| CH212 | 0.142 | 0.421** |
| CH492 | 0.119 | 0.100 |
| CH596 | 0.065 | 0.110 |
| CH728[§] | 0.346* | 0.136 |
| CH742 | 0.090 | 0.224 |

To assess to what extent viral isolates were representative of the virus present in the plasma of the chronically infected donors, we constructed maximum likelihood phylogenetic trees from 3' half genome or *env* gene[§] sequences and used these to assess the degree of segregation between single genome amplification derived plasma (Plasma SGA) and limiting dilution derived isolate (Plasma Isolates) sequences by determining their genealogical sorting index (gsi) (21). Gsi indices range between 0 (no segregation) and 1 (complete monophyly) and were calculated using the genealogical Sorting R package (http://molecularevolution.org/software/phylogenetics/gsi/download). Two gsi values, which were significantly higher than expected from random segregation, are indicated (* $p<0.05$; ** $p < 0.01$). For CH212, available plasma isolates represented only two of three diverse viral lineages present in this donor's quasispecies, indicating insufficient sampling (Fig. S3*F*). For CH728, the high gsi value was due to two clusters of near identical isolate sequences, indicating repeat culture of the same virus (Fig. S3*C*). Collapsing one of these clusters to a single sequence reduced the Plasma SGA and Plasma Isolate gsi values to non-significant values (0.3 and 0.116, respectively). For all other donors, limiting dilution isolates were fully representative of the viral diversity present in the plasma. Statistical significance was assessed by randomly permuting character states across the tips of the tree 10,000 times (p values were corrected for multiple tests).

**Table S2.2B.** Genealogical sorting index analysis of sequences from plasma and genital secretion isolates

| Donor | Genital Secretion Isolates | Plasma Isolates |
|---|---|---|
| CH492 | 0.142 | 0.225 |
| CH742 | 0.236 | 0.333* |

To assess the extent of segregation of plasma and CVL isolates for donor CH492, and plasma and semen isolates for donor CH742, we constructed maximum likelihood trees of full-length plasma and genital secretion isolate sequences, and used these to calculate the genealogical sorting index (http://molecularevolution.org/software/phylogenetics/gsi/download) (21). Gsi indices range between 0 (no segregation) and 1 (complete monophyly), and values that were significantly higher than expected from random segregation are indicated (* $p<0.05$). For CH742, the high gsi value was due to a pair of nearly identical semen isolate sequences, indicating repeat culture of the same virus (Fig. S3*A*). Collapsing this cluster to a single sequence reduced the genital secretion and plasma isolates gsi values to non-significant values (0.229 and 0.187, respectively). Statistical significance was assessed by randomly permuting character states across the tips of the tree 10,000 times. Non-significant values indicate the absence of compartmentalization (p values were corrected for multiple tests).

**Table S2.2C.** Genealogical sorting index analysis of sequences from IFN-selected and untreated plasma isolates

| Donor | IFN-Selected | Untreated |
|---|---|---|
| CH1064 | 0.175 | 0 |
| CH148 | 0.143 | 0 |
| CH492 | 0.136 | 0.112 |
| CH596 | 0.124 | 0.094 |
| CH742 | 0.095 | 0 |

To assess the extent of segregation of plasma isolates generated in IFN-selected and untreated CD4+ T cells, we constructed maximum likelihood trees of full-length isolate sequences and calculated their genealogical sorting index (gsi) (21) using the genealogical Sorting R package (http://molecularevolution.org/software/phylogenetics/gsi/download). Gsi indices range between 0 (no segregation) and 1 (complete monophyly). Statistical significance was assessed by randomly permuting character states across the tips of the tree 10,000 times. None of the values was significant, indicating complete interspersion (p values were corrected for multiple tests).

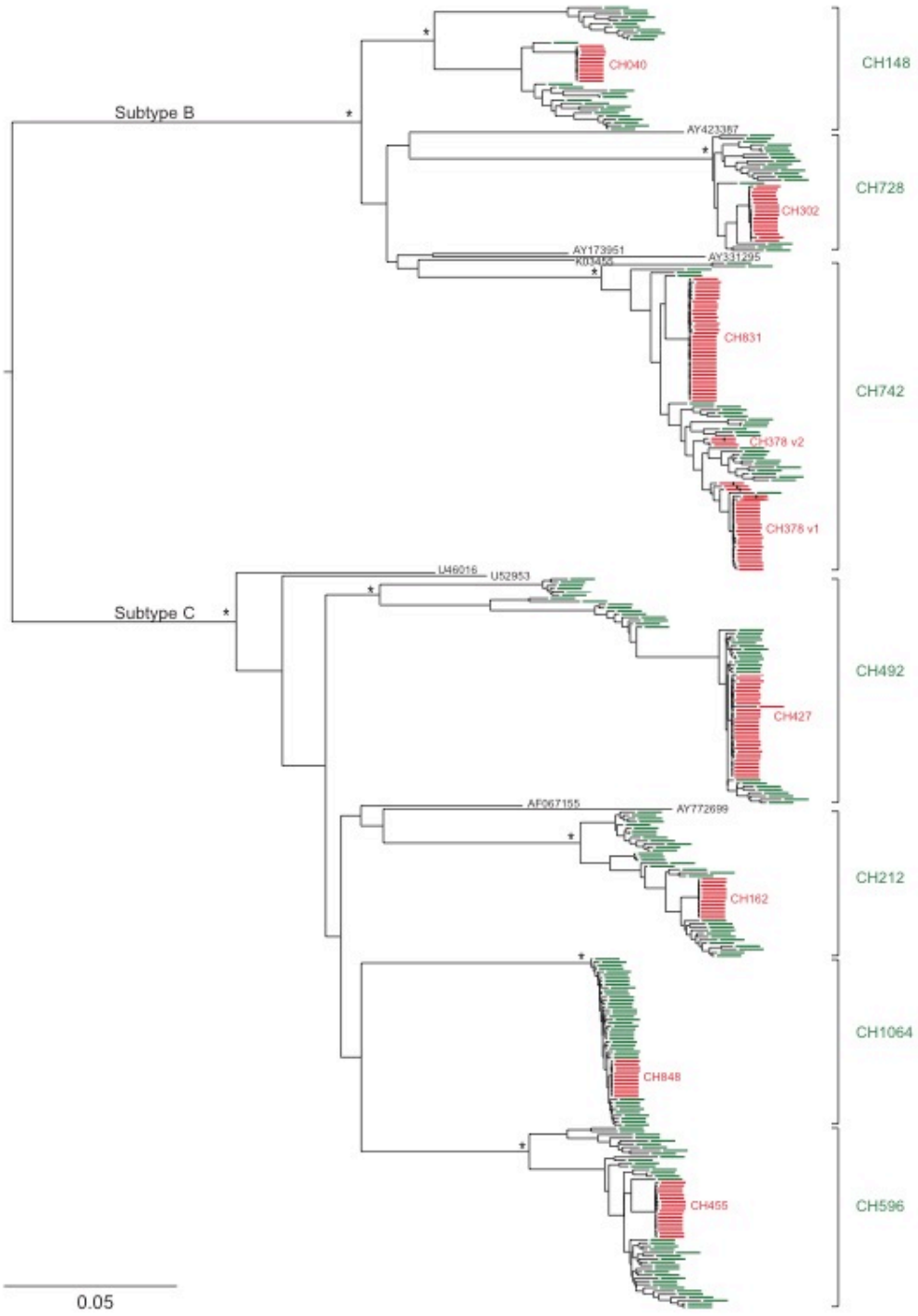
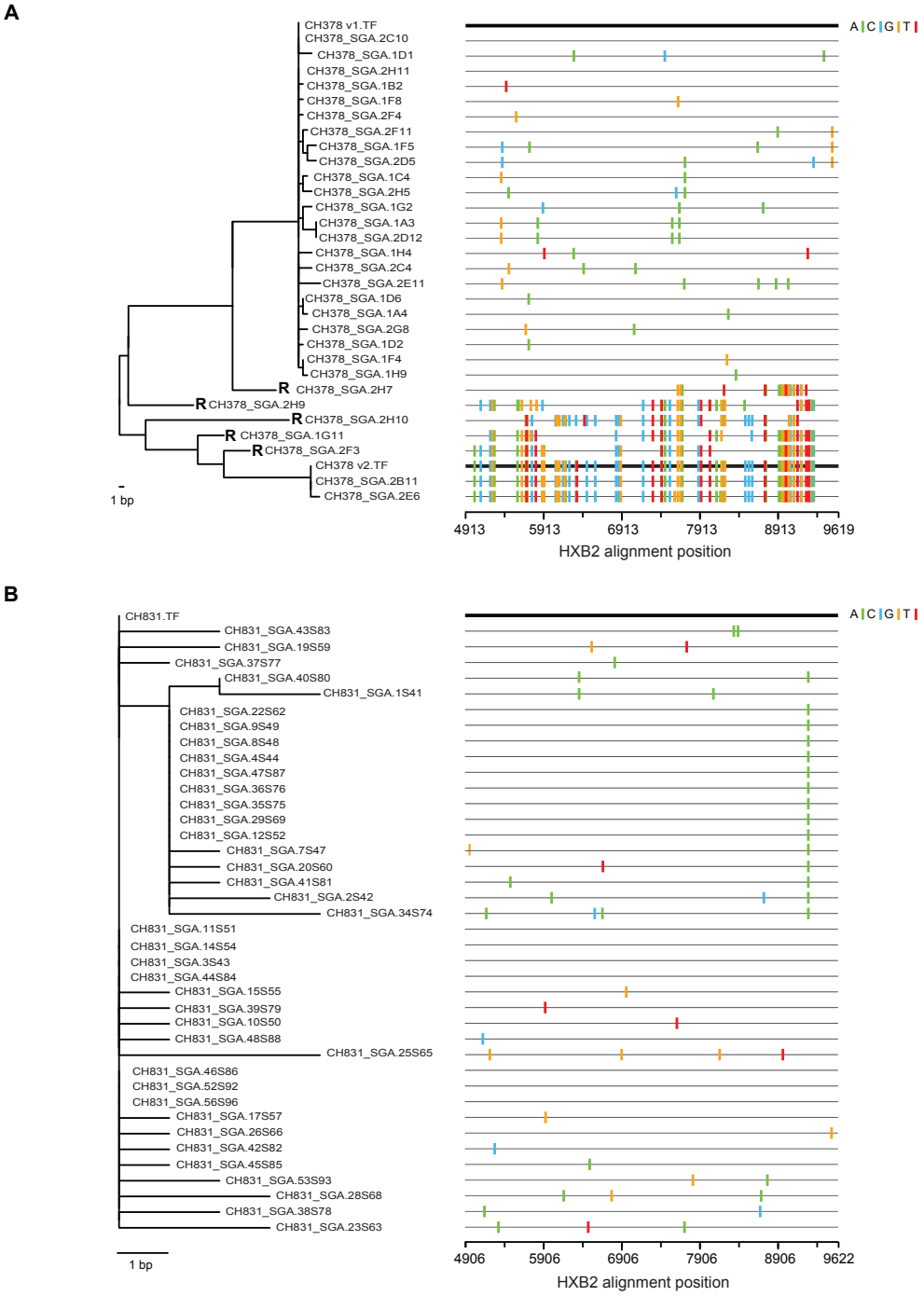

Subtype B
Subtype C
CH040
AY423387
CH302
AY173951
AY331295
K03455
CH831
CH378 v2
CH378 v1
U46016
U52953
CH427
AF067155
AY772699
CH162
CH848
CH455
CH148
CH728
CH742
CH492
CH212
CH1064
CH596
0.05

**Fig. S2.1. Confirmation of epidemiological linkage of transmission pairs.** 3' half genome sequences were generated by single genome amplification (SGA) of viral RNA from the plasma of respective donors and recipients. Nucleotide sequences of partial *tat*, partial *rev*, *vpu*, *env* and partial *nef* genes were aligned using CLUSTALW v. 2 (16), with ambiguous regions removed (the 2,654 bp alignment spans HXB2 coordinates 5,984-8,866). A maximum likelihood tree with bootstrap support (1,000 replicates) was constructed using RAxML v. 8.0.22 (20) with a GTRGAMMA evolutionary model. Sequences from donors and acutely infected recipients are indicated by green and red rectangles, respectively, with brackets denoting individual transmission pairs. Donor CH742 transmitted to two recipients (Fig. S2.2), one of whom (CH378) acquired two transmitted founder viruses (v1 and v2). Also shown for CH378 are sequences that are recombinant between v1 and v2 (red rectangles with black circles). Subtype B reference sequences (labeled by accession code) included HXB2 (K03455), BK132 (AY173951), 1058 (AY331295) and 671_00T36 (AY423387), while subtype C reference sequences included ETH2220 (U46016), BR025 (U52935), 95IN21068 (AF067155) and 04ZASK146 (AY772699). Asterisks indicate nodes with ≥ 95% bootstrap support (the scale bar represents 0.05 substitutions per site).


A
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CH378_SGA.1B2
CH378_SGA.1F8
CH378_SGA.2F4
CH378_SGA.2F11
CH378_SGA.1F5
CH378_SGA.2D5
CH378_SGA.1C4
CH378_SGA.2H5
CH378_SGA.1G2
CH378_SGA.1A3
CH378_SGA.2D12
CH378_SGA.1H4
CH378_SGA.2C4
CH378_SGA.2E11
CH378_SGA.1D6
CH378_SGA.1A4
CH378_SGA.2G8
CH378_SGA.1D2
CH378_SGA.1F4
CH378_SGA.1H9
R CH378_SGA.2H7
R CH378_SGA.2H9
R CH378_SGA.2H10
R CH378_SGA.1G11
R CH378_SGA.2F3
CH378 v2.TF
CH378_SGA.2B11
CH378_SGA.2E6
1 bp
A | C | G | T
4913
5913
6913
7913
8913
9619
HXB2 alignment position
B
CH831.TF
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CH831_SGA.19S59
CH831_SGA.37S77
CH831_SGA.40S80
CH831_SGA.1S41
CH831_SGA.22S62
CH831_SGA.9S49
CH831_SGA.8S48
CH831_SGA.4S44
CH831_SGA.47S87
CH831_SGA.36S76
CH831_SGA.35S75
CH831_SGA.29S69
CH831_SGA.12S52
CH831_SGA.7S47
CH831_SGA.20S60
CH831_SGA.41S81
CH831_SGA.2S42
CH831_SGA.34S74
CH831_SGA.11S51
CH831_SGA.14S54
CH831_SGA.3S43
CH831_SGA.44S84
CH831_SGA.15S55
CH831_SGA.39S79
CH831_SGA.10S50
CH831_SGA.48S88
CH831_SGA.25S65
CH831_SGA.46S86
CH831_SGA.52S92
CH831_SGA.56S96
CH831_SGA.17S57
CH831_SGA.26S66
CH831_SGA.42S82
CH831_SGA.45S85
CH831_SGA.53S93
CH831_SGA.28S68
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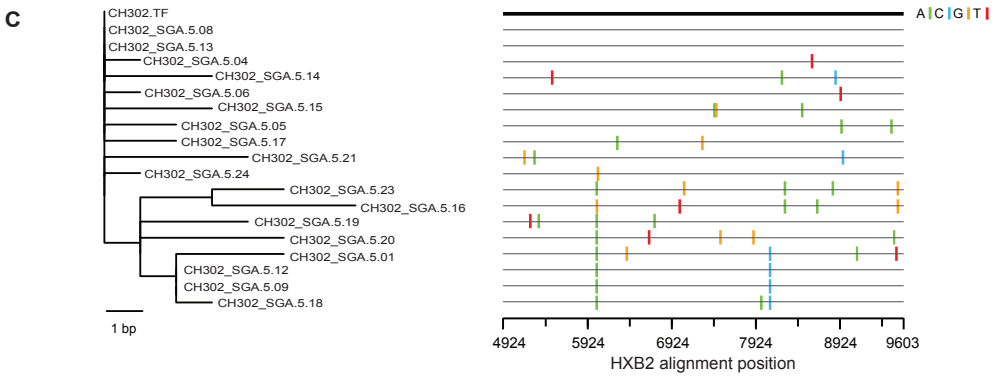
5906
6906
7906
8906
9622
HXB2 alignment position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CH302_SGA.5.04
CH302_SGA.5.14
CH302_SGA.5.06
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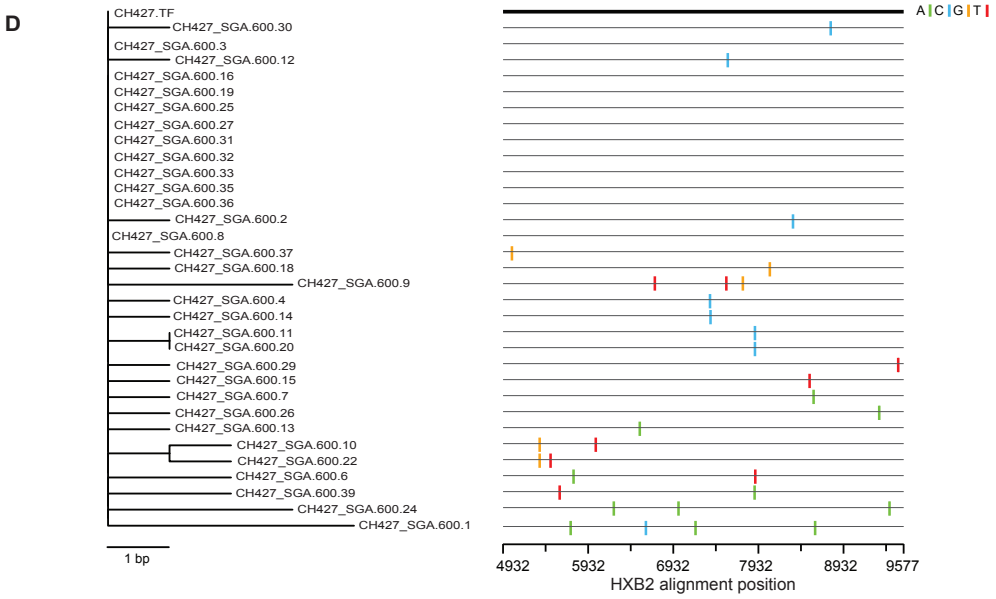
CH302_SGA.5.05
CH302_SGA.5.17
CH302_SGA.5.21
CH302_SGA.5.24
CH302_SGA.5.23
CH302_SGA.5.16
CH302_SGA.5.19
CH302_SGA.5.20
CH302_SGA.5.01
CH302_SGA.5.12
CH302_SGA.5.09
CH302_SGA.5.18
1 bp
A C G T
4924
5924
6924
7924
8924
9603
HXB2 alignment position


D
CH427.TF
CH427_SGA.600.30
CH427_SGA.600.3
CH427_SGA.600.12
CH427_SGA.600.16
CH427_SGA.600.19
CH427_SGA.600.25
CH427_SGA.600.27
CH427_SGA.600.31
CH427_SGA.600.32
CH427_SGA.600.33
CH427_SGA.600.35
CH427_SGA.600.36
CH427_SGA.600.2
CH427_SGA.600.8
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CH427_SGA.600.18
CH427_SGA.600.9
CH427_SGA.600.4
CH427_SGA.600.14
CH427_SGA.600.11
CH427_SGA.600.20
CH427_SGA.600.29
CH427_SGA.600.15
CH427_SGA.600.7
CH427_SGA.600.26
CH427_SGA.600.13
CH427_SGA.600.10
CH427_SGA.600.22
CH427_SGA.600.6
CH427_SGA.600.39
CH427_SGA.600.24
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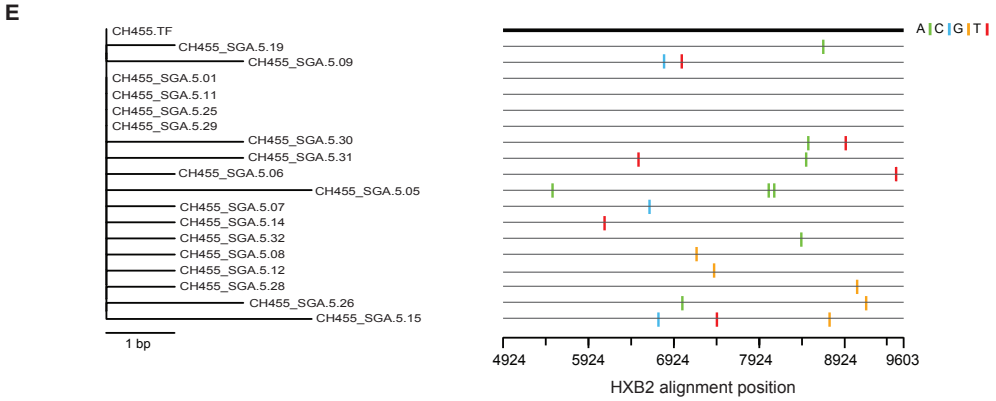
1 bp
A C G T
4932
5932
6932
7932
8932
9577
HXB2 alignment position


E
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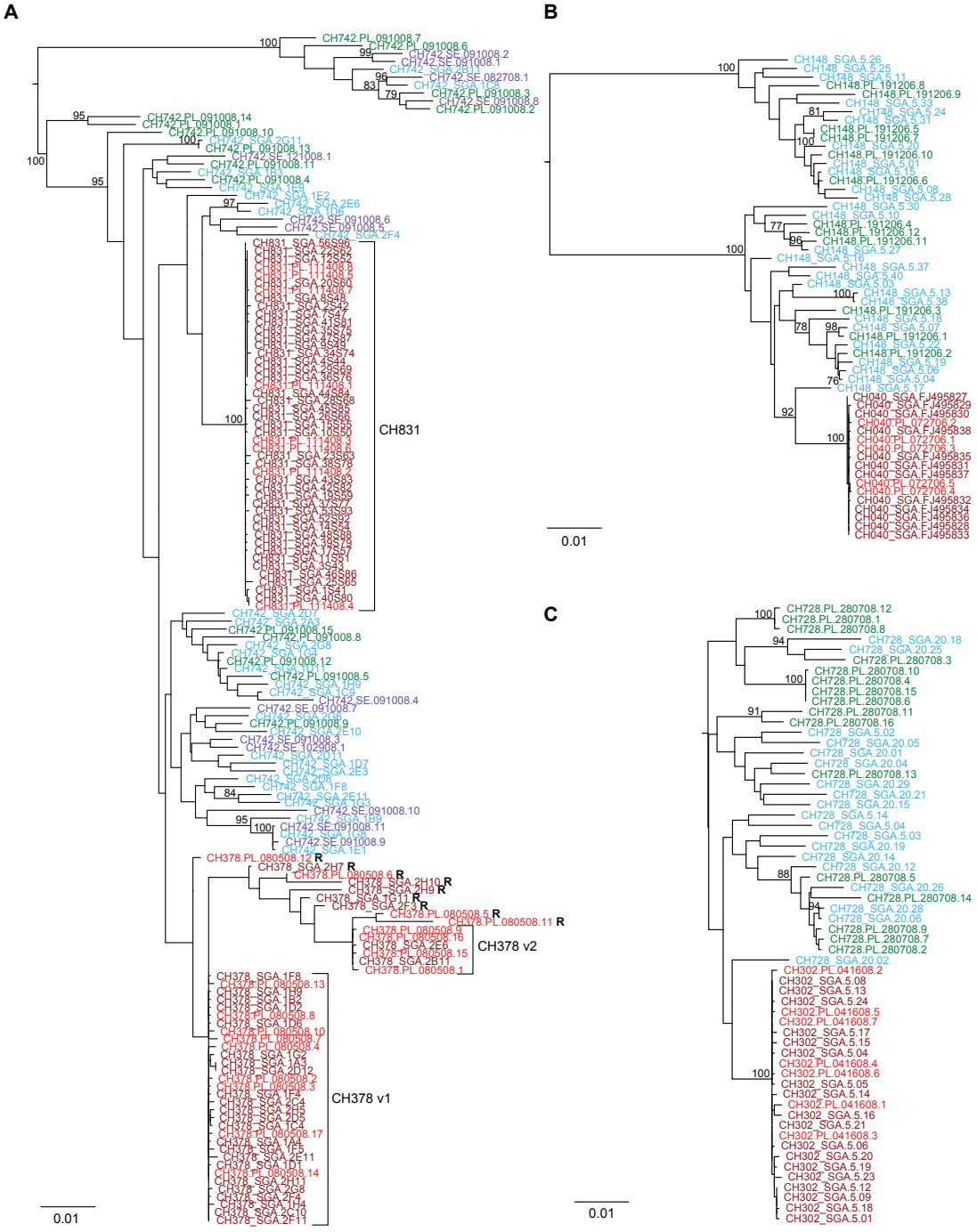
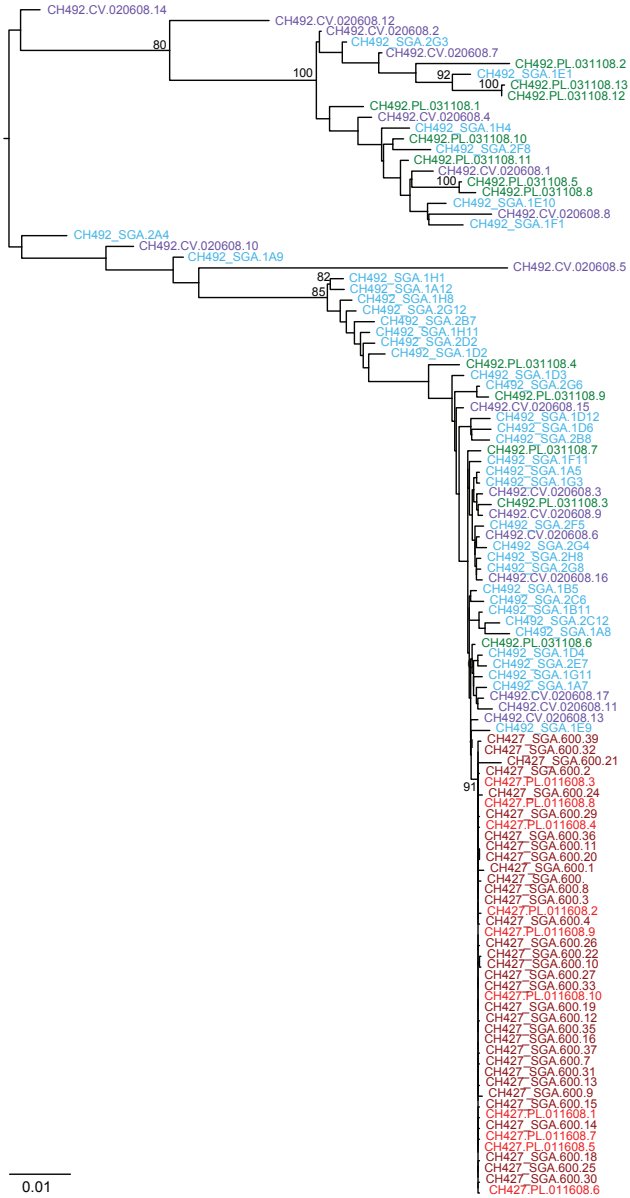
CH455_SGA.5.09
CH455_SGA.5.01
CH455_SGA.5.11
CH455_SGA.5.25
CH455_SGA.5.29
CH455_SGA.5.30
CH455_SGA.5.31
CH455_SGA.5.06
CH455_SGA.5.05
CH455_SGA.5.07
CH455_SGA.5.14
CH455_SGA.5.32
CH455_SGA.5.08
CH455_SGA.5.12
CH455_SGA.5.28
CH455_SGA.5.26
CH455_SGA.5.15
1 bp
A C G T
4924
5924
6924
7924
8924
9603
HXB2 alignment position

**Fig. S2.2**. **Transmitted founder sequence inference and enumeration.** Neighbor-joining phylogenetic trees (left) and highlighter plots (right) were generated for SGA derived 3' half genome sequences using the Los Alamos National Laboratory HIV Sequence Database Highlighter Tool (5). In the phylogenetic trees, the inferred transmitted founder (TF) sequence is shown at the top. The scale bar represents one base pair difference. In the highlighter plots, the TF sequence is used as the reference and indicated by a thick line. Thinner lines below correspond to sequences shown in the phylogenetic tree to the left. Tick marks indicate nucleotide differences from the TF sequence (green, A; blue, C; G orange; T, red). (*A*) Sequences from the acute recipient CH378 (4,774 bp) span HXB2 coordinates 4,913-9,619. For this recipient, two TF variants (v1 and v2) were identified. The former was used as a reference for the highlighter plot, but both variants are shown as thick lines. A bold-faced R preceding the sequence name indicates recombinants between these two variants. (*B*) Sequences from recipient CH831 (4,783 bp) span HXB2 coordinates 4906-9622. (*C*) Sequences from recipient CH302 (4,757 bp) span HXB2 coordinates 4924-9603. (*D*) Sequences from recipient CH427 (4,674 bp) span HXB2 coordinates 4932-9577. (*E*) Sequences from recipient CH455 (4,696 bp) span HXB2 coordinates 4924-9603. TF sequence inference and enumeration of the remaining three recipients (CH040, CH162, and CH848) have been published (4, 12, 27).


A
B
C
CH831
CH378 v2
CH378 v1
0.01
0.01
0.01

**D**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CH492.CV.020608.2
CH492_SGA.2G3
CH492.CV.020608.7
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CH492.PL.031108.13
CH492.PL.031108.12
CH492.PL.031108.1
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CH492_SGA.2D2
CH492_SGA.1D2
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CH492_SGA.1G3
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CH492_SGA.1A7
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CH492.CV.020608.11
CH492.CV.020608.13
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CH427_SGA.600.39
CH427_SGA.600.32
CH427_SGA.600.21
CH427_SGA.600.2
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CH427_SGA.600.24
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CH427_SGA.600.35
CH427_SGA.600.16
CH427_SGA.600.37
CH427_SGA.600.7
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CH427_SGA.600.9
CH427_SGA.600.15
CH427.PL.011608.1
CH427_SGA.600.14
CH427.PL.011608.7
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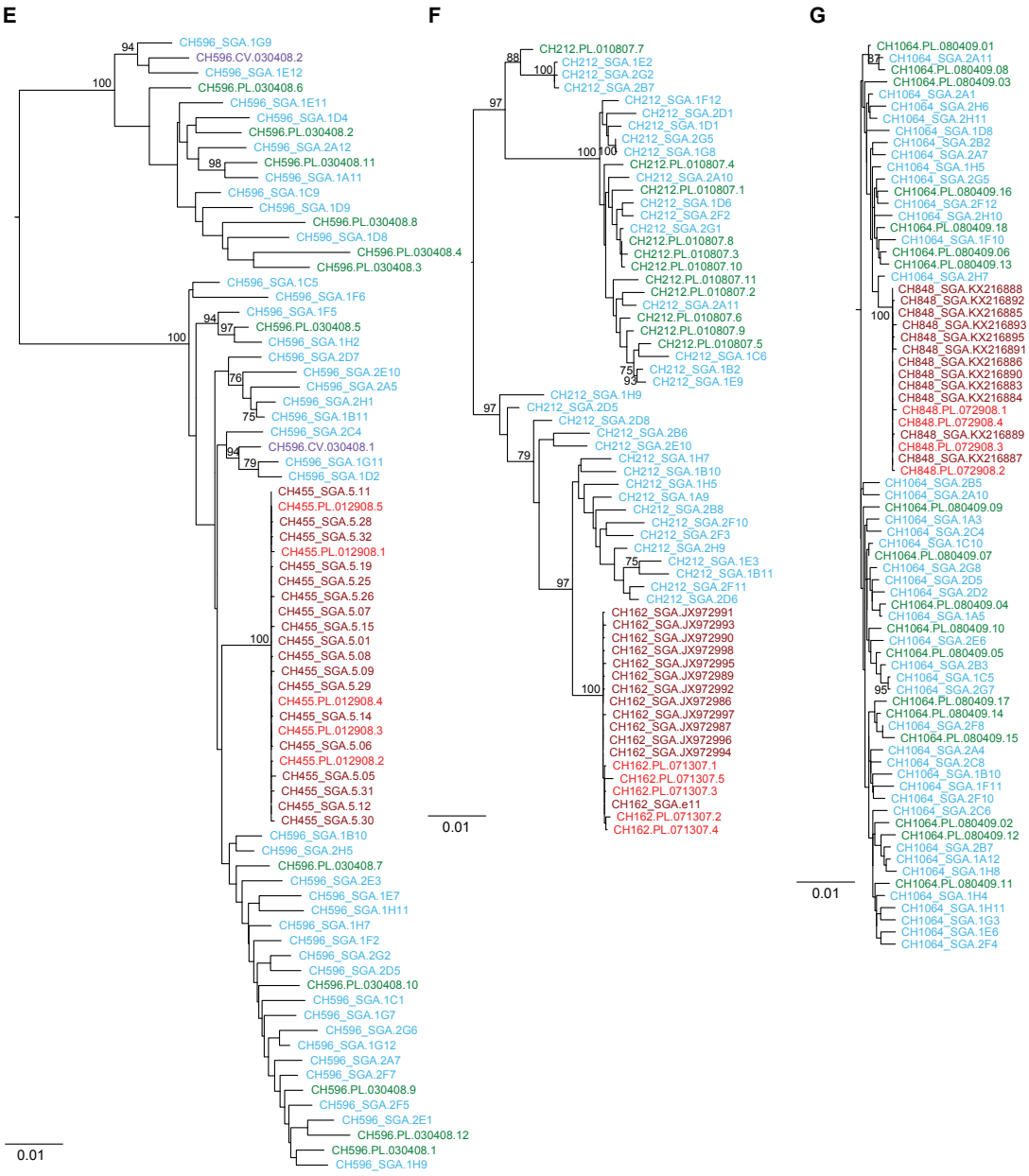
92
100
100
82
85
91
0.01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CH596_SGA.1A11
CH596_SGA.1C9
CH596_SGA.1D9
CH596.PL.030408.8
CH596_SGA.1D8
CH596.PL.030408.4
CH596.PL.030408.3
CH596_SGA.1C5
CH596_SGA.1F6
CH596_SGA.1F5
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CH596_SGA.2D7
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CH596_SGA.2H1
CH596_SGA.1B11
CH596_SGA.2C4
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CH455_SGA.5.28
CH455_SGA.5.32
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CH455_SGA.5.01
CH455_SGA.5.08
CH455_SGA.5.09
CH455_SGA.5.29
CH455.PL.012908.4
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CH455.PL.012908.3
CH455_SGA.5.06
CH455.PL.012908.2
CH455_SGA.5.05
CH455_SGA.5.31
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CH596_SGA.1F2
CH596_SGA.2G2
CH596_SGA.2D5
CH596.PL.030408.10
CH596_SGA.1C1
CH596_SGA.1G7
CH596_SGA.2G6
CH596_SGA.1G12
CH596_SGA.2A7
CH596_SGA.2F7
CH596.PL.030408.9
CH596_SGA.2F5
CH596_SGA.2E1
CH596.PL.030408.12
CH596.PL.030408.1
CH596_SGA.1H9
0.01
F
CH212.PL.010807.7
CH212_SGA.1E2
CH212_SGA.2G2
CH212_SGA.2B7
CH212_SGA.1F12
CH212_SGA.2D1
CH212_SGA.1D1
CH212_SGA.2G5
CH212_SGA.1G8
CH212.PL.010807.4
CH212_SGA.2A10
CH212.PL.010807.1
CH212_SGA.1D6
CH212_SGA.2F2
CH212_SGA.2G1
CH212.PL.010807.8
CH212.PL.010807.3
CH212.PL.010807.10
CH212.PL.010807.11
CH212.PL.010807.2
CH212_SGA.2A11
CH212.PL.010807.6
CH212.PL.010807.9
CH212.PL.010807.5
CH212_SGA.1C6
CH212_SGA.1B2
CH212_SGA.1E9
CH212_SGA.1H9
CH212_SGA.2D5
CH212_SGA.2D8
CH212_SGA.2B6
CH212_SGA.2E10
CH212_SGA.1H7
CH212_SGA.1B10
CH212_SGA.1H5
CH212_SGA.1A9
CH212_SGA.2B8
CH212_SGA.2F10
CH212_SGA.2F3
CH212_SGA.2H9
CH212_SGA.1E3
CH212_SGA.1B11
CH212_SGA.2F11
CH212_SGA.2D6
CH162_SGA.JX972991
CH162_SGA.JX972993
CH162_SGA.JX972990
CH162_SGA.JX972998
CH162_SGA.JX972995
CH162_SGA.JX972989
CH162_SGA.JX972992
CH162_SGA.JX972986
CH162_SGA.JX972997
CH162_SGA.JX972987
CH162_SGA.JX972996
CH162_SGA.JX972994
CH162.PL.071307.1
CH162.PL.071307.5
CH162.PL.071307.3
CH162_SGA.e11
CH162.PL.071307.2
CH162.PL.071307.4
0.01
G
CH1064.PL.080409.01
CH1064_SGA.2A11
CH1064.PL.080409.08
CH1064.PL.080409.03
CH1064_SGA.2A1
CH1064_SGA.2H6
CH1064_SGA.2H11
CH1064_SGA.1D8
CH1064_SGA.2B2
CH1064_SGA.2A7
CH1064_SGA.1H5
CH1064_SGA.2G5
CH1064.PL.080409.16
CH1064_SGA.2F12
CH1064_SGA.2H10
CH1064.PL.080409.18
CH1064_SGA.1F10
CH1064.PL.080409.06
CH1064.PL.080409.13
CH1064_SGA.2H7
CH848_SGA.KX216888
CH848_SGA.KX216892
CH848_SGA.KX216885
CH848_SGA.KX216893
CH848_SGA.KX216895
CH848_SGA.KX216891
CH848_SGA.KX216886
CH848_SGA.KX216890
CH848_SGA.KX216883
CH848_SGA.KX216884
CH848.PL.072908.1
CH848.PL.072908.4
CH848_SGA.KX216889
CH848.PL.072908.3
CH848_SGA.KX216887
CH848.PL.072908.2
CH1064_SGA.2B5
CH1064_SGA.2A10
CH1064.PL.080409.09
CH1064_SGA.1A3
CH1064_SGA.2C4
CH1064_SGA.1C10
CH1064.PL.080409.07
CH1064_SGA.2G8
CH1064_SGA.2D5
CH1064_SGA.2D2
CH1064.PL.080409.04
CH1064_SGA.1A5
CH1064.PL.080409.10
CH1064_SGA.2E6
CH1064.PL.080409.05
CH1064_SGA.2B3
CH1064_SGA.1C5
CH1064_SGA.2G7
CH1064.PL.080409.17
CH1064.PL.080409.14
CH1064_SGA.2F8
CH1064.PL.080409.15
CH1064_SGA.2A4
CH1064_SGA.2C8
CH1064_SGA.1B10
CH1064_SGA.1F11
CH1064_SGA.2F10
CH1064_SGA.2C6
CH1064.PL.080409.02
CH1064.PL.080409.12
CH1064_SGA.2B7
CH1064_SGA.1A12
CH1064_SGA.1H8
CH1064.PL.080409.11
CH1064_SGA.1H4
CH1064_SGA.1H11
CH1064_SGA.1G3
CH1064_SGA.1E6
CH1064_SGA.2F4
0.01

**Fig. S2.3. Limiting dilution-derived isolates are representative of the viral quasispecies present *in vivo*.** The phylogenetic relationships of limiting dilution-derived isolate and SGA-derived plasma viral sequences are shown for all transmission pairs. Maximum likelihood trees with bootstrap support (1,000 replicates) were constructed using PhyML v. 3.1 (17) with evolutionary models selected using jModelTest v. 2.1.4 (19). For transmitting donors, plasma isolates (labeled PL, followed by the date and the isolate number), genital secretion isolates (labeled SE for semen and CV for cervicovaginal lavage, followed by the date and isolate number), and plasma vRNA derived SGA sequences (labeled SGA, followed by the amplicon number) are indicated in green, purple and blue, respectively. For acutely infected recipients, plasma isolate and SGA sequences (labeled like donor isolates) are shown in red and brown, respectively. Nodes with ≥ 75% bootstrap support are indicated (the scale bars represent 0.01 substitutions per site). (*A*) 3' half genome sequences from donor CH742 and his two recipients CH831 and CH378 denoted by brackets (4,358 bp). CH378 acquired two TF viruses, termed v1 and v2 (recombinants of these are labeled R). (*B*) 3' half genome sequences from donor CH148 and recipient CH040 (4,481 bp). SGA sequences for CH040 are available under GenBank accession codes: FJ495827 - FJ495838. (*C*) *tat*/*rev*, *env* and *nef* sequences from donor CH728 and recipient CH302 (2,829 bp). (*D*) 3' half genome sequences from donor CH492 and recipient CH427 (4,539 bp). (*E*) 3' half genome sequences from donor CH596 and recipient CH455 (4,501 bp). (*F*) 3' half genome sequences from donor CH212 and recipient CH162 (4,463 bp). SGA sequences for CH162 are available under GenBank accession codes: JX972986 - JX972998. (*G*) 3' half genome sequences from donor CH1064 and recipient CH848 (4,550 bp). SGA sequences for CH848 are available under GenBank accession codes: KX216883 - KX216893 and KX216895.

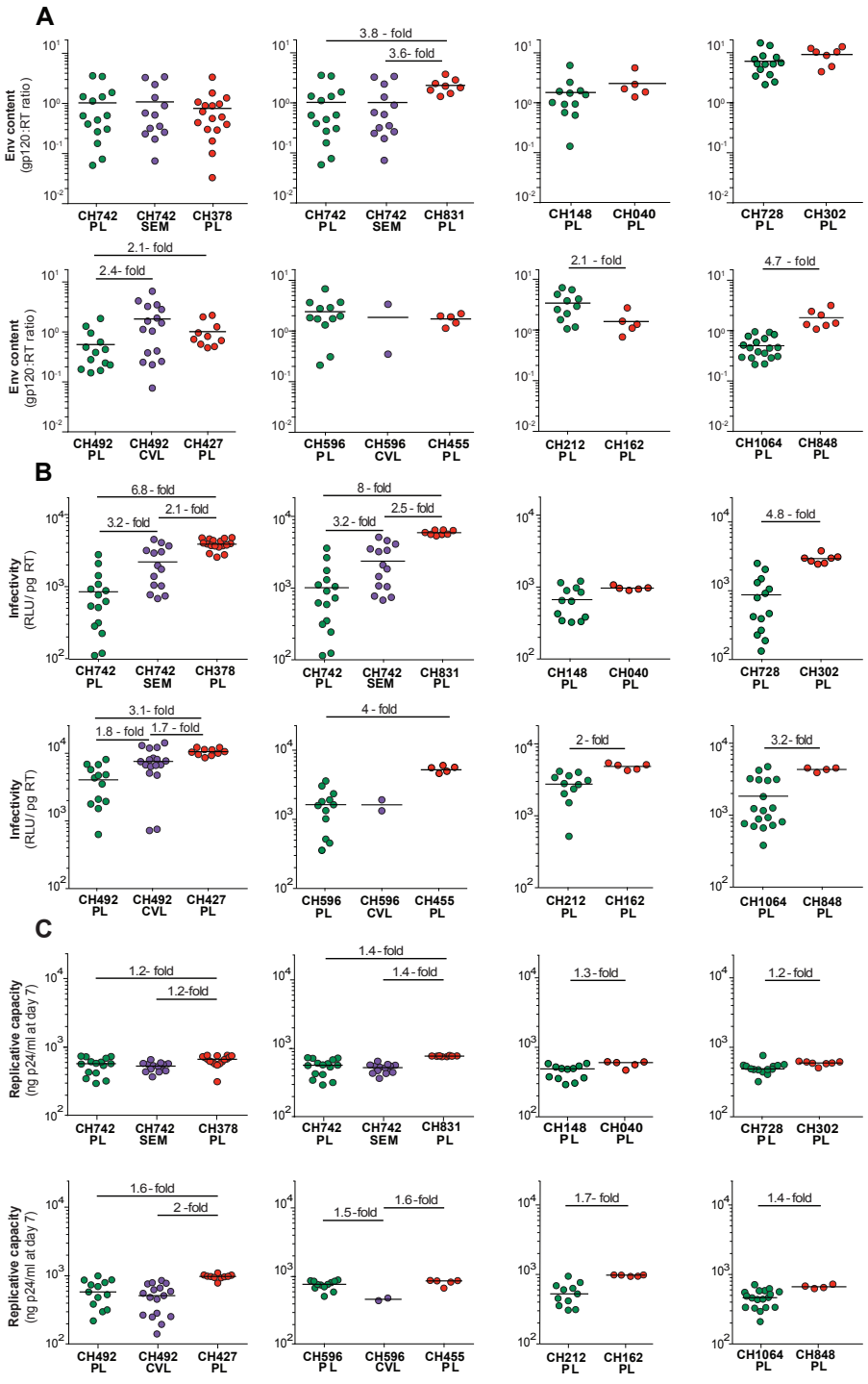

A
Env content (gp120:RT ratio)
3.8 - fold
3.6- fold
2.1- fold
2.4- fold
2.1 - fold
4.7 - fold
CH742 PL
CH742 SEM
CH378 PL
CH831 PL
CH148 PL
CH040 PL
CH728 PL
CH302 PL
CH492 PL
CH492 CVL
CH427 PL
CH596 PL
CH596 CVL
CH455 PL
CH212 PL
CH162 PL
CH1064 PL
CH848 PL
B
Infectivity (RLU/ pg RT)
6.8 - fold
2.1 - fold
3.2 - fold
8 - fold
2.5 - fold
4.8 - fold
3.1- fold
1.8 - fold
1.7 - fold
4 - fold
2 - fold
3.2- fold
C
Replicative capacity (ng p24/ml at day 7)
1.2- fold
1.2-fold
1.4 - fold
1.3 - fold
1.6 - fold
2 -fold
1.5 - fold
1.7- fold

**Fig. S2.4. Env content, particle infectivity and replicative capacity of limiting dilution-derived isolates from matched donor and recipient pairs.** (*A*) Env content (gp120:RT mass ratio), (*B*) particle infectivity (relative light units [RLU] in the TZM-bl assay per picogram of RT), and (*C*) replicative capacity (ng of p24 per ml of CD4 T cell culture supernatant at day 7 post infection) are shown for each limiting dilution-derived isolate of each transmission pair. Each dot represents an individual isolate derived from donor plasma (PL, green), donor genital secretions (CVL or SEM, purple), or recipient plasma (PL, red). Black lines denote the geometric mean, and fold changes are listed above groups when significant ($P < 0.05$).

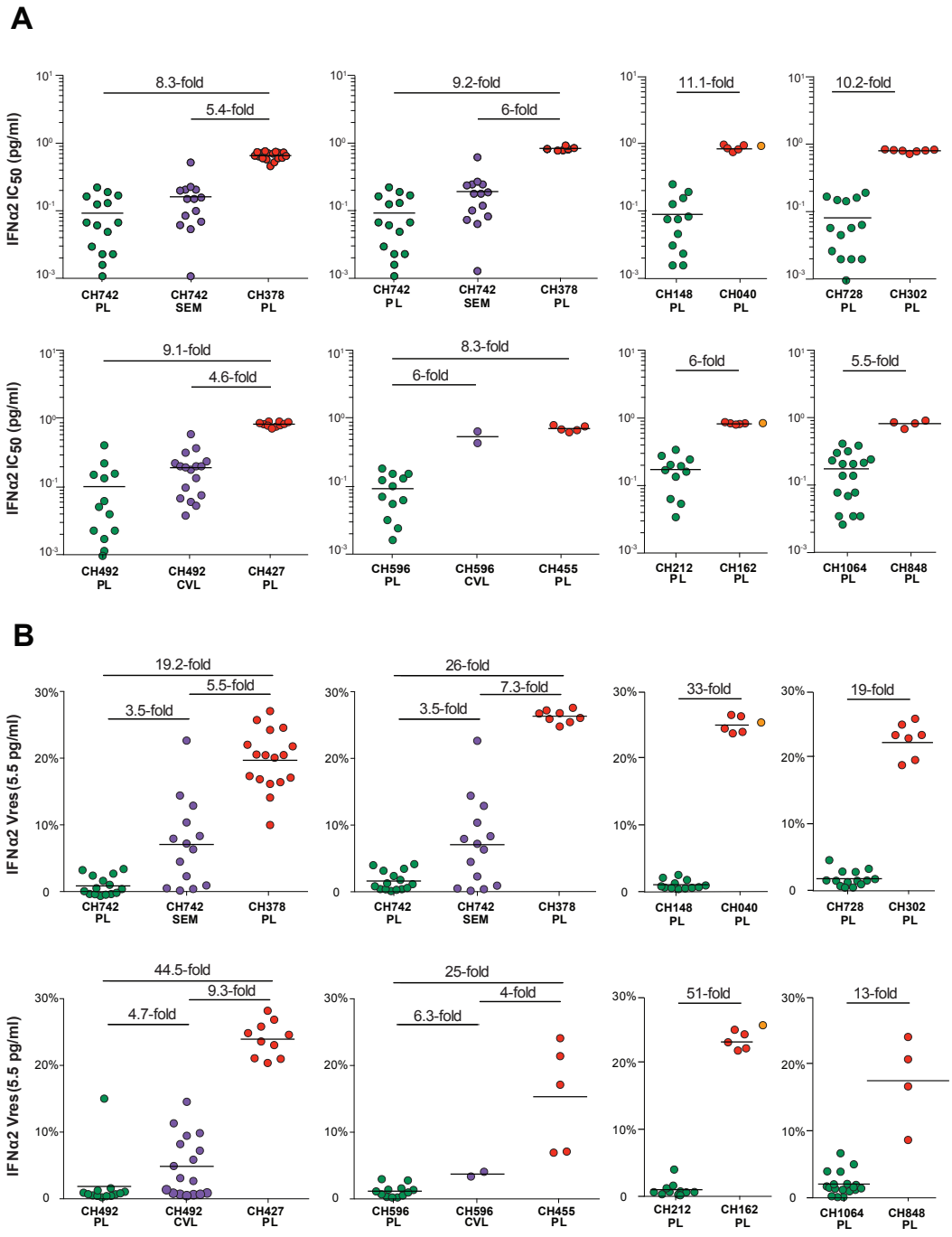

A
IFNα2 IC50 (pg/ml)
8.3-fold
5.4-fold
CH742 PL
CH742 SEM
CH378 PL
9.2-fold
6-fold
CH742 PL
CH742 SEM
CH378 PL
11.1-fold
CH148 PL
CH040 PL
10.2-fold
CH728 PL
CH302 PL
9.1-fold
4.6-fold
CH492 PL
CH492 CVL
CH427 PL
8.3-fold
6-fold
CH596 PL
CH596 CVL
CH455 PL
6-fold
CH212 PL
CH162 PL
5.5-fold
CH1064 PL
CH848 PL
B
IFNα2 Vres(5.5 pg/ml)
19.2-fold
5.5-fold
3.5-fold
CH742 PL
CH742 SEM
CH378 PL
26-fold
7.3-fold
3.5-fold
CH742 PL
CH742 SEM
CH378 PL
33-fold
CH148 PL
CH040 PL
19-fold
CH728 PL
CH302 PL
44.5-fold
9.3-fold
4.7-fold
CH492 PL
CH492 CVL
CH427 PL
25-fold
4-fold
6.3-fold
CH596 PL
CH596 CVL
CH455 PL
51-fold
CH212 PL
CH162 PL
13-fold
CH1064 PL
CH848 PL

Figure S5

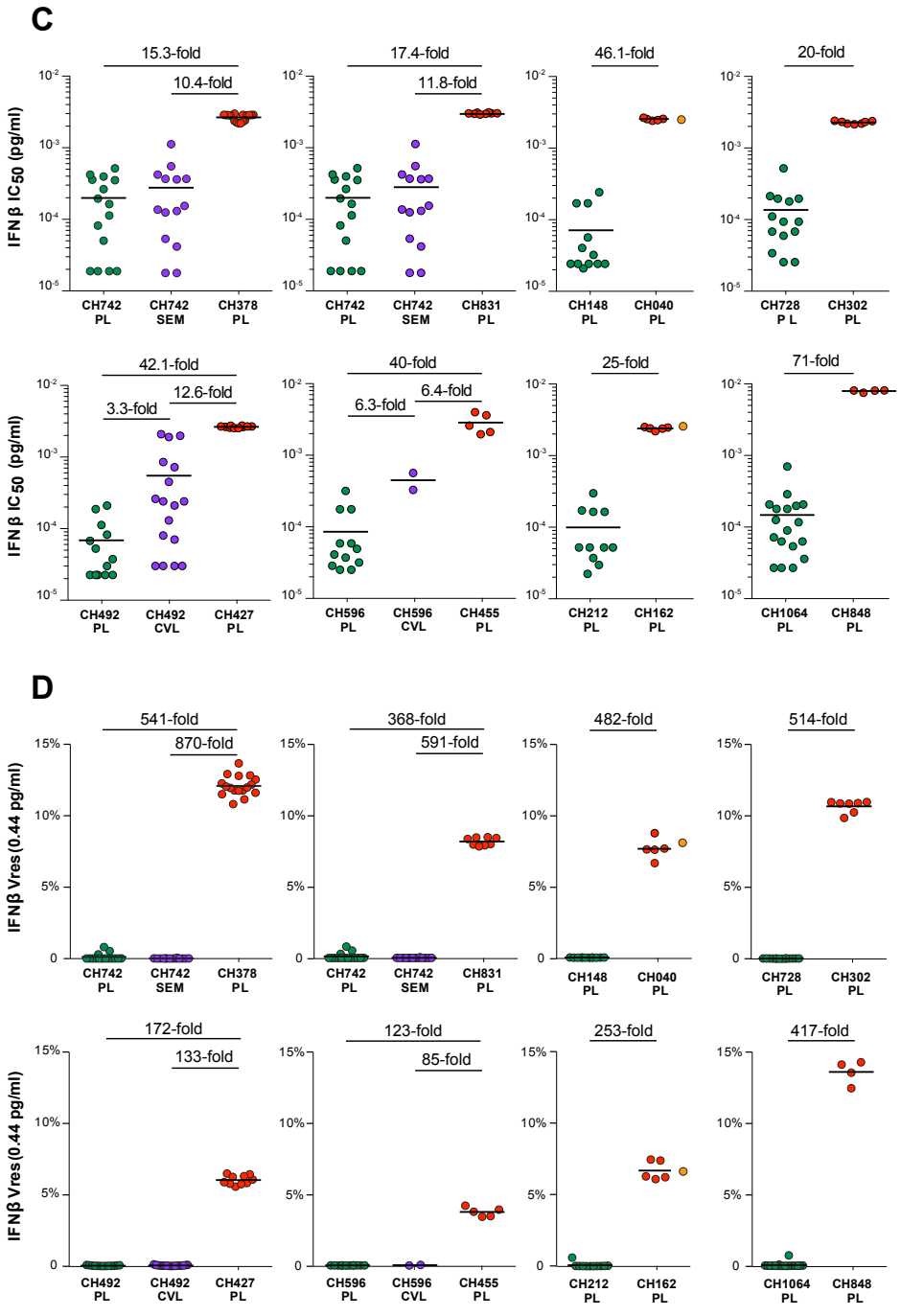

C
IFN β IC50 (pg/ml)
15.3-fold
10.4-fold
17.4-fold
11.8-fold
46.1-fold
20-fold
CH742 PL
CH742 SEM
CH378 PL
CH831 PL
CH148 PL
CH040 PL
CH728 P L
CH302 PL
42.1-fold
12.6-fold
3.3-fold
40-fold
6.4-fold
6.3-fold
25-fold
71-fold
CH492 PL
CH492 CVL
CH427 PL
CH596 PL
CH596 CVL
CH455 PL
CH212 PL
CH162 PL
CH1064 PL
CH848 PL
D
IFNβ Vres (0.44 pg/ml)
541-fold
870-fold
368-fold
591-fold
482-fold
514-fold
172-fold
133-fold
123-fold
85-fold
253-fold
417-fold

**Fig. S2.5. IFN resistance of limiting dilution-derived isolates from matched donor and recipient pairs.** Donor and recipient isolates were tested for their sensitivity to inhibition by type 1 IFNs. (*A*) Half-maximal inhibitory concentrations ($IC_{50}$) for IFN$\alpha$2 (pg/ml); (B) residual viral replication (Vres) at the highest (5.5 pg/ml) IFN$\alpha$2 dose (Vres); (*C*) Half-maximal inhibitory concentrations ($IC_{50}$) for IFN$\beta$ (pg/ml); (*D*) residual viral replication (Vres) at the highest (0.44 pg) IFN$\beta$ dose. Each dot represents an individual isolate derived from donor plasma (PL, green), donor genital secretions (CVL or SEM, purple), or recipient plasma (PL, red). Black lines denote the geometric mean, and fold changes are listed above groups when significant ($P < 0.05$). Orange dots indicating IFN$\alpha$2 and IFN$\beta$ $IC_{50}$ and Vres values for two available (CH040 and CH162) TF virus infectious molecular clones (4, 12) are shown for control.

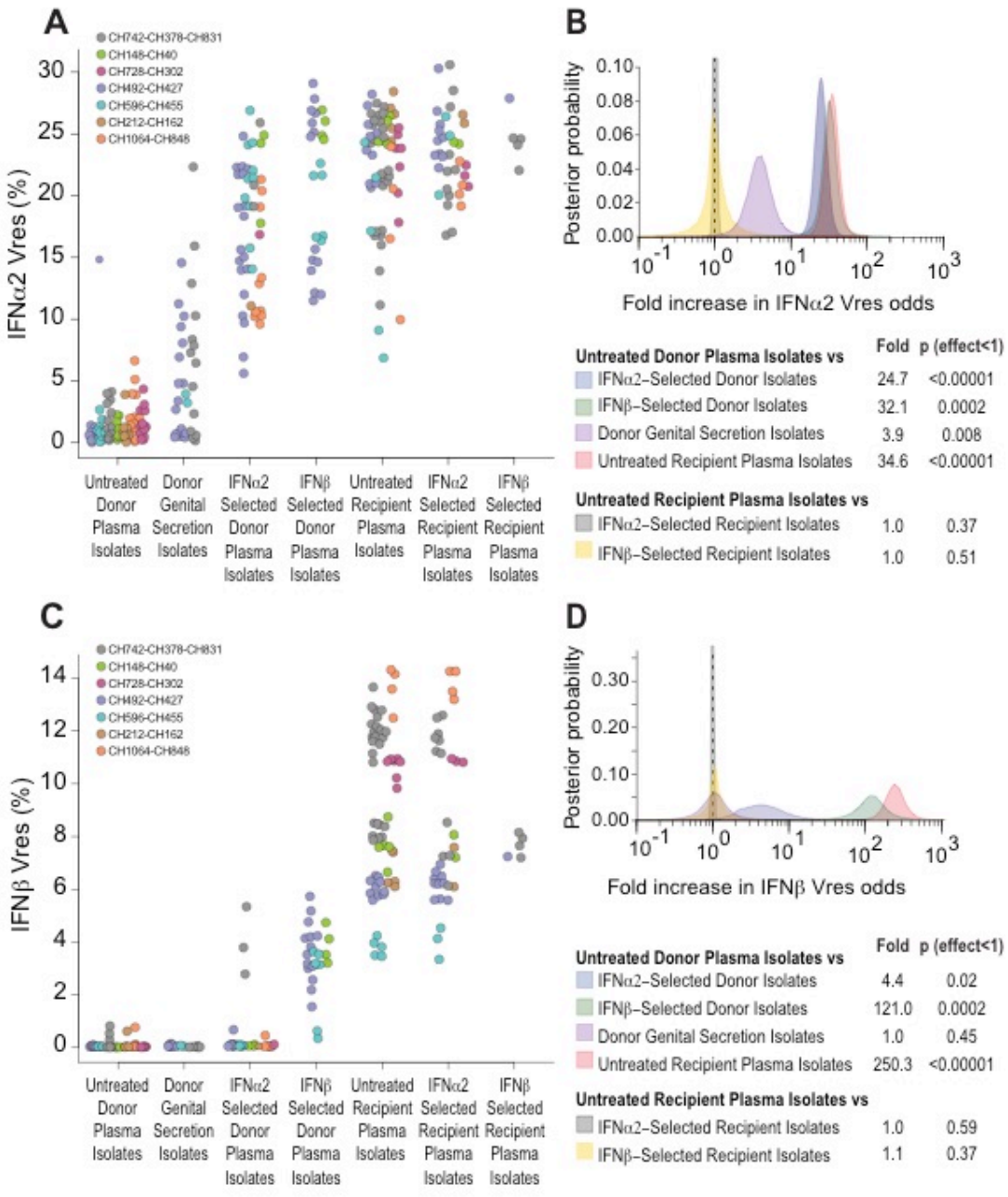
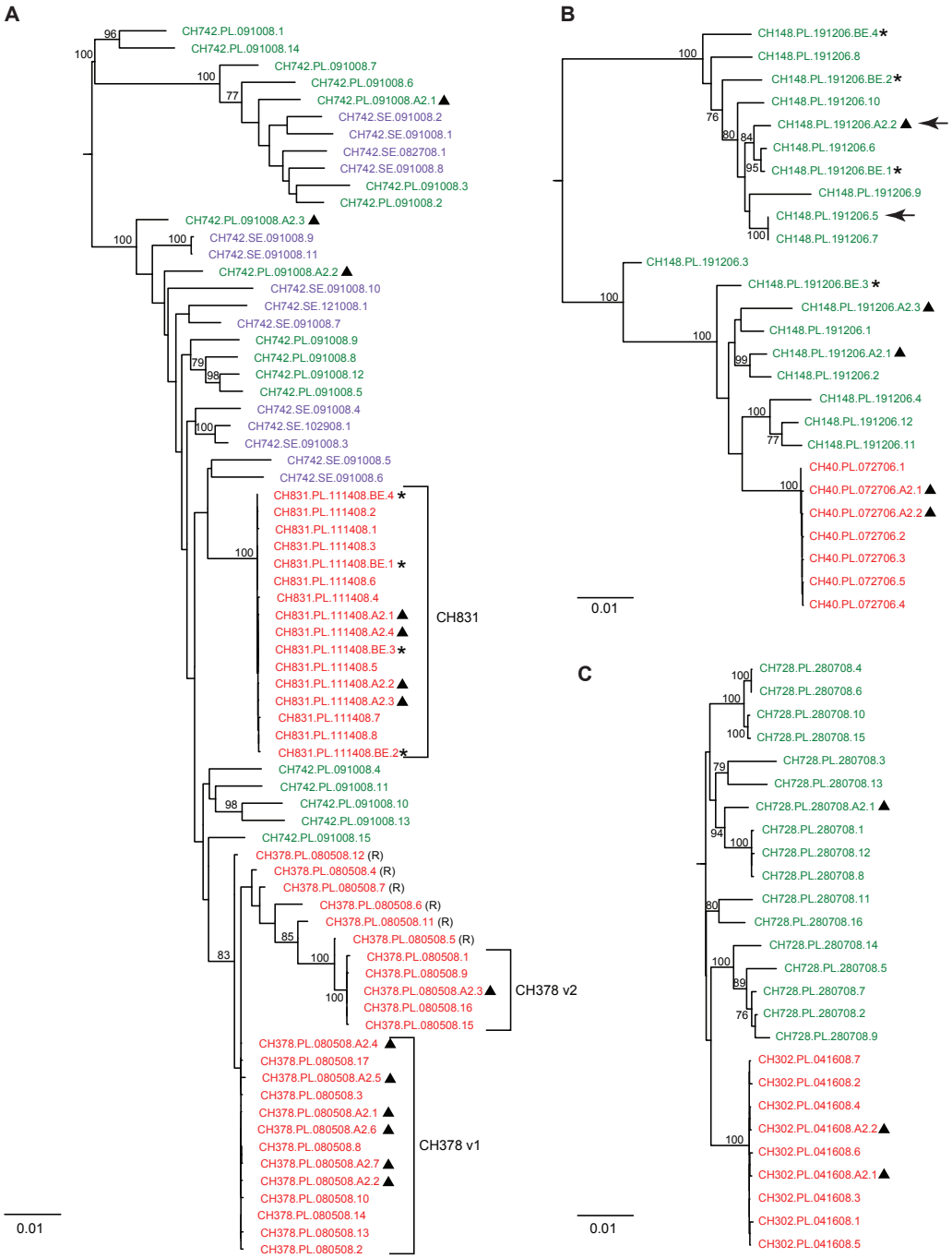
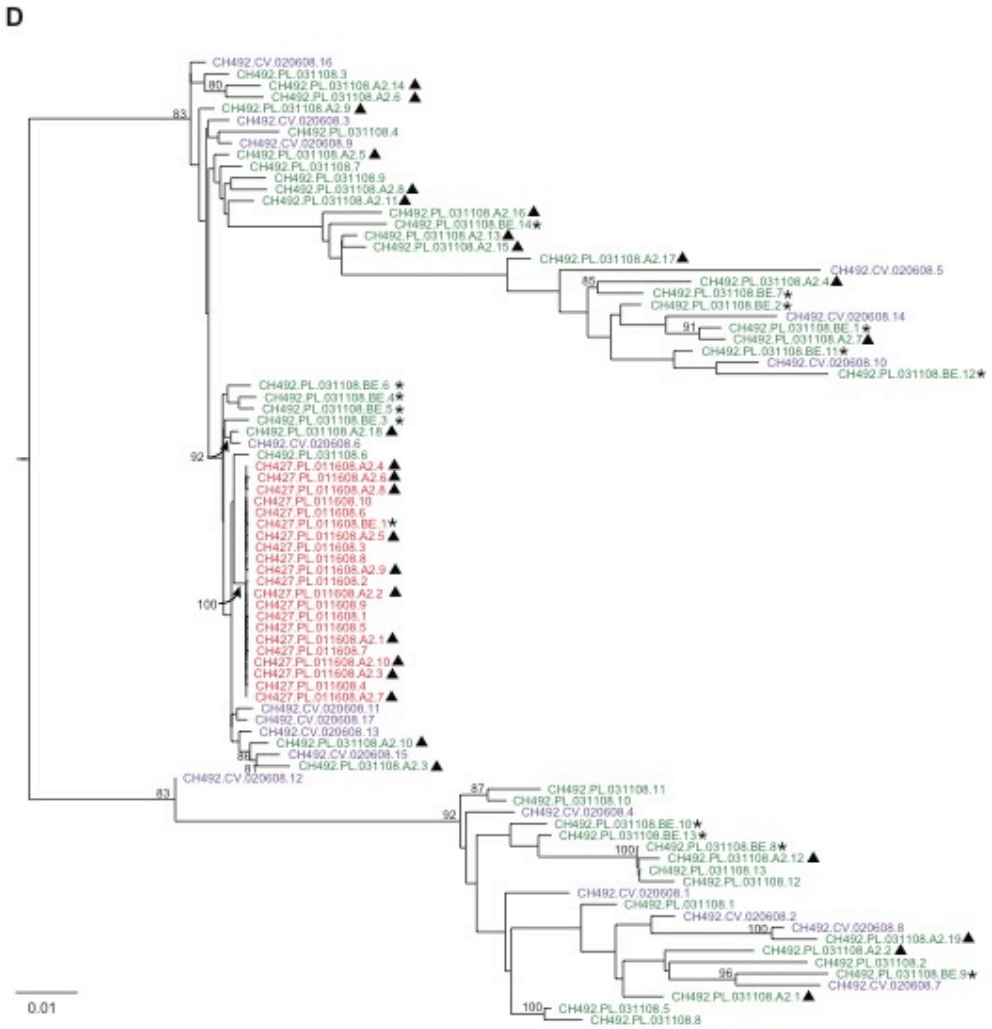

A
CH742-CH378-CH831
CH148-CH40
CH728-CH302
CH492-CH427
CH596-CH455
CH212-CH162
CH1064-CH848
IFNα2 Vres (%)
Untreated Donor Plasma Isolates
Donor Genital Secretion Isolates
IFNα2 Selected Donor Plasma Isolates
IFNβ Selected Donor Plasma Isolates
Untreated Recipient Plasma Isolates
IFNα2 Selected Recipient Plasma Isolates
IFNβ Selected Recipient Plasma Isolates
B
Posterior probability
Fold increase in IFNα2 Vres odds
Untreated Donor Plasma Isolates vs
Fold
p (effect<1)
IFNα2–Selected Donor Isolates 24.7 <0.00001
IFNβ–Selected Donor Isolates 32.1 0.0002
Donor Genital Secretion Isolates 3.9 0.008
Untreated Recipient Plasma Isolates 34.6 <0.00001
Untreated Recipient Plasma Isolates vs
IFNα2–Selected Recipient Isolates 1.0 0.37
IFNβ–Selected Recipient Isolates 1.0 0.51
C
IFNβ Vres (%)
D
Fold increase in IFNβ Vres odds
Untreated Donor Plasma Isolates vs
IFNα2–Selected Donor Isolates 4.4 0.02
IFNβ–Selected Donor Isolates 121.0 0.0002
Donor Genital Secretion Isolates 1.0 0.45
Untreated Recipient Plasma Isolates 250.3 <0.00001
Untreated Recipient Plasma Isolates vs
IFNα2–Selected Recipient Isolates 1.0 0.59
IFNβ–Selected Recipient Isolates 1.1 0.37

**Fig. S2.6**. **Residual viral replication (Vres) of untreated and IFN-selected isolates from matched donor and recipient pairs.** (*A, C*) Donor and recipient isolates were tested for their ability to replicate in CD4+ T cells in the presence of maximal doses of IFN$\alpha$2 (*A*) and IFN$\beta$ (*C*), expressed as the percentage of viral growth retained relative to growth in the absence of IFN. Viruses are colored by transmission pair and include untreated as well as IFN$\alpha$2-selected and IFN$\beta$-selected isolates from both donors and recipients. *(B, D*) A hierarchical Bayesian regression model was used to estimate the population-wide fold change in the odds of retaining replication in the presence of maximal (non-toxic) doses of IFN$\alpha$2 (*B*) or IFN$\beta$ (*D*), when comparing untreated and IFN$\alpha$2-selected donor plasma isolates (blue), untreated and IFN$\beta$-selected donor plasma isolates (green), untreated donor plasma and genital secretion isolates (purple), untreated donor and recipient plasma isolates (red), untreated and IFN$\alpha$2-selected recipient plasma isolates (grey) and untreated and IFN$\beta$-selected recipient plasma isolates (yellow). The dashed vertical line marks a fold change of 1, indicating no effect. The estimated posterior probability distribution for each parameter is shown along with a table summarizing the expected fold change, and the probability that the effect is less than 1.


A
CH742.PL.091008.1
CH742.PL.091008.14
CH742.PL.091008.7
CH742.PL.091008.6
CH742.PL.091008.A2.1
CH742.SE.091008.2
CH742.SE.091008.1
CH742.SE.082708.1
CH742.SE.091008.8
CH742.PL.091008.3
CH742.PL.091008.2
CH742.PL.091008.A2.3
CH742.SE.091008.9
CH742.SE.091008.11
CH742.PL.091008.A2.2
CH742.SE.091008.10
CH742.SE.121008.1
CH742.SE.091008.7
CH742.PL.091008.9
CH742.PL.091008.8
CH742.PL.091008.12
CH742.PL.091008.5
CH742.SE.091008.4
CH742.SE.102908.1
CH742.SE.091008.3
CH742.SE.091008.5
CH742.SE.091008.6
CH831.PL.111408.BE.4
CH831.PL.111408.2
CH831.PL.111408.1
CH831.PL.111408.3
CH831.PL.111408.BE.1
CH831.PL.111408.6
CH831.PL.111408.4
CH831.PL.111408.A2.1
CH831.PL.111408.A2.4
CH831.PL.111408.BE.3
CH831.PL.111408.5
CH831.PL.111408.A2.2
CH831.PL.111408.A2.3
CH831.PL.111408.7
CH831.PL.111408.8
CH831.PL.111408.BE.2
CH831
CH742.PL.091008.4
CH742.PL.091008.11
CH742.PL.091008.10
CH742.PL.091008.13
CH742.PL.091008.15
CH378.PL.080508.12 (R)
CH378.PL.080508.4 (R)
CH378.PL.080508.7 (R)
CH378.PL.080508.6 (R)
CH378.PL.080508.11 (R)
CH378.PL.080508.5 (R)
CH378.PL.080508.1
CH378.PL.080508.9
CH378.PL.080508.A2.3
CH378.PL.080508.16
CH378.PL.080508.15
CH378 v2
CH378.PL.080508.A2.4
CH378.PL.080508.17
CH378.PL.080508.A2.5
CH378.PL.080508.3
CH378.PL.080508.A2.1
CH378.PL.080508.A2.6
CH378.PL.080508.8
CH378.PL.080508.A2.7
CH378.PL.080508.A2.2
CH378.PL.080508.10
CH378.PL.080508.14
CH378.PL.080508.13
CH378.PL.080508.2
CH378 v1
0.01
B
CH148.PL.191206.BE.4
CH148.PL.191206.8
CH148.PL.191206.BE.2
CH148.PL.191206.10
CH148.PL.191206.A2.2
CH148.PL.191206.6
CH148.PL.191206.BE.1
CH148.PL.191206.9
CH148.PL.191206.5
CH148.PL.191206.7
CH148.PL.191206.3
CH148.PL.191206.BE.3
CH148.PL.191206.A2.3
CH148.PL.191206.1
CH148.PL.191206.A2.1
CH148.PL.191206.2
CH148.PL.191206.4
CH148.PL.191206.12
CH148.PL.191206.11
CH40.PL.072706.1
CH40.PL.072706.A2.1
CH40.PL.072706.A2.2
CH40.PL.072706.2
CH40.PL.072706.3
CH40.PL.072706.5
CH40.PL.072706.4
0.01
C
CH728.PL.280708.4
CH728.PL.280708.6
CH728.PL.280708.10
CH728.PL.280708.15
CH728.PL.280708.3
CH728.PL.280708.13
CH728.PL.280708.A2.1
CH728.PL.280708.1
CH728.PL.280708.12
CH728.PL.280708.8
CH728.PL.280708.11
CH728.PL.280708.16
CH728.PL.280708.14
CH728.PL.280708.5
CH728.PL.280708.7
CH728.PL.280708.2
CH728.PL.280708.9
CH302.PL.041608.7
CH302.PL.041608.2
CH302.PL.041608.4
CH302.PL.041608.A2.2
CH302.PL.041608.6
CH302.PL.041608.A2.1
CH302.PL.041608.3
CH302.PL.041608.1
CH302.PL.041608.5
0.01


D
CH492.CV.020608.16
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CH492.PL.031108.A2.6
CH492.PL.031108.A2.9
CH492.CV.020608.3
CH492.PL.031108.4
CH492.CV.020608.9
CH492.PL.031108.A2.5
CH492.PL.031108.7
CH492.PL.031108.9
CH492.PL.031108.A2.8
CH492.PL.031108.A2.11
CH492.PL.031108.A2.16
CH492.PL.031108.BE.14
CH492.PL.031108.A2.13
CH492.PL.031108.A2.15
CH492.PL.031108.A2.17
CH492.CV.020608.5
CH492.PL.031108.A2.4
CH492.PL.031108.BE.7
CH492.PL.031108.BE.2
CH492.CV.020608.14
CH492.PL.031108.BE.1
CH492.PL.031108.A2.7
CH492.PL.031108.BE.11
CH492.CV.020608.10
CH492.PL.031108.BE.12
CH492.PL.031108.BE.6
CH492.PL.031108.BE.4
CH492.PL.031108.BE.5
CH492.PL.031108.BE.3
CH492.PL.031108.A2.18
CH492.CV.020608.6
CH492.PL.031108.6
CH427.PL.011608.A2.4
CH427.PL.011608.A2.6
CH427.PL.011608.A2.8
CH427.PL.011608.10
CH427.PL.011608.6
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CH427.PL.011608.A2.5
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CH427.PL.011608.7
CH427.PL.011608.A2.10
CH427.PL.011608.A2.3
CH427.PL.011608.4
CH427.PL.011608.A2.7
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CH492.CV.020608.15
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CH492.CV.020608.12
CH492.PL.031108.11
CH492.PL.031108.10
CH492.CV.020608.4
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CH492.PL.031108.BE.13
CH492.PL.031108.BE.8
CH492.PL.031108.A2.12
CH492.PL.031108.13
CH492.PL.031108.12
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CH492.PL.031108.1
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CH492.PL.031108.A2.2
CH492.PL.031108.2
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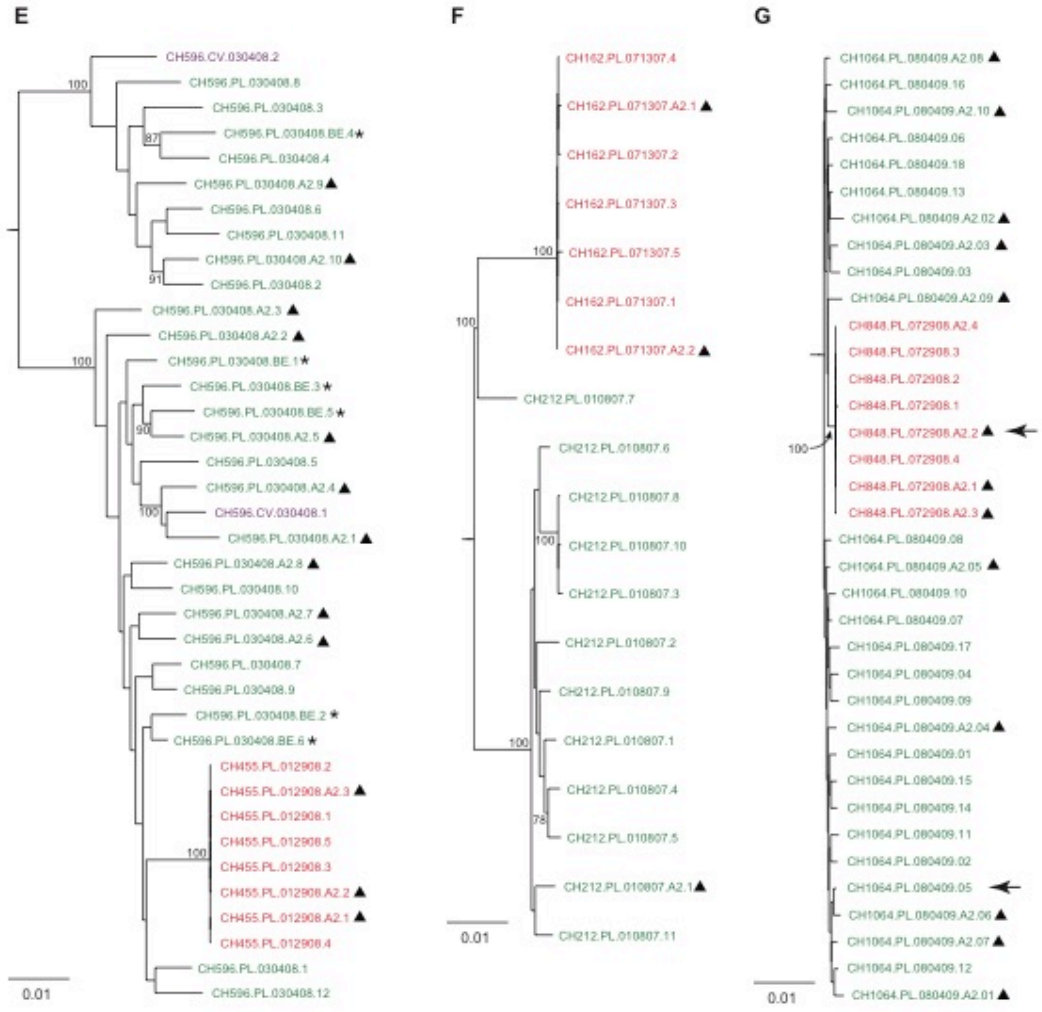
CH492.PL.031108.8
0.01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CH596.CV.030408.2
CH596.PL.030408.8
CH596.PL.030408.3
CH596.PL.030408.BE.4
CH596.PL.030408.4
CH596.PL.030408.A2.9
CH596.PL.030408.6
CH596.PL.030408.11
CH596.PL.030408.A2.10
CH596.PL.030408.2
CH596.PL.030408.A2.3
CH596.PL.030408.A2.2
CH596.PL.030408.BE.1
CH596.PL.030408.BE.3
CH596.PL.030408.BE.5
CH596.PL.030408.A2.5
CH596.PL.030408.5
CH596.PL.030408.A2.4
CH596.CV.030408.1
CH596.PL.030408.A2.1
CH596.PL.030408.A2.8
CH596.PL.030408.10
CH596.PL.030408.A2.7
CH596.PL.030408.A2.6
CH596.PL.030408.7
CH596.PL.030408.9
CH596.PL.030408.BE.2
CH596.PL.030408.BE.6
CH455.PL.012908.2
CH455.PL.012908.A2.3
CH455.PL.012908.1
CH455.PL.012908.5
CH455.PL.012908.3
CH455.PL.012908.A2.2
CH455.PL.012908.A2.1
CH455.PL.012908.4
CH596.PL.030408.1
CH596.PL.030408.12
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F
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CH162.PL.071307.A2.1
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CH162.PL.071307.3
CH162.PL.071307.5
CH162.PL.071307.1
CH162.PL.071307.A2.2
CH212.PL.010807.7
CH212.PL.010807.6
CH212.PL.010807.8
CH212.PL.010807.10
CH212.PL.010807.3
CH212.PL.010807.2
CH212.PL.010807.9
CH212.PL.010807.1
CH212.PL.010807.4
CH212.PL.010807.5
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CH212.PL.010807.11
0.01
G
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CH1064.PL.080409.A2.03
CH1064.PL.080409.03
CH1064.PL.080409.A2.09
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CH848.PL.072908.A2.2
CH848.PL.072908.4
CH848.PL.072908.A2.1
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CH1064.PL.080409.09
CH1064.PL.080409.A2.04
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CH1064.PL.080409.15
CH1064.PL.080409.14
CH1064.PL.080409.11
CH1064.PL.080409.02
CH1064.PL.080409.05
CH1064.PL.080409.A2.06
CH1064.PL.080409.A2.07
CH1064.PL.080409.12
CH1064.PL.080409.A2.01
0.01

**Fig. S2.7. Phylogenetic relationships of IFN-selected and unselected isolates from matched donor and recipient pairs.** Nucleotide sequences were aligned using CLUSTALW v. 2 (16), with ambiguous regions removed. Maximum likelihood trees with bootstrap support (1,000 replicates) were constructed using PhyML v. 3.1 (17) with evolutionary models selected using jModelTest v. 2.1.4 (19). Donor plasma isolate sequences (labeled PL, followed by the date and the isolate number) and genital tract isolate sequences (labeled SE for semen and CV for cervicovaginal lavage, followed by the date and isolate number) are shown in green and purple, respectively, while recipient plasma isolate sequences are shown in red (labeled PL, followed by the date and the isolate number). Isolates obtained in CD4+ T cells pretreated with IFNα2 (A2) or IFNβ (BE) are highlighted by asterisks and triangles, respectively. Bootstrap values ≥ 75% are shown (scale bars represent 0.01 substitutions per site). (*A*) Phylogenetic tree of near complete isolate sequences (8,812 bp) from donor CH742 and recipients CH831 and CH378 (denoted by brackets). CH378 was infected by two TF viruses (v1 and v2; recombinants of v1 and v2 are indicated with an "R"). (*B*) Phylogenetic tree of near complete isolate sequences (8,737 bp) from donor CH148 and recipient CH040; (*C*) Phylogenetic tree of near complete isolate sequences (8,872 bp) from donor CH728 and recipient CH302; (*D*) Phylogenetic tree of near complete isolate sequences (8,582 bp) from donor CH492 and recipient CH427; (*E*) Phylogenetic tree of near complete isolate sequences (8,674 bp) from donor CH596 and recipient CH455; (*F*) Phylogenetic tree of near complete isolate sequences (8,795 bp) from donor CH212 and recipient CH162; (*G*) Phylogenetic tree of near complete isolate sequences (8,861 bp) from donor CH1064 and recipient CH848. Arrows indicate examples of isolates with identical *vpu* sequences that differed in their p24 release capacity.

## CHAPTER 3

# VPU-MEDIATED COUNTERACTION OF TETHERIN IS A MAJOR DETERMINANT OF HIV-1 INTERFERON RESISTANCE

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SSI and DK contributed equally to this work

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**Section 3.1- Abstract**

HIV-1 groups M, N, O and P are the result of independent zoonotic transmissions of SIVs infecting great apes in Africa. Among these, only Vpu proteins of pandemic HIV-1 group M strains evolved potent activity against the restriction factor tetherin, which inhibits virus release from infected cells. Thus, effective Vpu-mediated tetherin antagonism may have been a prerequisite for the global spread of HIV-1. To determine whether this particular function enhances primary HIV-1 replication and interferon resistance, we introduced mutations into the *vpu* gene of HIV-1 group M and N strains to specifically disrupt their ability to antagonize tetherin, but not other Vpu functions, such as degradation of CD4, down-modulation of CD1d and NTB-A, and suppression of NF-kB activity. Lack of particular human-specific adaptations reduced the ability of HIV-1 group M Vpu proteins to enhance virus production and release from primary CD4+ T cells at high levels of type I IFN from about 5-fold to 2-fold. Interestingly, transmitted founder HIV-1 strains exhibited higher virion release capacity than chronic control HIV-1 strains irrespective of Vpu function, and group M viruses produced higher levels of cell-free virions than an N group HIV-1 strain. Thus, efficient virus release from infected cells seems to play an important role in the spread of HIV-1 in the human population and requires a fully functional Vpu protein that counteracts human tetherin.

**Section 3.2 – Importance**

Understanding which human-specific adaptations allowed HIV-1 to cause the AIDS pandemic is of great importance. One feature that distinguishes pandemic HIV-1 group M strains from non-pandemic or rare group O, N and P viruses is the acquisition of mutations in the accessory Vpu protein that confer potent activity against human tetherin. Adaptation was required because human tetherin has a deletion that renders it resistant to the Nef protein used by the SIV precursor of HIV-1 to antagonize this antiviral factor. It has been suggested that these adaptations in Vpu were critical for the effective spread of HIV-1 M strains, but direct evidence has been lacking. Here, we show that these changes in Vpu significantly enhance virus replication and release in human CD4+ T cells, particularly in the presence of IFN, thus supporting an important role in the spread of pandemic HIV-1.

**SECTION 3.3 – Introduction**

Pandemic HIV-1 emerged following the transmission of a simian immunodeficiency virus (SIV) from chimpanzees (cpz) to humans early in the 20$^{th}$ century (1). Since then, this major (M) group of HIV-1 has infected more than 70 million people and caused more than 30 million deaths. In contrast, HIV-1 groups O, N and P, which also resulted from zoonotic transmissions of chimpanzee (N) and gorilla (O, P) SIVs, have spread far less efficiently in the human population. Group O viruses have been found in about 100,000 individuals in Cameroon and surrounding countries (2), while HIV-1 groups N and P viruses are rare and have only been detected in a handful of individuals (3, 4).

One possible reason for why only HIV-1 group M became pandemic is the acquisition of potent anti-tetherin activity by its Vpu protein (5). Tetherin is an antiviral restriction factor that inhibits virus release by tethering nascent virus particles to the surface of infected cells (6, 7). Most primate lentiviruses including SIVcpz and SIVgor use their Nef protein to antagonize this antiviral factor (5, 8, 9). A deletion in the cytoplasmic domain of human tetherin, however, confers resistance to SIV Nefs and thus represents a significant barrier for successful zoonotic transmission (10, 11). Pandemic group M and (to a much lesser extent) rare group N strains acquired Vpu-mediated anti-tetherin activity (5, 12), while HIV-1 group O strains evolved the ability to counteract human tetherin by adapting their Nef protein to target a region adjacent to the deletion (13). However, neither of the two known group P viruses acquired significant anti-human tetherin activity (14, 15).

It has been shown that specific amino acid residues in the transmembrane domain (TMD) allow HIV-1 group M Vpus to interact directly with the TMD of tetherin and to counteract this restriction factor (16-18). In contrast, other Vpu functions are conserved between HIV-1 and its simian precursors. For example, SIVcpz and SIVgor Vpus are active in degrading human CD4 (5) and in suppressing the transcription factor NF-kB and interferon induction in human cells (19).

Furthermore, SIVcpz Vpu proteins downregulate cell surface expression of human NTB-A and CD1d (12), which suppress NK cell-mediated lysis of virally infected cells (20) and antigen presentation by virally infected dendritic cells, respectively (21). Complete abrogation of Vpu impairs HIV-1 replication in primary CD4+ T cells and humanized mice, and renders the virus hypersensitive to IFN-α inhibition (22-24). However, it remains unknown how much the more recently acquired Vpu-mediated anti-tetherin activity contributes to replication fitness and IFN resistance of HIV-1 group M. To address this, we introduced mutations in the TMD of the Vpu proteins of six group M infectious molecular clones (IMCs) that specifically abrogated their ability to antagonize human tetherin. We show that these changes significantly decrease HIV-1 replication and increase IFN sensitivity in primary human CD4+ T cells. Thus, human-specific adaptation of SIVcpz Vpu was likely required to gain maximal replication fitness of group M viruses in the new host and facilitate the successful colonization of humans.

**SECTION 3.4 – Results**

**Generation of HIV-1 Vpu mutants that are selectively impaired in tetherin antagonism.** Efficient counteraction of human tetherin by Vpu distinguishes HIV-1 group M strains from other group O, N and P strains (5). To examine the effect of Vpu-mediated tetherin antagonism on HIV-1 replication and IFN sensitivity in human CD4+ T cells, we generated a panel of infectious molecular clones (IMCs) that lacked this specific Vpu function. We achieved this by mutating two alanines in the TMD of Vpu, which have previously been shown to be critical for antagonism of human tetherin (16-18), to leucines (Fig. 3.1A). These mutations were introduced into two (CCR5-tropic) transmitted founder (TF) (CH058-TF, CH077-TF) and two chronic control (CC) viruses (STCO-CC, CH167-CC) (25). The T-cell line adapted (CXCR4-tropic) NL4-3 clone served as a control. All IMCs represented clade B viruses, except for CH167-CC, which is a clade C strain (Table 1). For comparison, we also generated a mutant of the group N HIV-1 clone DJO0131 (26) to determine whether the modest gain of anti-tetherin activity by this viral lineage (5, 12) is sufficient to promote virus replication and release in primary CD4+ T cells.

To verify that the introduced mutations abrogated Vpu`s ability to counteract human tetherin, we cotransfected HEK293T cells with vectors expressing wild-type (wt) or TMD mutant (Tmut) Vpu proteins and eGFP (or eGFP alone for control) together with a construct expressing human tetherin. The TMD mutations did not affect Vpu expression levels (Fig. S3.1A), but significantly impaired the ability of all HIV-1 M Vpus to reduce tetherin cell surface expression (Fig. 3.1B, S3.1B). In agreement with published data (12), the DJO0131 N-Vpu was poorly expressed (Fig. S3.1A) and had only a modest effect on tetherin, which was entirely abolished by the TMD mutations (Fig. 3.1B, S3.1B). To further examine the effect of these TMD mutations, we analyzed the efficiency of virus release from HEK293T cells cotransfected with *vpu*-defective HIV-1 NL4-3 together with constructs expressing wt or TMD-mutated Vpu proteins or eGFP only, as well as

plasmids expressing human tetherin at different doses. The results showed that the TMD mutations completely disrupted the ability of Vpu to enhance virus release (Fig. 1C). Notably, HIV-1 M subtype C CH167-CC Vpu antagonized tetherin more efficiently than subtype B and group N Vpus (Fig. 3.1C).

To determine the specificity of the TMD mutations for the anti-tetherin activity of Vpu, we transfected HEK293T cells with vectors co-expressing Vpu and eGFP together with constructs expressing CD4, NTB-A or CD1d. All HIV-1 M Vpus strongly reduced cell surface expression of CD4, while the group N Vpu had little effect (Fig. S3.1B, S3.1C). Although only Vpus from the two CC HIV-1 M strains STCO-CC and CH167-CC significantly reduced NTB-A and CD1d cell surface expression (Fig. S3.1D, S3.1E), the effect of Vpu on these receptors was not significantly impaired by the TMD mutations. HIV-1 M Vpus also suppressed antiviral gene expression and immune activation by inhibiting NF-κB activation (19, 27). Cotransfection of HEK293T cells with vectors coexpressing Vpu and eGFP together with an NF-κB-dependent firefly luciferase reporter construct and a constitutively active mutant of IKKβ showed that the CH058-TF, CH077-TF and STCO-CC Vpus suppressed IKKβ-mediated NF-κB activation by ~80%, whereas the NL4-3 Vpu achieved ~40% inhibition and the HIV-1 N Vpu was inactive (Fig. S2A). Thus, in agreement with previous data (19, 28), primary HIV-1 M Vpus inhibited NF-κB more efficiently than the NL4-3 or group N Vpu proteins. It has been reported that Vpu may suppress NF-κB activation by at least two different mechanisms: antagonism of tetherin (27) and stabilization of IκB and prevention of nuclear translocation of p65 (19). Thus, we also analyzed whether the TMD mutations affect the ability of Vpu to inhibit tetherin-mediated NF-κB stimulation. Consistent with previous data (27), tetherin expression induced NF-κB activation in a dose-dependent manner (Fig. S3.2B). However, wt and Tmut Vpu proteins suppressed tetherin-mediated NF-κB activation with similar potencies (Fig. S3.2B). This result is in agreement with our previous finding that primate lentiviral Vpu proteins efficiently prevent NF-κB activation independent of their anti-tetherin activity (19).

To determine the effect of the TMD mutations on the ability of Vpu to reduce tetherin and CD4 surface expression levels in primary human cells, PHA-stimulated peripheral blood mononuclear cells (PBMCs) were infected with the six sets of HIV-1 infectious molecular clones (IMCs) containing wt, TMD mutated or defective *vpu* genes. The latter contained either a 120 bp deletion (NL4-3) or two premature stop codons at positions two and three of the reading frame (all other IMCs). Three days later, the cells were stained for surface tetherin and CD4, permeabilized, and stained for intracellular p24 expression. On average, wt group M Vpus reduced the surface levels of tetherin by ~50%, while HIV-1 N Vpu achieved 34% (Fig. 3.2). Since no specific antibodies are available, we could not determine whether the modest activity of the N-Vpu was the result of poor activity or low expression levels. The two Ala to Leu substitutions in the TMD domain of Vpu generally disrupted tetherin downmodulation in HIV-1-infected primary cells (Fig. 3.2). In contrast, all 18 HIV-1 IMCs efficiently reduced CD4 cell surface expression, irrespective of the Vpu allele (Fig. S3.3). This is because these proviral HIV-1 constructs express functional Env and Nef proteins and particularly the latter is highly effective in down-modulating CD4 in HIV-1-infected T cells (29).

**Tetherin antagonism is critical for effective HIV-1 production in CD4+ T cells.** CD4+ T cells are the first productively infected cell type detected in primary HIV-1 infection (30) and TF HIV-1 strains, which establish *de novo* clinical infection, are less sensitive to inhibition by type I interferon (IFN) than chronic control HIV-1 (31, 32). To determine the role of Vpu-mediated tetherin antagonism in virus production and sensitivity to IFN, we infected activated CD4+ T cells with equivalent amounts of virus in the presence and absence of IFN$\alpha$a and determined the levels of p24 antigen production in culture supernatants on day 7 post-infection. Because of their importance in HIV-1 transmission, we focused on the CH058-TF and CH077-TF viruses and used the NL4-3 and chronic CH167-CC IMCs as controls.

In agreement with published data (31, 32), the CH058-TF and CH077-TF HIV-1 IMCs produced substantially higher levels of cell-free virus than the chronic CH167-CC or the T-cell line adapted NL4-3 construct in the presence, but not in the absence, of IFNαa (Fig. 3.3A). IFNαa treatment reduced cell-free p24 yield of wt TF HIV-1 IMCs by ~9-fold. This reduction was significantly lower than that observed for NL4-3 (58.2-fold) and the CC CH167-CC IMC (44.1-fold) (Fig. 3.3B). Point mutations in the TM domain of Vpu increased IFN sensitivity to a similar extent (~3.1-fold) as the complete lack of Vpu (~3.5-fold) (Fig. 3.3B). In the absence of IFNαa, wt CH058-TF and CH077-TF Vpus enhanced p24 production by 85% and 189%, whereas the corresponding Tmut Vpus achieved only 38% and 121% (Fig. 3.3C). The ability of Vpu to enhance cell-free p24 levels was more pronounced in the presence of IFNαa: wt CH058-TF, CH077-TF and NL4-3 Vpu proteins increased cell-free p24 antigen yield about 5-fold (Fig. 3.3C). In agreement with its potent anti-tetherin activity in transient transfection assays (Fig. 3.1C), the CH167-CC Vpu achieved a 9-fold enhancement (Fig. 3.3C), although the corresponding IMC produced only low levels of cell-free virus (Fig. 3.3A). Tmut Vpus increased the levels of p24 antigen in the supernatants of IFNαa CD4+ T cell cultures only marginally compared to HIV-1 IMCs lacking Vpu function entirely (Fig. 3.3C). Our finding that wt Vpu proteins enhanced the levels of cell-free HIV-1 TF viruses in the presence of IFNa substantially more efficiently than Tmut Vpus is consistent with a relevant role of tetherin antagonism for viral spread *in vivo*.

**IFNαa treatment impairs release of *vpu* mutant but not wt HIV-1 strains.** To assess the effects of the TMD mutations on total virus production and the efficiency of virion release, we determined the levels of cell-associated and total p24 antigen in the HIV-1-infected cultures (Fig. S3.4A, S3.4B). Total p24 was determined as the sum of both cell-free and cell-associated p24. The impact of Vpu on the levels of cell-associated p24 varied (Fig. S3.4C), most likely because functional *vpu* genes may also enhance viral replication and thus increase the total number of

infected cells. Fully functional wt Vpus increased the total amount of p24 antigen produced in IFN-treated cultures by ~3-fold and this enhancement was severely impaired by the TMD mutations in Vpu (Fig. 3.3D). We quantified released p24 as the ratio of cell-free p24 divided by total p24. IFNαa treatment generally decreased the efficiency of virus release (Fig.3.3E). TMD mutations or the lack of Vpu function reduced virion release efficiency by ~20% in the absence and by ~50% in the presence of IFNa treatment (Fig. 3F). Although the Tmut Vpus failed to enhance virion release (Fig. 3.3E, 3.3F), they significantly enhanced total (Fig. 3.3D, S3.4B) p24 production in the infected cultures.

**TF IMCs produce high titers of cell-free virus even in the absence of Vpu function.** The data outlined above suggest that in addition to the anti-tetherin function, other activities of M-Vpus contribute to efficient viral replication in primary CD4+ T cells. However, the results shown in Figure 3.3 were only derived from a single time point (day 7) following HIV-1 infection. To further examine the importance of Vpu-mediated tetherin antagonism for HIV-1 replication, we monitored virus production in primary CD4+ T cells infected with wt and *vpu* mutant HIV-1 IMCs over a period of nine days (Fig. 3.4A). In addition, we included another CC HIV-1 IMC (STCO-CC) and the HIV-1 N DJO0131 clone in the analyses. As expected, TF HIV-1 strains CH058-TF and CH077-TF exhibited substantially higher levels of virus production than the remaining IMCs in the presence of IFNαa (Fig. 3.4A, 3.4B). On average, IFNαa treatment decreased virus yield of these two TF viruses ~9-fold, whereas that of the CC HIV-1 strains CH167-CC and STCO-CC was 47- and 75-fold, and that of the group N virus even >100-fold reduced (Fig. 3.4C).

The TMD mutations in Vpu resulted in cell-free virus yields that were intermediate between wt and *vpu*-defective HIV-1 group M strains in the absence of IFNαa treatment (Fig. 3.4B). In the presence of IFNαa, the Tmut Vpus failed to enhance the p24 levels in cultures infected with NL4-3 or the CC strains, and had only modest effects on the two TF strains (Fig. 3.4B). Mutations in

the TMD or lack of Vpu function enhanced sensitivity of most HIV-1 M IMCs to IFNαa inhibition ~3- to 4-fold (Fig. 3.4C). The exception was the STCO-CC strain, which showed low levels of replication and was highly susceptible to IFNα inhibition irrespective of Vpu function (Fig. 3.4C). The single group N virus was also very susceptible to IFN inhibition. Notably, the ~4-fold enhancement of p24 production by HIV-1 N Vpu in the presence of IFN was not impaired by the TMD mutations (Fig. 3.4A-C). Thus, N-Vpu appears to promote HIV-1 replication independent of its modest anti-tetherin activity. In contrast, the 5- to 9-fold enhancing effect of group M Vpus was disrupted by the TMD mutations (Fig. 3.4D). We ranked the HIV-1 IMCs based on their efficacy to produce cell-free virus (Fig. 3.4E). In the absence of IFN, the T-cell line adapted NL4-3 construct showed the highest virus yield and functional *vpu* genes had only modest effects on the levels of cell-free p24 (Fig. 3.4E). In contrast, TF HIV-1 IMCs produced the highest levels of cell-free p24 in the presence of IFN. Mutations in the TMD domain or entire loss of Vpu function reduced cell-free p24 yield from TF IMCs by 2.5- and 5-fold, respectively. However, even the *vpu* mutated or *vpu*-defective TF IMCs showed higher virus yields than the CC HIV-1 M and the group N strains (Fig. 3.4E, right). Thus, Vpu-mediated tetherin antagonism is critical for high virus yield from infected CD4+ T cells in the presence of IFNαa, but additional *vpu*-independent functions also play a role.

**TF IMC infected cells release virions with high efficacy even in the absence of Vpu function.** Next, we determined the levels of cell-free and total p24 antigen in the cultures (Fig. S3.5) to calculate the efficiency of virus release. Unexpectedly, the Tmut HIV-1 M IMCs produced total quantities of p24 antigen that were as high (CH058-TF, STCO-CC) or slightly higher (NL4-3; CH077-TF, CH167-CC) than the p24 antigen amounts produced by the respective wt viruses (Fig. S3.5B), which may be due to more effective cell-to-cell spread and/or Vpu-mediated degradation of CD4 in Tmut infected cultures. In agreement with data shown in Figure 3.3, IFNαa

treatment reduced the efficiency of virus release, particularly in the absence of a functional Vpu. Furthermore, release of the TF HIV-1 IMCs was more efficient than that of CC viruses, while release of the HIV-1 group N DJO0131 IMC was markedly reduced relative to the five group M viruses (Fig. 3.5A). These differences in virion release were highly reproducible in independent experiments (Fig. S3.6). Interestingly, Tmut as well as *vpu*-defective TF HIV-1 strains showed significantly higher efficiencies of virion release than the wt CC HIV-1 strains in the presence of IFNαa (Fig. 3.5A, 3.5B). In general, the differences in virion release capacity were much more pronounced in IFNαa-treated than in untreated CD4+ T cell cultures (Fig. 3.5C), but the relative efficiencies of the 18 HIV-1 IMCs measured under both conditions showed a highly significant correlation (Fig. 3.5D). CC HIV-1 IMCs containing disrupted or mutated *vpu* genes and all HIV-1 group N constructs exhibited very low (<10% of wt TF HIV-1 IMCs) efficiencies of virion release (Fig. 3.5C, right). For all viruses, there was a significant correlation between p24 production and release, particularly in IFNαa-treated cultures (Fig. 3.5E, 3.5F), although other factors clearly also influence virus production. Finally, we examined whether the human specific adaptations in Vpu affected the infectiousness of viral particles produced in the infected CD4+ T cells cultures. We found that the TF derived virions were substantially more infectious than the CC and group N derived particles (Fig. S3.7A). The mutations in Vpu, however, had no significant effect on virion infectivity (Fig. S3.7B). Altogether, these results suggest that high infectivity and efficient virion release might represent hallmarks of TF HIV-1 strains and that the latter is only partly dependent on potent Vpu-mediated tetherin antagonism.

**Section 3.5 – Discussion**

Great apes transmitted SIVs to humans on at least four independent occasions. However, only one of these transmission events resulted in a pandemically spreading pathogen (1). Elucidating the viral properties that mediate efficient spread of HIV-1 is important for preventive strategies. It has been suggested that the acquisition of Vpu-mediated tetherin antagonism promoted efficient spread of HIV/AIDS (10, 11). However, direct evidence for this hypothesis has been lacking because thus far only T-cell line adapted viruses have been characterized that were completely vpu-deficient. Here, we show that amino acid mutations in the TMD domain of Vpu, which are critical for anti-tetherin activity, reduce virion production and release in the presence of IFN$\alpha$a by about 50%. Tmut TF viruses were released about 3-fold more efficiently than Tmut CC viruses, and >10-fold more efficiently than the Tmut group N virus in IFNa-treated human CD4+ T cells, although this release was only partly dependent on Vpu (Fig. 3.5C). Thus, our data support the hypothesis that adaptation at key Vpu residues that confer effective tetherin antagonism were indeed important for the spread of HIV/AIDS. Moreover, our data suggest that TF HIV-1 M strains have evolved additional yet-to-be-defined Vpu-independent functions to ensure efficient virus release and replication in the face of an innate antiviral response.

The TMD mutations in Vpu resulted in HIV-1 M virus levels that were intermediate between wt and *vpu*-defective IMCs, although in the presence of exogenous IFN$\alpha$a this phenotype was almost identical to that of HIV-1 lacking Vpu entirely (Fig. 3.4A, 3.5B). However, in the absence of IFN$\alpha$a, Tmut Vpus had little if any reducing effect on the total levels of HIV-1 p24 antigen production (Fig. S3.3B, S3.4B). The remaining activity of Tmut Vpus is unlikely due to residual anti-tetherin activity, since there were no significant differences in the amounts of particle release from TMD-mutated and Vpu deficient IMC infected cultures (Fig. 3.3F, 3.5A). Together, these data suggest that both the newly acquired anti-tetherin activity and other Vpu functions that are

conserved between HIV-1 and SIVcpz Vpus, such as degradation of CD4 or inhibition of NF-kB activation, increase viral replication fitness in primary CD4+ T cells. Lentiviral accessory proteins are well known for their multi-functionality, only some of which might be lost after cross-species transmission.

Although mutations in the TMD domain of Vpu and complete lack of Vpu function reduced the replication potential and particle release of TF viruses, particularly upon treatment with IFN$\alpha$a, their growth rates and virion production capacity remained significantly higher than those of wt CC HIV-1 strains (Fig. 3.4, 3.5). Tetherin-independent effects on virus release are further supported by the reduced replication capacity of the CH167-CC IMC compared to the two TF viruses and HIV-1 NL4-3 (Fig. 3A, 4A), although its Vpu showed the highest potency in antagonizing tetherin (Fig. 3.1C) and enhancing p24 production (Fig. 3.3C, 3.4D). Moreover, the Vpu proteins of TF viruses are equally potent at antagonizing human tetherin than those derived from CC HIV-1 strains (33, 34). Thus, other as-yet-unknown viral properties that promote efficient release of virions from infected T cells likely contribute to virus spread. One of them is the ability to potently degrade and downmodulate CD4. It has been shown that CD4 inhibits virus release (35) and reduces virion infectivity (36, 37). The phenotype of the group HIV-1 N DJO0131 strain that lacks a Vpu-mediated CD4 degradation function (12) supports these findings. Potent CD4 downmodulation are also consistent with previous data showing that TF virions are slightly more infectious and contain about two-fold more Env per particle than CC viruses (32). Although all IMCs efficiently down-modulated cell-surface CD4 due to functional Nef and Env expression, Vpu-mediated CD4 degradation may contribute to potent virus release and replication by preventing intracellular interaction between CD4 and the viral Env glycoprotein (35-37). However, other cellular factors that affect virion release efficacy, such as T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins (38), may also play a role, and it will be interesting to determine whether they are efficiently counteracted by TF HIV-1.

It is still unclear whether cell-free or cell-associated virus predominates in sexual HIV-1 transmission (39, 40), although multiple studies found a correlation between the efficiency of transmission and levels of cell-free virus in blood or genital secretions (41-43). We found that TF viruses produced much higher levels of cell-free virus than CC HIV-1 M, whereas the levels of cell-associated virus were higher in the T cell cultures infected with the CH167-CC and group N DJO0131 viruses (Fig. S3.5A). Thus, it is possible that cell-free HIV-1 plays an important role in sexual virus transmission.

The group N HIV-1 molecular clone was highly sensitive to IFN inhibition and produced very little cell-free virus in the presence of IFN (Fig. 3.4E), although the levels of cell-associated p24 antigen and total produced virus were comparable to that of the two TF HIV-1 M strains both in the presence and absence of IFN$\alpha$a (Fig. S3.5). However, in the presence of IFN both wt and TMD mutated group N Vpus increased cell-free virus production about 4-fold (Fig. 3.4D). Thus, it seems clear that the DJO (N) Vpu promotes virus production by yet-to-be-defined tetherin-independent mechanisms. Whether these effects of N-Vpu contribute to viral pathogenesis remains to be determined but it is noteworthy that HIV-1 N strains can cause CD4+ T cell depletion and AIDS (44-46).

In summary, our results demonstrate that Vpu-mediated tetherin antagonism enhances virus production and release from primary CD4+ T cells by about 5-fold in the presence of high levels of type I IFN. We further show that even *vpu*-defective or mutated TF HIV-1 strains exhibit higher virion release capacity than wt CC HIV-1 strains in IFN$\alpha$a-treated primary T cells. Thus, TF HIV-1 M *vpu* genes appear to encode functions in addition to effective tetherin antagonism that enhance viral replication and release in the presence IFN. Finally, CD4 T cell infected with wt group N virus produced about 4-fold less cell-free virions compared to CC HIV-1 M strains and about 13-fold less virions compared to TF HIV-1 M IMCs. Thus, the efficiency with which virus is released from infected CD4 T cells appears to be correlated with the ability of HIV-1 to spread in humans, with anti-tetherin activity playing a major role at least for group M viruses.

**Section 3.6 - Materials and methods**

**HIV-1 proviral constructs.** Generation of NL4-3, CH058, CH077, CH167, STCO and group N DJO0131 HIV-1 IMCs has been previously described (12, 25, 26, 32, 47) (Table S1). Site-directed mutagenesis of *vpu* was performed by splice overlap extension PCR and all constructs were verified by sequence analysis. TMD mutations in Vpu are shown in Fig. 3.1A. Grossly *vpu*-defective IMCs contained a premature stop codon at amino acid positions two and three of the *vpu* reading frame, except NL4-3 that contained a 120 bp deletion in *vpu*.

**Expression vectors.** Cloning of HIV-1 *vpu* genes and human *tetherin*, *CD4*, *NTB-A* and *CD1d* alleles into the bi-cistronic CMV promoter-based pCG expression vector coexpressing the green fluorescent protein (GFP) was performed as described previously (5, 12).

**Cell culture.** HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 350 µg/ml L-glutamine, 100 µg/ml streptomycin sulfate and 100 U/ml penicillin. HEK293T cells were transfected by the calcium phosphate method. Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using lymphocyte separation medium (Biocoll Separating Solution, Biochrom), stimulated for 3 days with PHA (1 µg/ml) and cultured in RPMI1640 medium with 10% FCS and 10 ng/ml IL-2 prior to infection.

**Flow cytometric analysis.** To determine the effect of Vpu on CD4, CD1d, NTB-A and tetherin cell surface expression, HEK293T cells were transfected by the calcium phosphate method with 1

µg of a CD4, CD1d, NTB-A, or tetherin expression vector and 5 µg of pCG eGFP/Vpu constructs expressing eGFP alone or together with Vpu. Two days post-transfection CD4, CD1d, NTB-A or tetherin expression was examined by FACS analysis. An allophycocyanin-conjugated anti-human tetherin antibody (Biolegend), allophycocyanin-conjugated anti-human CD4 antibody (Invitrogen; MHCD0405), a phycoerythrin-conjugated anti-CD1d antibody (BD 550255) or an APC-conjugated anti-SLAM6 antibody (R&D FAB19081A) was used for staining. Fluorescence of stained cells was detected by two-color flow cytometry and Vpu-mediated CD4, CD1d, NTB-A or tetherin down-modulation was calculated as described previously for the functional analysis of *nef* alleles (48). To determine the effect of Vpu on tetherin surface expression levels in primary cells, PHA-stimulated PBMCs were transduced by spinoculation (2 h at 37°C, 1300 x g) with VSVg-pseudotyped HIV-1 proviral constructs. Three days after transduction, PBMCs were dual stained for surface tetherin (allophycocyanin-conjugated anti-human tetherin antibody from Biolegend) and CD4 (phycoerythrin-conjugated anti-human CD4 from Invitrogen MHCD0404), permeabilized and stained intracellularly for p24 with a FITC-conjugated antibody (Beckman coulter).

**Western blot.** To monitor Vpu expression, 293T cells were transfected with 5 µg of vector DNA co-expressing eGFP and AU-1 tagged Vpus. The *vpu* alleles were not codon-optimized. Two days post-transfection cells were harvested, lysed in CO-IP buffer (150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 0.1% NP40, 0.5 mM sodium orthovanadate, 0.5 mM NaF, pH 7,5) and cell lysates were separated in 4-12% Bis-Tris gels (Invitrogen). After gel electrophoresis, proteins were transferred onto PVDF membranes and probed with AU-1 antibody (Covance, MMS-130P). Subsequently, blots were probed with anti-mouse or anti-rabbit IRDye Odyssey antibodies (926-32210, 926-32221) and proteins detected using a LI-COR Odyssey scanner. For internal controls, blots were incubated with antibodies specific for eGFP (290-50, Abcam) and β-actin (8227-50, Abcam).

**Tetherin antagonism in HEK293 cells.** To determine the capability of Vpu to antagonize tetherin, 293T cells were seeded in six-well plates and transfected with 2 µg of NL4-3 ΔVpu IRES

eGFP, 500 ng Vpu expression plasmid and different dilutions of tetherin expression plasmid. A pCGCG vector expressing eGFP only was used to equalize the DNA concentrations. At two days post-transfection supernatants were harvested and the yield of infectious HIV-1 was determined by a 96-well infection assay on TZM-bl indicator cells as described previously (49).

**Inhibition of NF-kB activity.** To determine the effect of Vpu on NF-κB activity, HEK293T cells in 96-well format were co-transfected in triplicates with 0.1 µg firefly luciferase reporter construct under the control of three NF-κB binding sites, 0.025 µg Gaussia luciferase construct under the control of a minimal pTAL promoter for normalization, and 0.04 µg expression vectors for a mutant of IKKβ containing two phosphomimetic changes (S177E, S181E) in the activation loop that render the expressed protein constitutively active or increasing concentration of tetherin, as well as 0.025 µg pCGCG eGFP/Vpu. Dual luciferase assays were performed 48 h post-transfection and the firefly luciferase signals were normalized to the internal Gaussia luciferase control as described (19).

**Viral replication in CD4+ T cells.** To assess the contribution of tetherin antagonism to the IFN resistance of full-length IMCs, we generated virus stocks of wildtype, Tmut and vpu- IMCs by transfection of 293T cells. CD4+ T-cells were positively selected (Miltenyi Biotec) from buffy coats of 3 healthy donors (Research blood components). Cells were activated by anti- CD2/CD3/CD28 beads (Miltenyi Biotec) and cultured in cell culture media (RPMI 15% FBS, 1X PSG + IL-2 (30 U/ml)) for 4 days at 37 $^{o}$C and 5% CO2. Cells were pooled and either treated with 500 U/ml of IFN$\alpha$2 (PBL Assay Science) or left untreated. Cells were infected with normalized amounts of virus in small volumes (250 µl) overnight (12-15 h). Cells were washed with PBS (3x) and resuspended in cell culture media. Every 48 hours, supernatants were sampled for cell- free p24 measurements, and media (-/+ IFN) was added back. To quantify cell- associated p24, we harvested cells at days 7 and 9 and resuspended cells in lysis buffer. Cell-free and cell-associated p24 antigen levels were quantified using the commercially available p24 AlphaLisa

(Perkin Elmer). Each virus was tested in duplicate per experiment and experiments were repeated twice in two separate pools of CD4+ T-cells.

**Virion infectivity.** 8.300 TZM-bl cells were seeded per well in a 96-well plate. At a confluence of ~ 40%, the cells were infected with 100 µl of cell-free supernatant of infected CD4+ T cells obtained 7 days post-infection in the presence of DEAE-dextran (final 40ug/ml). 48 hours later, the cells were lysed (Promega E153A), lysates were frozen at -80 $^{o}$C for 2 hours and relative light units (RLU) were determined using the luciferase assay system (Promega). The RLUs obtained were normalized to the capsid antigen p24 levels to obtain RLUs per pg p24 capsid antigen. Each measurement was performed in duplicate.

**Ethics statement.** Ethical approval for the utilization of human-derived cells was obtained from the Ethics Committee of Ulm University Medical Center.

**Statistical analysis.** Statistical calculations were performed using two-tailed unpaired (for comparison of different groups) or paired Student's-t-tests using Graph Pad Prism Version 5.0.

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**Section 3.9 – Chapter Tables and Figures**

**Table 3.1.** Infectious molecular clones of HIV-1 analyzed.

| Clone | Group | Subtype | Type | Tropism | Vpu length | Mutations | Reference |
|---|---|---|---|---|---|---|---|
| HIV-1 NL4-3 | M | B | lab-ad. | X4 | 81aa | A14L, A18L | (44) |
| HIV-1 CH058 | M | B | T/F | R5 | 80aa | A14L, A18L | (32) |
| HIV-1 CH077 | M | B | T/F | R5 | 81aa | A15L, A19L | (32) |
| HIV-1 STCO | M | B | CC | R5 | 81aa | A15L, A19L | (32) |
| HIV-1 CH0167 | M | C | CC | R5 | 84aa | A20L, A24L | (32) |
| HIV-1 DJO0131 | N | - | n.k. | n.k. | 74aa | A12L, A16L | (12) |

n.k., not known

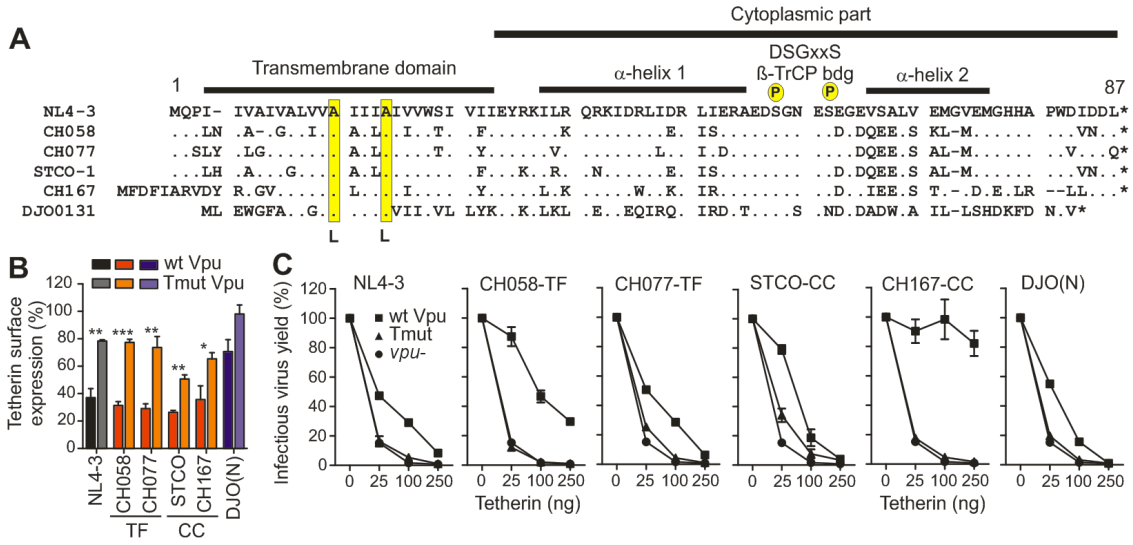


**FIG 3.1.** Mutant Vpus selectively defective in tetherin antagonism. (A) Alignment of Vpu amino acid sequences analyzed. The NL4-3 Vpu sequence is shown on top for comparison. Important functional domains are indicated and the mutated Ala residues are highlighted in yellow. Dots specify amino acid identity and dashes represent gaps introduced to optimize the alignment. (B) Downmodulation of human tetherin by wt and mutant Vpu proteins in HEK293T cells cotransfected with vectors coexpressing eGFP and Vpu and a construct expressing human tetherin. Shown are the levels of tetherin cell surface expression relative to those measured in cells transfected with the eGFP only control vector (100%). Shown are mean values (±SEM) derived from three experiments. Wt Vpu alleles are color coded in dark colors and mutant Vpus in light colors. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (C) Virus release from HEK293T cells following transfection with *vpu*-defective HIV-1 NL4-3, expression constructs for the indicated Vpu proteins or eGFP only and varying amounts of plasmid expressing human tetherin. Infectious virus was determined by infection of TZM-bl indicator cells and is shown as a percentage of that detected in the absence of tetherin (100%). Infections were performed in triplicate and the results were confirmed in an independent experiment.

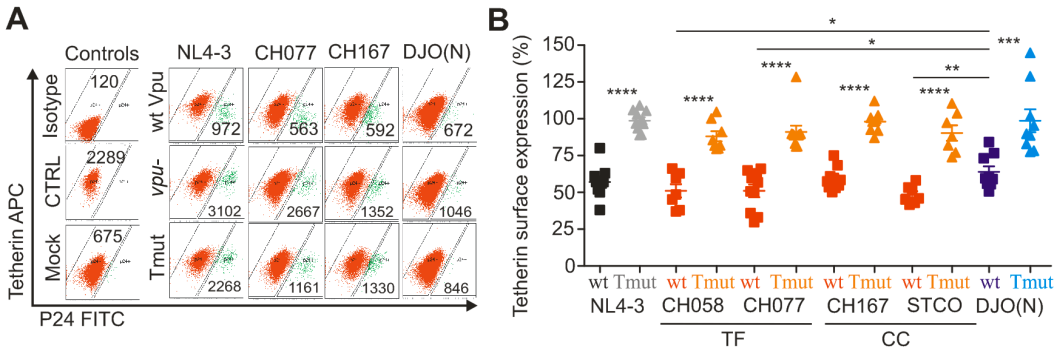


**FIG 3.2.** TMD mutations in Vpu disrupt tetherin downmodulation in HIV-1 infected primary T cells. PHA-activated PBMCs were infected with HIV-1 constructs containing wt, TMD mutated or grossly defective *vpu* alleles and examined for tetherin surface expression 3 days later. Panel A provides examples of primary data and the panel B the levels of tetherin surface expression in cells infected with the wt and Vpu mutant constructs relative to those infected with the *vpu*-defective HIV-1 constructs (100%). Each symbol represents the result obtained for one individual PBMC donor investigated. The numbers in panel A give the mean fluorescence intensity (MFI) of tetherin expression in the HIV-1 infected (p24+) cell population. *** indicates that wt Vpus are significantly ($p < 0.001$) more active than the TMD mutant Vpus.

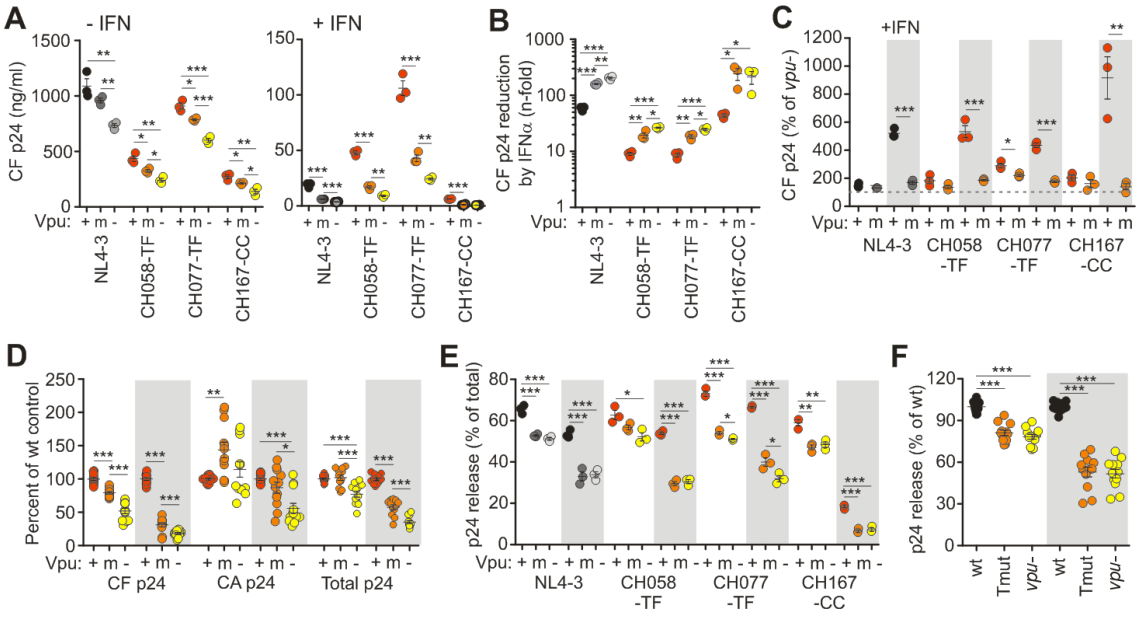


**FIG 3.3.** Effect of alterations in *vpu* on HIV-1 yield and release in CD4+ T cells in the presence or absence of IFNα. (A) p24 antigen levels in the supernatant of CD4+ T cells at day 7 post-infection with HIV-1 IMCs expressing wt (+), Tmut (m) or no (-) Vpu proteins. Virus yield was determined after triplicate HIV-1 infection in the absence (left) and presence (right) of 500 U/ml IFN-α. (B) Reduction of cell-free p24 antigen yield by IFN-α treatment. For calculation of n-fold reduction, the levels of p24 antigen obtained in the absence of IFN were divided by those obtained in the presence of IFN-α. (C) Enhancement of p24 release by wt and Tmut Vpu proteins in the absence or presence (shaded) of exogenous IFN-α. Data were derived from the experiment shown in panel A. Shown are the levels of cell-free p24 antigen relative to the cultures infected with the respective *vpu*-defective HIV-1 IMCs (100%, indicated by the dashed line). (D) Cell-free, cell-associated and total p24 yield in CD4+ T cells infected with HIV-1 NL4-3, CH058-TF, CH077-TF and CH167 IMCs containing wt, mutant or grossly defective *vpu* genes. The average values obtained for the respective wt IMCs were set to 100%. (E) Efficiency of p24 release in CD4+ T cells infected with the indicated HIV-1 IMCs. Values present percentages of cell-free p24 antigen out of the total p24 detected in the absence and presence (shaded) of IFN-α. Cell-free and cell-associated p24 antigen were quantified by ELISA at day 7 post-infection. (F). Effect of TMD mutations in Vpu or entire lack of Vpu function on the efficiency of virion release. Values obtained for all four IMCs analyzed are shown relative to the respective wt viruses (100%).

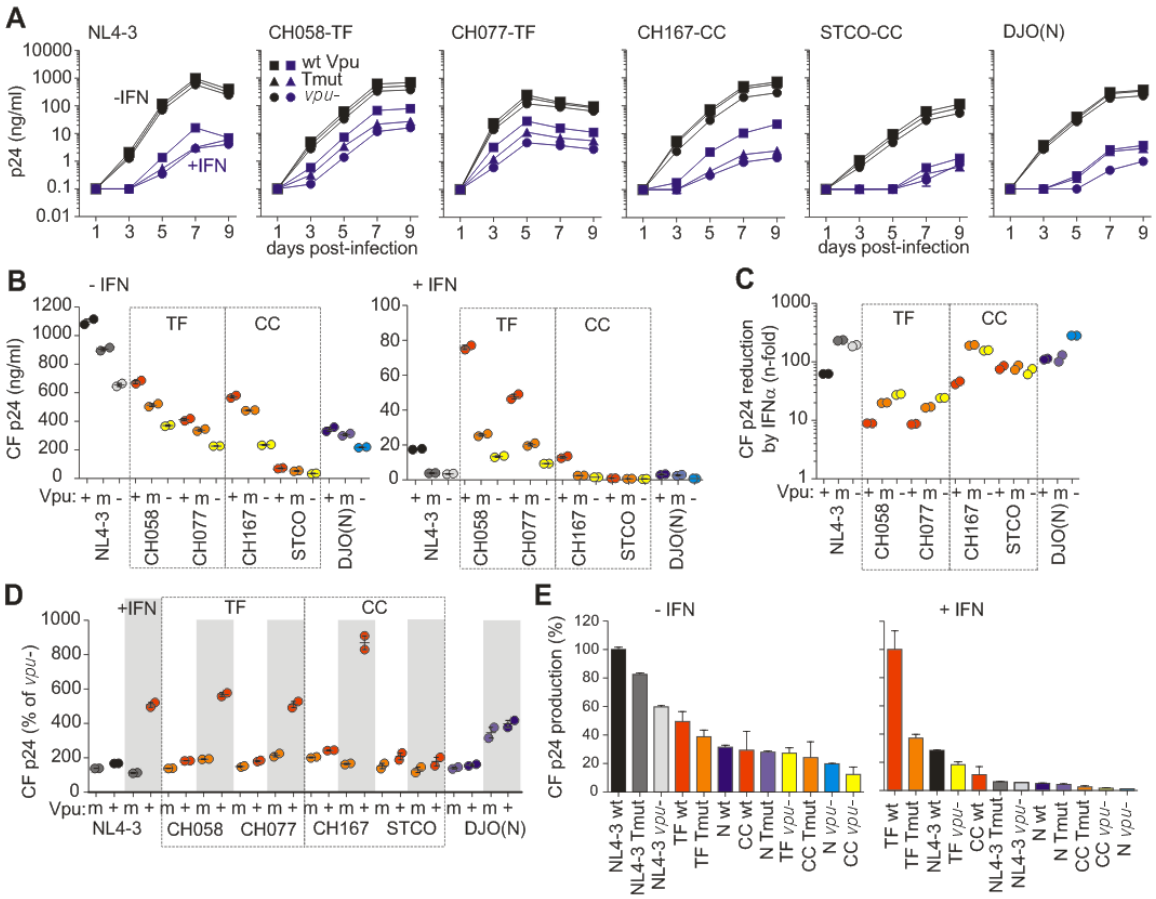


**FIG 3.4.** Replication of wt and *vpu* mutant HIV-1 constructs in CD4+ T cells in the presence and absence of IFN-α. (A) Replication kinetics of HIV-1 IMCs expressing wt, TMD mutant or no Vpu proteins in CD4+ T cells in the absence (black lines) and presence (blue lines) of 500 U/ml IFNα. Results show median values of p24 antigen production (n=3) from two different donors. (B) Cumulative p24 antigen levels in the absence (left) and presence (right) of IFN-α measured at 1, 3, 5, 7 and 9 days post-infection. Panels B, C and D show the results obtained from two different blood donors. (C) Reduction of cumulative cell-free p24 antigen yield by IFN-α treatment. (D) Enhancement of cumulative p24 yield by wt and Tmut Vpu proteins in the absence or presence (shaded) of exogenous IFN-α. Data were derived from the experiment shown in panel A. Values present total cell-free virus yield relative to the respective *vpu*-defective HIV-1 IMC (100%). (E) Ranking of wt and *vpu* mutant or defective HIV-1 IMCs according to their efficiency in cell-free

p24 production. The levels achieved for the most potent IMC were set to 100%. Shown are median values of p24 antigen production (±SEM, n=3).

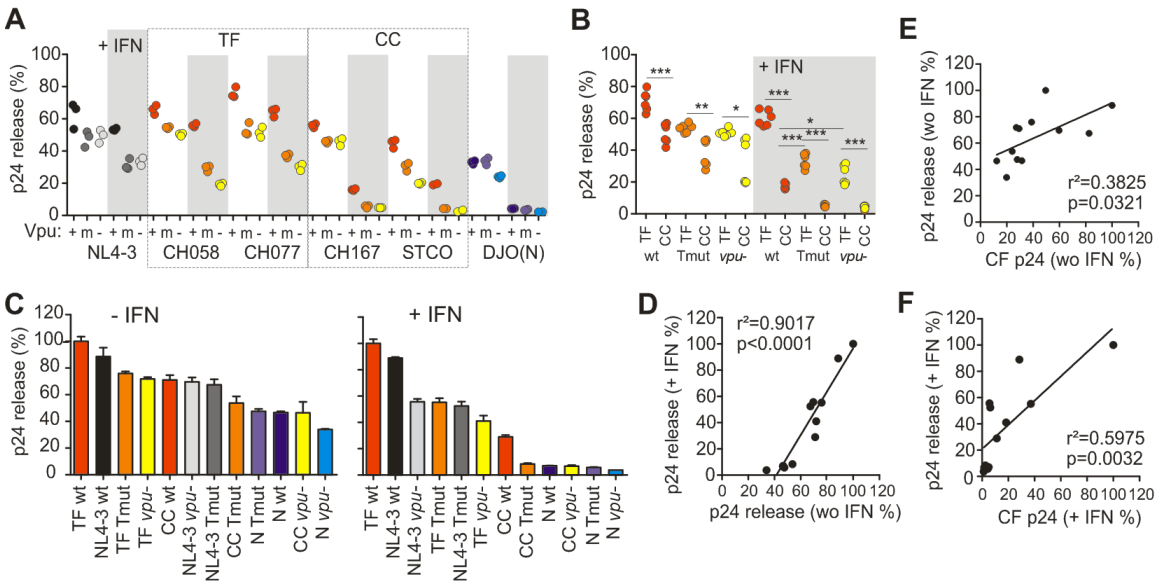


**FIG 3.5.** Release of wt and *vpu* mutant HIV-1 constructs in CD4+ T cells in the presence and absence of IFN-α. (A) Values present percentages of cell-free p24 antigen out of the total p24 detected in the presence and absence of IFN-α. Triplicate infections of T cells derived from three PBMC donors are shown. Cell-free and cell-associated p24 antigen was quantified by ELISA at day 5 post-infection. (B) Efficiency of TF and CC virus release in CD4+ T cells infected with the indicated HIV-1 IMCs. Values present percentages of cell-free p24 antigen out of the total p24 detected in the absence and presence (shaded) of IFN-α. (C) Ranking of wt and *vpu* mutant or defective HIV-1 IMCs according to their release efficiency. The levels achieved by the most potent IMCs were set to 100%. Shown are median values of release efficacy (±SEM, n=3). (D) Correlation between the virus release efficiencies measured in the absence and presence of IFN-a. (E, F) Correlation between the virus release efficiencies (values derived from panel C) and p24 antigen yield (values derived from Fig. 3.4E) in the absence (E) and presence (F) of IFN-a.

**Section 3.10 Chapter supplemental material**

**SUPPLEMENTAL MATERIAL**

**FIG S3.1.** Expression and tetherin, CD4, NTB-A and CD1a downmodulation activities of TMD mutant Vpu proteins.

**FIG S3.2.** Inhibition of NF-κB activation by wt and Tmut Vpu proteins.

**FIG S3.3.** Downmodulation of CD4 in PBMCs infected with HIV-1 IMCs differing in their *vpu* coding sequences.

**FIG S3.4.** Effect of alterations in *vpu* on cell-associated and total HIV-1 yield in the presence and absence of IFNα.

**FIG S3.5.** Effect of alterations in *vpu* on cumulative cell-associated and total p24 production in the presence and absence of IFNα.

**FIG S3.6.** Differences in virion release efficacy are highly reproducible.

**FIG S3.7.** Infectivity of HIV-1 IMCs produced in CD4+ T cells

**Supplemental Figures**

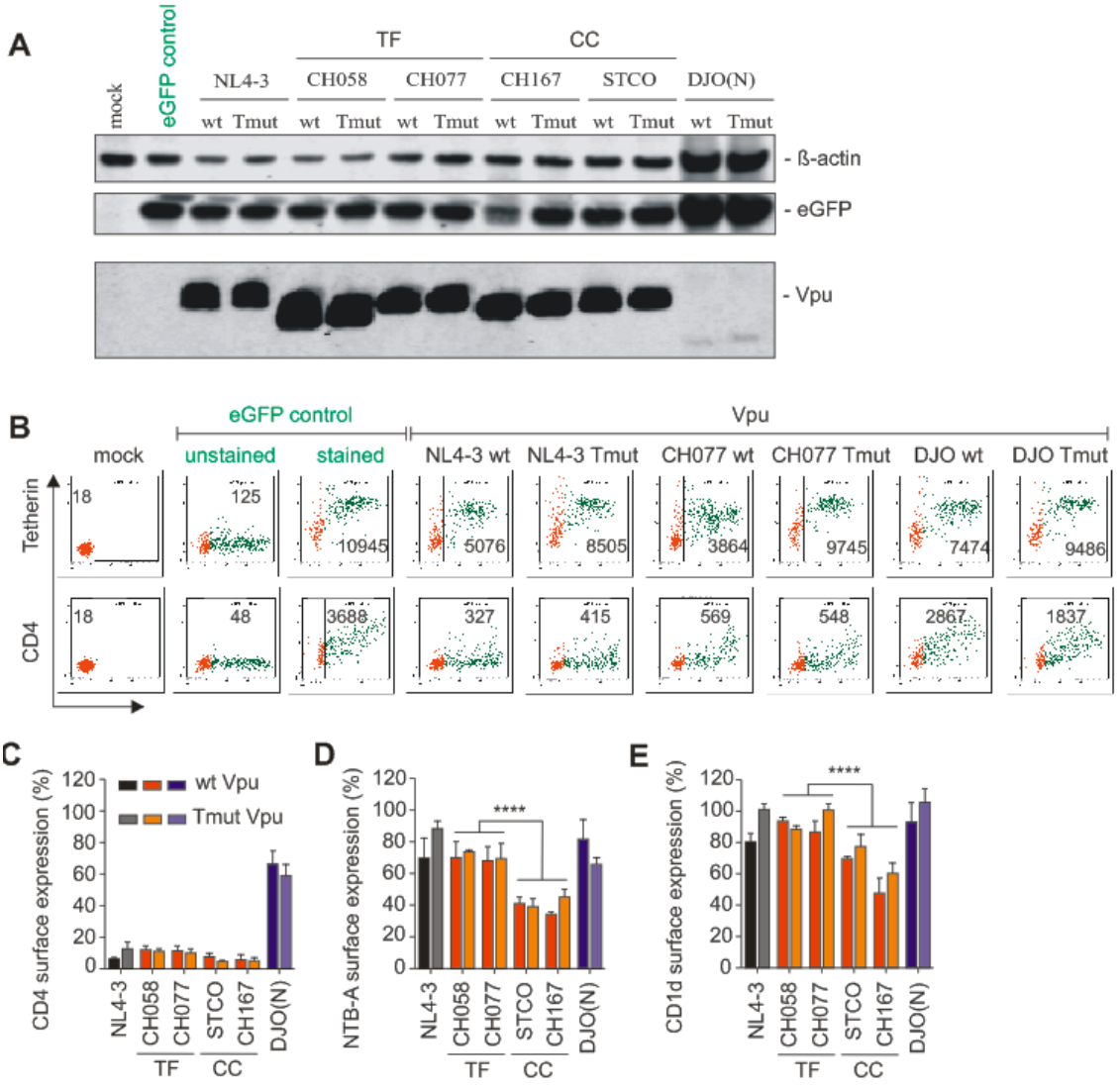


**FIG S3.1.** Expression and tetherin, CD4, NTB-A and CD1a downmodulation activities of TMD mutant Vpu proteins. (A) HEK293T cells were transfected with plasmids encoding the indicated AU1-tagged Vpus and analyzed by Western blot. An empty vector and mock transfected cells were used as negative controls. The *vpu* alleles were not codon-optimized. (B) FACS analysis of HEK293T cells cotransfected with tetherin or CD4 expression vectors and pCG plasmids

expressing eGFP alone (lanes 2 and 3) or together with the indicated *vpu* allele. The mean fluorescence intensities (MFIs) are indicated. (C-E) Vpu-dependent reduction of (C) CD4, (D) NTB-A and (E) CD1d and surface expression in HEK293T cells. Shown are the levels of receptor cell surface expression relative to those measured in cells transfected with the eGFP control vector. Shown are mean values (±SEM) derived from three experiments. Wt *vpu* alleles are indicated by dark and Tmut Vpu by light colors.

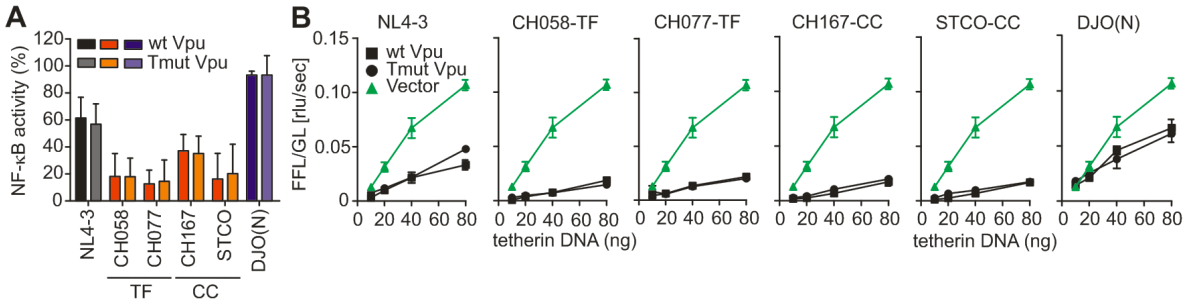


**FIG S3.2.** Inhibition of NF-κB activation by wt and Tmut Vpu proteins. (A) HEK293T cells were cotransfected with the indicated *vpu* alleles, a firefly luciferase reporter construct under the control of three NF-kB binding sites, a Gaussia luciferase construct for normalization, and expression vectors for a constitutively active mutant of IKKb as inducer of NF-κB. Luciferase activities were determined 48 h post-transfection. Shown are mean values (±SEM) derived from 3 experiments. (B) HEK293T cells were transfected as described in panel A, except that different quantities of tetherin expression vectors were used to induce NF-κB activation.

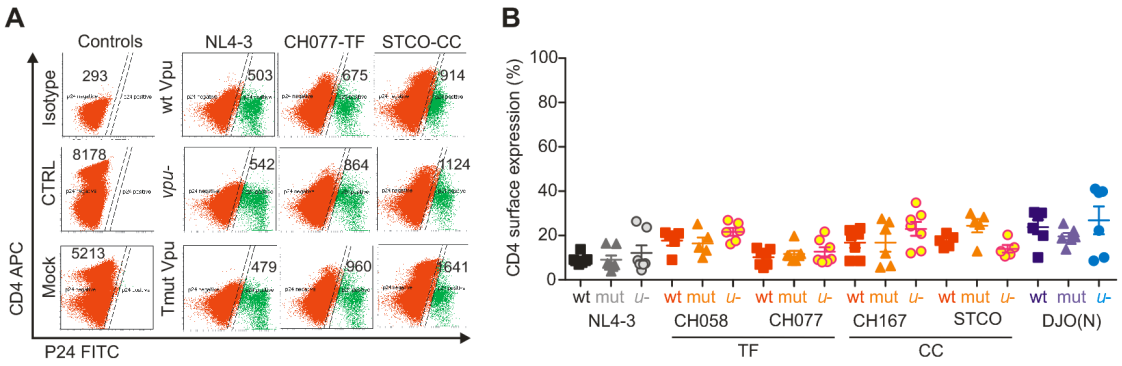


**FIG S3.3.** Downmodulation of CD4 in PBMCs infected with HIV-1 IMCs differing in their *vpu* coding sequences. PHA-activated PBMCs were transduced with the indicated VSV-G pseudotyped HIV-1 IMCs and examined for CD4 surface expression 3 days later. Panel A shows examples of primary FACS data. Numbers give mean fluorescence intensities (MFI) of CD4 expression in the HIV-1 infected (p24+) cell population. Panel B shows the levels of surface expression in virally infected (p24+) cells relative to uninfected cells (100%). Each symbol provides the result obtained for one individual PBMC donor.

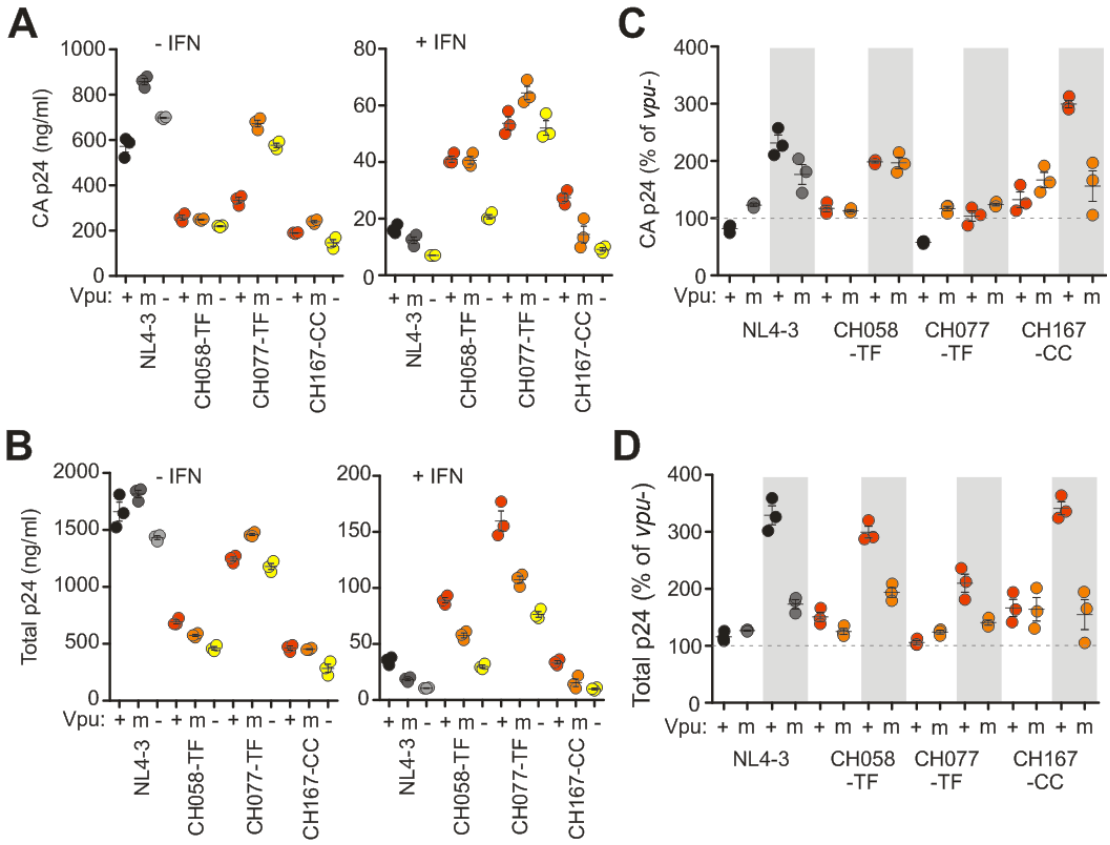


**FIG S3.4.** Effect of alterations in *vpu* on cell-associated and total HIV-1 yield in the presence and absence of IFNα. (A) Cell-associated and (B) total p24 antigen levels in CD4+ T cells at day 7 post-infection with HIV-1 IMCs expressing wt (+), Tmut (m) or no (-) Vpu proteins. P24 levels were determined by ELISA after triplicate HIV-1 infection in the absence (left) and presence (right) of 500 U/ml IFN-α. (C, D) Enhancement of (C) cell-associated and (D) total p24 antigen levels by wt and Tmut Vpu proteins in the absence or presence (shaded) of exogenous IFN-α. Data were derived from the experiment shown in panels A and B. Shown are the levels of cell-associated and total p24 antigen relative to the cultures infected with the respective *vpu*-defective HIV-1 IMCs (100%, indicated by the dashed line.

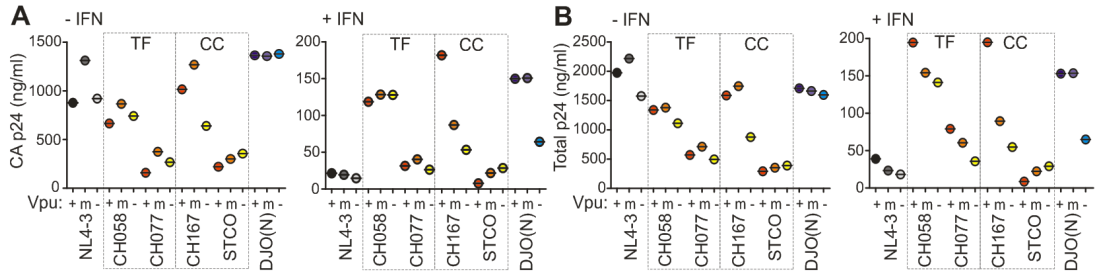


**FIG S3.5.** Effect of alterations in *vpu* on cumulative cell-associated and total p24 production in the presence and absence of IFNα. (A) Cumulative cell-associated and (B) total p24 antigen levels in CD4+ T cells at 5, 7 and 9 days post-infection with HIV-1 IMCs expressing wt (+), Tmut (m) or no (-) Vpu proteins. P24 levels were determined by ELISA in the absence (left) and presence (right) of 500 U/ml IFN-α.

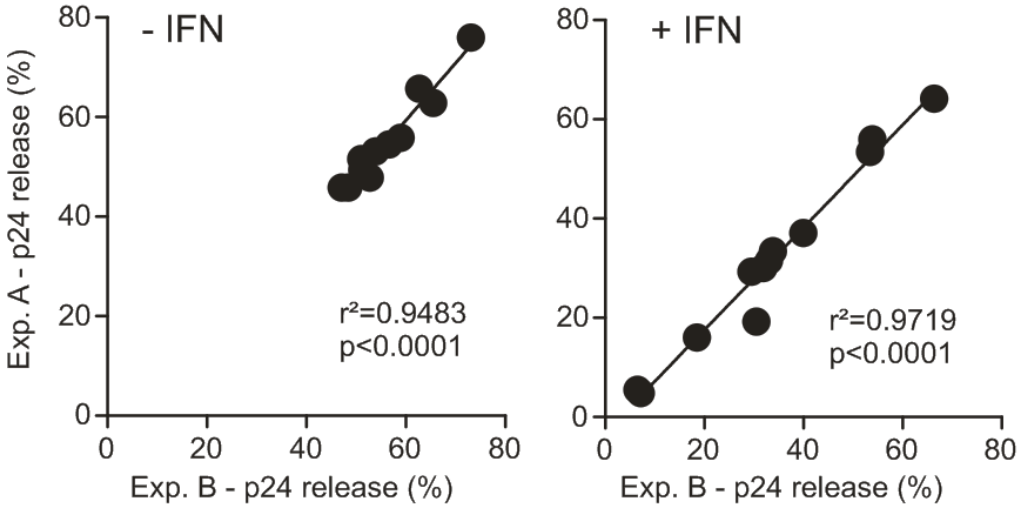


**FIG S3.6.** Differences in virion release efficacy are highly reproducible. Correlation between the release efficiencies at day 7 post-infection in the experiment shown in Fig. 3.3E and average values obtained at 5, 7 and 9 days post-infection in an independent experiment (Fig. 3.5A) in the absence (left) and presence (right) of IFN-a treatment.

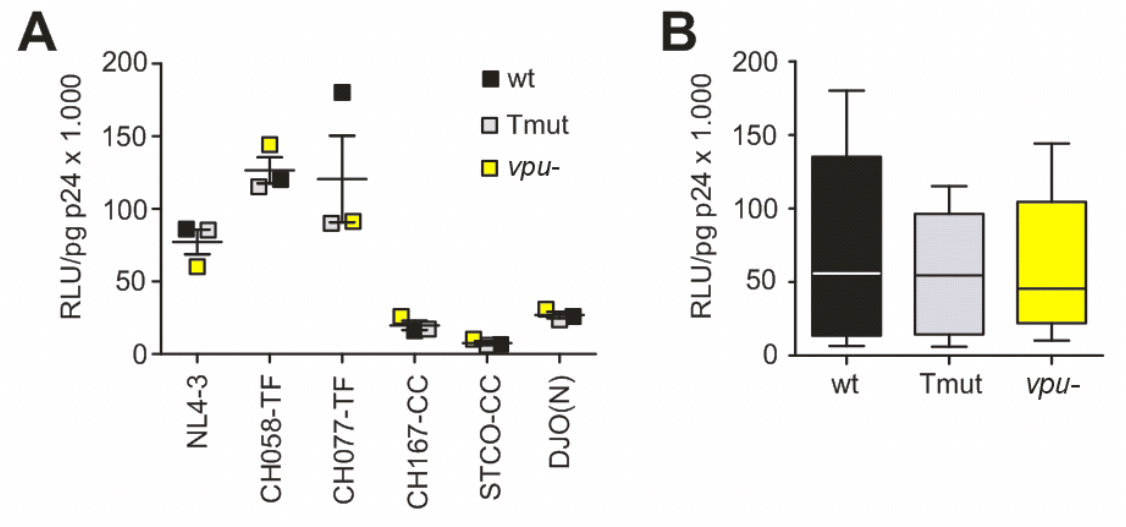


**FIG S3.7.** Infectivity of HIV-1 IMCs produced in infected CD4+ T cells. (A) Infectivity of HIV-1 IMCs expressing wt, Tmut or no (-) Vpu proteins obtained from infected CD4+ T cells at day 7 post-infection. Values represent averages of duplicate infection and were obtained in the absence of IFN-α treatment. (B) Infectivity of the HIV-1 IMCs shown in panel A grouped based on their *vpu* coding sequences. Shown are minimum and maximum values, 25% and 75% percentiles, and median values.

**CHAPTER 4**

# CONCLUSIONS AND FUTURE DIRECTIONS

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**Section 4.1**

**Mucosal HIV-1 transmission and resistance to type I Interferons**

Mucosal transmission of HIV-1 is characterized by a stringent bottleneck where ~80% of infections are founded by a single variant (1-4). This finding coupled with the observation that more ancestral viral genomes are preferentially transmitted, led to the hypothesis that mucosal infection selects for viruses with increased fitness (5-8). However, the determinants of this viral fitness have not been identified.

To determine biological properties of transmitted founder (TF) viruses, it is important to have full-length replication competent viruses and a good set of controls. The inference of the TF sequence is generally straight forward (1, 3, 9-12), however the selection of the chronic viruses for comparision is not (13-15). Parrish and colleagues identified clusters of near identical sequences in chronically infected individuals (rakes) and constructed infectious molecular clones (IMCs) matching the consensus sequences of these rakes(16). This approach makes the reasonable assumption that common ancestors of each rake represents a replication-competent and therefore biologically relevant virus. In two subsequent studies using transmission pairs (17, 18), the authors also constructed IMCs, but picked random viral genomes from chronically infected individuals to compare to TF IMCs. With this method, there is no evidence of the in-vivo functionality and relevance of the chronic control viruses (19).

In the pursuit of transmitted phenotypes, transmission pairs are ideal, as they enable the direct comparison of the TF virus to the quasispecies that it was derived from. These comparisons are thus controlled for virological and host factors with the caveat that sampling at the time of transmission is not feasible and hence a temporal gap exists between transmission and the availability of samples for study. The next consideration is how to represent the genetic and therefore, biological diversity of the viruses infecting a chronic donor. Previous studies have compared a limited number (1-2) of chronic viruses to the matched TF in many assays (17, 18). Under these conditions, limited sampling can cause inaccurate or incomplete results. The ideal study would assemble panels of multiple chronic viruses to compare to each TF virus.

To this end, in chapter 2, I compared the biological properties of 300 viruses from donors and recipients of mucosal transmission pairs. Subjects with stringent mucosal bottlenecks characterized by the transmission of a single (7 out of 8) or two (1 out of 8) variants were selected. In all but one pair, the acutely infected recipient was identified first and the donor was identified and enrolled retrospectively. In the last pair, the donor transmitted to two recipients, and the second recipient was sampled shortly after the donor (median time between the donor and recipient samples was 65 days). The selected transmission pairs represented different routes of transmission (male to female, MTF; female to male, FTM; and men who have sex with men, MSM) and both, subtype B and C infections.

The construction of IMCs is time-consuming and expensive, and requires the authors to choose a method to generate chronic controls. To derive viruses while avoiding these concerns, I generated virus isolates by limiting-dilution of donor and recipient plasma. Isolates generated by this method are derived from a single viral variant and in addition, I was able to generate similar

viral isolates from genital secretion samples of 3 donors. This study is the first to characterize the biology of full-length viruses from the genital compartment.

In my analysis, I found that recipient viruses had comparable levels of envelope glycoprotein, yet were more infectious, replicated to higher titers in primary cells, were released more efficiently from infected cells and displayed enhanced resistance to the antiviral effects of IFN$\alpha$2 and IFN$\beta$ when compared to donor plasma viruses. In addition, recipient viruses replicated more efficiently in the presence of maximal doses of IFN$\alpha$2 and IFN$\beta$ than donor plasma viruses. Interestingly, donor genital tract viruses were more infectious and were more resistant to IFN$\alpha$2 than donor plasma viruses. Thus there were biological differences in plasma and genital tract viruses, despite the lack of compartmentalization of the viral sequences. I reasoned that chronically infected donors must harbor IFN resistant viruses so as to transmit them to the matched recipients. To identify these viruses, and in part re-create hurdles observed in-vivo, I pre-treated CD4+ T-cells with IFN$\alpha$2 and IFN$\beta$ prior to generating chronic donor plasma isolates. I found that IFN b selected isolates, and not IFN$\alpha$2 selected ones were phenotypically similar to recipient viruses. Thus transmitted viruses are phenotypically distinct, and resistance to type I IFNs is their most characteristic property and these findings are summarized in Fig 1 (modified from (20)).

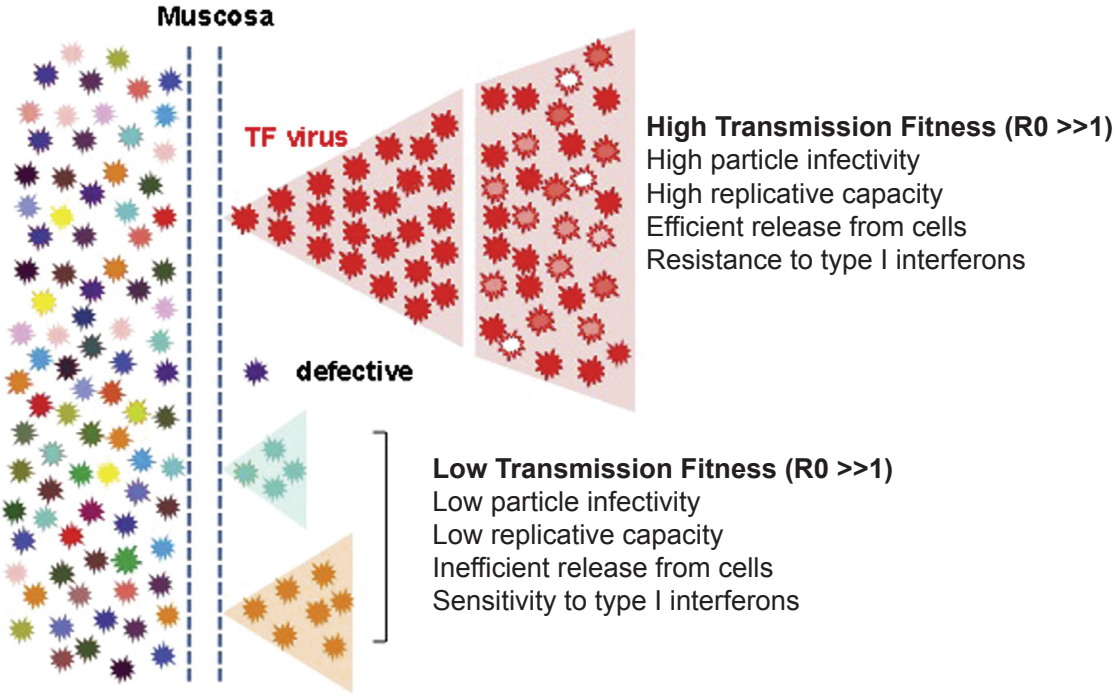
Muscosa
TF virus
defective
High Transmission Fitness (R0 >>1)
High particle infectivity
High replicative capacity
Efficient release from cells
Resistance to type I interferons
Low Transmission Fitness (R0 >>1)
Low particle infectivity
Low replicative capacity
Inefficient release from cells
Sensitivity to type I interferons

**Figure 4.1** Model of the HIV-1 mucosal bottleneck. Mucosal transmission of HIV-1 is associated with a stringent genetic bottleneck, where only one or very few variants from the donor quasispeces found infection in the new recipient. Viruses with enhanced particle infectivity, replicative capacity, IFN$\alpha$ and IFN$\beta$ resistance and release are selected for during mucosal transmission.

In contrast to results from Parrish and colleagues, I did not find all recipient viruses to incorporate more Env than matched donor viruses and hence this property is not a generalizable feature of TFs. However, in 3 out of 8 pairs, recipient viruses did contain more Env. This suggests that under certain circumstances, variants with increased Env content have an advantage at transmission. Increased levels of Env could cause more stable attachment to target cells(21), or make up for loss due to shedding or overcome inactivation by genital secretions (22, 23). I observed that recipient viruses replicated more efficiently than matched donor viruses, suggesting that interventions that reduce the reproductive ratio (R0) by even a modest degree could impact early steps following transmission (24). The observation that recipient viruses are released more efficiently from infected cells is intriguing. Differences between donor and recipient viruses at multiple stages of the viral life cycle can contribute to the observed result. There could be a difference in the number of infected cells, which would determine the initial foci of infection. In addition, each infected cell could produce different numbers of viral particles (burst sizes), resulting in differences in both particle accumulation and subsequent rounds of infection (25-28). Modest differences in either or both of these could impact in-vivo outcomes substantially, especially given the exponential replication in the acute phase of infection (29-33).

Previous studies suggest that the TF is a minor variant in the chronic quasispecies (34), and our results in Chapter 2, show that TF viruses are phenotypically distinct. It will thus be

important to understand the basis for these differences. A large amount of sequence information was generated in this study, and it will be interesting to attempt to identify signatures that associate with IFN resistance and other biological properties. Signature analysis for HIV-1 is complicated and requires a large number of sequences (35, 36), however, analysis by transmission pair could reduce inter-person variability.

Consistent with the cytokine storm observed during acute HIV-1 infection (32), transmitted viruses were more resistant to IFN$\alpha$2 and IFN$\beta$. In the earliest stages of viral infection, elevations in the levels of these antiviral cytokines can extinguish foci of IFN sensitive viruses (37)(Estes personal communication). Under these conditions, the residual viral replication at the maximal IFN dose is particularly telling, where recipient viruses are able to replicate, while donor viruses cannot. The ability to isolate IFN selected viruses suggest that IFN resistant viruses are maintained during chronic infection. Mutations associated with the loss of IFN resistance arise in part, due to the escape from neutralizing antibody (38) and cytotoxic T-lymphocyte pressure (SI unpublished). Given this, it is important to identify the reason for the maintenance of these IFN resistant viruses. It is possible that these viruses are present in the latent reservoir, and thus shielded from adaptive immune pressure. A recent study showed that the latent reservoir is seeded very early in infection (39, 40) and might be continually re-seeded during the course of infection and the genetic diversity we observed among IFN selected viruses would support the latter. Current HIV cure efforts aim to combine IFN therapy with broadly neutralizing antibodies (bNAbs) (41) after the activation of viruses from latency, to block subsequent infection and reduce the size of the latent reservoir (42). Hence, it would be very important to determine the level of pre-existing IFN resistance in the latent reservoir.

In our previous study (43) we observed a difference between TF viruses from subtypes B and C. While TF HIV-1 from subtype B were ~60-fold more resistant to IFN$\alpha$2 than chronic

viruses, subtype C TFs were comparable to subtype-matched chronic controls, with chronic viruses displaying unexpectedly high levels of IFN resistance. This raised the possibility that there might be differences between virus subtypes or differences between the cohorts studied. However, in chapter 2, I did not find a difference between subtype B and C transmission pairs, and hence the previously observed difference is likely patient-specific. It is possible that the chronically infected individuals studied by Parrish and colleagues were late in their disease course allowing for the reacquisition of IFN resistance (44, 45). In fact, our unpublished results support this hypothesis, with 4 longitudinally followed individuals displaying the expected rapid loss of IFN resistance within the first year of infection (37)(own unpublished observations), followed by the reacquisition of IFN resistance ~3 years following infection (Gondim and Hahn, unpublished observations). Thus, these data would suggest that IFN resistance is dynamic over the course of infection, and that the acute phase and late stage of infection are highly transmissible with high viral loads (46-49) and IFN resistant viruses.

Many of our observations were consistent for both IFN$\alpha$2 and IFN$\beta$, yet I also observed differences in the antiviral potencies of the two IFN subtypes tested. Despite signaling through the same receptor (50), IFN$\alpha$2 and IFN$\beta$ are reported to have varied downstream effects (51-54), consistent with what I observed in this study. I hypothesized that the two IFN subtypes would induce the expression of different sets of interferon stimulated genes, therefore leading to the different downstream outcomes. To test this, I performed a microarray experiment where I treated CD4+ T cells with both IFN subtypes for 24 hours and used untreated cells as a control. To my surprise, I did not find large differences in the numbers and nature of induced transcripts. In fact, only 15 genes were differentially induced (> 1.5-fold) by the two IFN subtypes. There are a few potential explanations for these observations. It is possible that the differences between the IFN subtypes are not visible at the level of RNA transcripts, but are due to differences in protein expression or RNA stability. However, our preliminary data suggests that protein levels of an ISG,

protein kinase R, are comparable across IFN subtypes (Persephone Borrow, personal communication). While this might not be generalizable for all ISGs, it suggests that other mechanisms must exist to account for the different downstream effects of interferon receptor (IFNAR) binding. One possibility is the modification of proteins by ubiquitylation or ISGylation. Both of these involve the non-covalent attachment of a small moiety (either ubiquitin or ISG15, respectively) to the protein of interest. The consequence of this modification can range from degradation to enhanced functionality (55-58). Type I IFNs can induce the expression of non-coding RNA and therefore by extension, different IFN subtypes might induce distinct non-coding RNAs (59-66). Similar to protein modification, non-coding RNAs can have diverse downstream effects including impacting the stability of transcripts and efficiency of translation (67)m(68). In cases of invading pathogens and the necessity of a prompt immune response, the use of non-coding RNA and protein modification to alter or enhance existing transcripts and proteins, and to subtly direct different responses by different IFN subtypes might be advantageous (66). To determine if there are differences in protein modification due to different IFN subtypes, we could use mass spectrometry or SILAC and to identify non-coding RNA species differentially modulated by IFN subtypes, RNA sequencing (RNAseq) could also be utilized. It is likely that cells respond to IFN in a heterogenous manner. This could obscure differences at the DNA, RNA or protein level unless analyzed at the level of the responding cell. In order to detect more subtle changes, it will be important to differentiate IFN responsive cells and perform downstream analysis on these cells to maximize the likelihood of identifying IFN subtype specific effects.

I found that recipient viruses were on average 5-fold more resistant to IFN$\alpha$2, but ~40-fold more resistant to IFN$\beta$ than donor viruses. Harper and colleagues have quantified the IFN subtypes produced after incubating plasmacytoid DC (pDC) with HIV-1 (69). It will be both interesting and important to determine if recipient viruses are uniformly resistant to all the produced subtypes, and whether there are certain subtypes of IFN to which donor and recipient

viruses have equal levels of resistance. If we identify such subtypes, we can identify differentially modulated ISGs that are important during transmission. The identification of a set of critical ISGs can then be used to assess vaccination strategies that can selectively upregulate them in target CD4+ T cells or to define small molecules that interfere with viral counteraction of the ISG (70, 71). As previously mentioned, type I IFNs, particularly IFN$\alpha$2 is being evaluated in combination with bNAbs for a functional cure for HIV. Our data, consistent with data from Veazey and colleagues (72) indicates that IFN$\beta$ is more potently antiviral, and that chronic viruses have increased sensitivity to IFN b. Thus, it would be important to evaluate IFN$\beta$ and possibly, other IFN subtypes for their efficacy in reducing the HIV-1 reservoir.

Lastly, it will be useful to determine if the selection of IFN resistant viruses at transmission can be recapitulated in the bone marrow liver thymus (BLT) mouse model. This humanized mouse model is repopulated with T and B cells, DC and natural killer (NK) cells of human origin, and is considered the best mouse model for HIV-1 transmission studies (73). I have previously shown that we can infect these mice (n=30) by vaginal routes and reproduce the bottleneck, characteristic of mucosal HIV-1 transmission in humans. However, in contrast to acutely infected humans, I observed only a modest increase in plasma IFN levels upon infection. This might be due to less efficient signaling from the mouse epithelium to recruit human immune cells upon contact with virus. I next determined if exogenous administration of IFN would be effective. I found that intraperitoneal injection of pegylated IFN$\alpha$2 resulted in the induction of anti-HIV-1 ISGs in the vaginal mucosa in a dose-dependent manner. Since I found IFN$\beta$ to distinguish recipient and donor viruses more strongly, I propose to administer IFN$\beta$ to the BLT mice prior to an intra-vaginal exposure to a pool of IFN sensitive and resistant viruses. I predict that in mice treated with IFN$\beta$, only IFN resistant viruses will be selected for during transmission. Additionally, from our previous efforts to identify critical ISGs that impact transmission, we can transduce the

cells used to repopulate the BLT mice to over express candidate ISGs. Thus, we can evaluate their importance to transmission.

In summary, my works shows that TF viruses are characterized by a set of biological properties that distinguish them from non-transmitted chronic viral variants. This argues against the stochastic nature of transmission. The most distinguishing property of TF viruses is their resistance to IFN. This would imply, that both the mucosal barrier and forces acting on the transmitted virus beyond the mucosal barrier in combination, select for the TF virus. The increased resistance to IFN indicates that TF viruses overcome restriction by interferon-stimulated genes more effectively than chronic non-transmitted viruses. We explored this hypothesis in Chapter 3 and the results and future directions are discussed in section 4.2 below.

**Section 4.2**

**Viral accessory proteins and their counteraction of interferon stimulated genes**

Continuous interactions with various viral pathogens have shaped the evolution of antiviral restriction factors (74, 75). These host factors are induced by Type I IFNs and act to inhibit multiple steps of the viral life cycle. Underscoring the importance of these restriction factors, are the multiple viral proteins dedicated to their counteraction (75, 76). The interactions between host restriction factors and their viral antagonists can influence both within species and cross-species transmission events. In fact, it has been suggested that the ability to overcome the host protein tetherin was an important factor in the spread of Group M HIV-1 (77).

Pandemic group M HIV-1 strains use Vpu to counteract tetherin resulting in efficient released from infected cells(78, 79). Multiple studies have shown that the antiviral state established by type I IFNs inhibits HIV-1 replication (37, 76, 80-82). Different ISGs have been identified with varying anti-HIV-1 potency and it is assumed that the overall viral inhibition is a sum of the activities of individual ISGs. However, it has not been determined to what extent each of these ISGs contribute to the observed antiviral state. In chapter 3, I showed that mutations in critical residues (Tmut) in Vpu critical for tetherin antagonism reduce the production and release of virions by approximately 50% in the presence of IFN$\alpha$2. In addition, Tmut chronic viruses were released 3-fold less efficiently than Tmut TF viruses. Consistent with my observations in Chapter 2, I found that TF viruses were released more efficiently from CD4+ cells than chronic control viruses. Intriguingly, this finding was independent of the presence of Vpu, with TF viruses lacking Vpu released more efficiently than chronic viruses lacking Vpu. This suggests that other viral proteins can influence release independently of the Vpu- tetherin interaction. In this study, I also found that a Group N virus was very sensitive to IFN$\alpha$2, and was released ~30% less efficiently than TF viruses in the presence of IFN, comparable to chronic viruses. Interestingly, the Group N Vpu promoted viral replication independent of its modest antagonism of tetherin. In the absence of IFN$\alpha$2, Tmut viruses had similar levels of replication and particle release as wildtype viruses, and both (wildtype and Tmut) replicated to higher titers than viruses lacking Vpu. This suggests that functions of Vpu other than counteracting tetherin, such as the degradation of CD4 or the inhibition of NFkB mediated signaling might impact viral replication in primary cells.

The observation that TF viruses are released more efficiently from infected cells deserves additional investigation. It is possible that TF viruses combine the ability to infect more target cells and higher burst sizes and thus have a higher reproductive ratio (R0) than viruses from chronic

infection (83). To specifically dissect this, one would need to determine the number of infected cells after a given number of cycles of replication, and then determine the number of infectious particles produced per infected cell after the addition of a fusion inhibitor to prevent subsequent rounds of infection. It will also be important to determine which viral protein (s) that contribute to this phenotype and what role they play. The observation that Vpu- deficient TF strains are released more efficiently than Vpu-deficient chronic strains implicates non-Vpu proteins. This property might be due to differences in protein function between TF and chronic viruses. Alternatively, non-coding regions like transcription factor binding sites, especially the NFkB binding sites in the long terminal repeat (LTR) can influence viral replication (84-86). The observation that Tmut TF strains are still released more efficiently than Tmut chronic viral strains suggest that this is independent of tetherin, but that there are differences in Vpu function between TF and chronic viruses. CD4 interacts with newly synthesized viral Env glycoprotein in the endoplasmic reticulum and has been shown to inhibit virus release and reduce virion infectivity (87-93). Other cellular proteins like T-cell immunoglobulin and mucin domain proteins can also affect virion release efficiency (94) and it would be interesting to assess if any of these are more effectively overcome by TF viruses.

I observed that TF viruses were released more efficiently in the presence of IFN$\alpha$2 than chronic viruses. As mentioned above, multiple cellular factors can affect and influence the measurements of particle release in our assay. However, it is tempting to speculate that TF viruses overcome tetherin-mediated restriction more efficiently than chronic viruses. A previous report compared the ability of TF and chronic Vpu alleles to downmodulate surface tetherin and promote viral release and did not find significant differences (95). However, these experiments were performed in cell lines using transient transfections. It is possible that more physiologically relevant levels of tetherin such as those observed in primary cells, and of Vpu expressed in the context of a full-length virus enable the observation of differences in activity. An alternative

possibility is that TF viruses have evolved another viral protein to counteract tetherin in addition to Vpu. A recent study found that certain HIV-1 group M viruses encoded Nef proteins with anti-tetherin activity (96). Similarly, HIV-1 group O viruses, including the most recent common ancestor of the group O viruses, have been demonstrated to encode Nef proteins with anti-tetherin activity (97). Thus, it is possible that TF Nef proteins are capable of overcoming tetherin in addition to their Vpu proteins allowing more efficient particle release. Vpu has been demonstrated to downmodulate NFkB activity (85), thus reducing the induction of antiviral genes. It is possible, that in addition to overcoming tetherin more efficiently, TF Vpu proteins inhibit NFkB activation more efficiently than chronic viruses.

It would be extremely interesting to map the viral determinants of IFN resistance. To do this, one would require a panel of viruses that differ at very few nucleotide positions but differed greatly in their resistance to IFN. I assembled a panel of such virus clones from longitudinally followed individuals (n=13). These clones represented the TF virus and the consensus virus present six months following infection. Thus, the virus pairs from an individual were related genetically, and thus did not differ at too many positions across the genome (6-20 nucleotides). I demonstrated that the TF virus in each case was more resistant to IFN than the corresponding virus at 6-months, and in some cases this difference was up to 10-fold. I constructed chimeric viruses by swapping regions of the genome between the IFN resistant TF and the sensitive 6-month virus and used site-directed mutagenesis to identify specific amino acid residues that confer IFN sensitivity to TF viruses. It will be very interesting to continue this analysis and extend the identification of sensitivity-conferring mutations to all the virus pairs. Initial analysis did not identify common polymorphisms in all 13 IFN sensitive 6-month viruses, thus the determinants of IFN sensitivity are likely to be complex and multi-factorial.

Complimentary to the identification of the viral determinants, it would be interesting to assess the contribution of known anti-HIV-1 ISGs to the IFN-mediated antiviral state. In fact, once we identify the mutations responsible for conferring IFN sensitivity to the 6-month virus, I propose to use tools like mass spectrometry and proteomic analysis (in collaboration with Nevan Krogan) to determine whether TF and mutant viruses bind ISGs with different efficiencies, or whether mutant viruses are unable to interact with the ISG. These analyses will identify both viral mutations that cause sensitivity to IFN and determine which ISGs are responsible for the observed sensitivity of the 6-month virus. These experiments can be followed by studies like Chapter 3 to identify the contribution of other ISGs like A3G, MxB and IFITM to the restriction of viral replication observed in IFN- treated primary cells. It would be interesting to silence these host genes in primary target cells and assess the effect of IFN in restricting replication. These experiments will enable the description of the key ISGs responsible for HIV-1 restriction in IFN-stimulated primary CD4+ T cells.

Thus, in summary, tetherin is clearly an important part of the anti-HIV-1 IFN$\alpha$2 response, and it is intriguing that Group M viruses with adaptions at key anti-tetherin residues of Vpu have spread successfully in humans, while viruses with suboptimal adaptations like group N viruses have had limited spread. Additionally, TF viruses are released efficiently from infected cells. This is consistent with observations that initial small foci of infection rapidly result in millions of infected cells in acutely infected individuals. The efficient particle release from infected cells could explain these observations and the observed high R0 in acute infection. Lastly, the observation that TF viruses are released efficiently could provide evidence for the cell-free virus that initiates infection. Conversely, this could mean that chronic viruses are better at navigating cell-cell spread as previous reports suggest that tetherin-mediated restriction could enhance cell-cell spread.

**Section 4.3 - Chapter References**


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