In vivo Serial Passaging of Human–Simian Immunodeficiency Virus Clones Identifies Characteristics for Persistent Viral Replication

Rajesh Thippeshappa¹, Patricia Polacino², Shaswath S. Chandrasekar³, Khanghy Truong³, Anisha Misra⁴, Paula C. Aulicino⁴, Shiu-Lok Hu⁵, Deepak Kaushal* and Jason T. Kimata*¹

¹Disease Intervention and Prevention Program, Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, United States, ²Washington National Primate Research Center, University of Washington, Seattle, WA, United States, ³Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States, ⁴Laboratorio de Biología Celular y Retrovirus, Hospital de Pediatría “Juan P. Garrahan”-CONICET, Buenos Aires, Argentina, ⁵Department of Pharmaceutics, University of Washington, Seattle, WA, United States, ⁶Host–Pathogen Interactions Program, Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, United States

We previously reported that a human immunodeficiency virus type 1 with a simian immunodeficiency virus vif substitution (HSIV-vifNL4-3) could replicate in pigtailed macaques (PTMs), demonstrating that Vif is a species-specific tropism factor of primate lentiviruses. However, infections did not result in high-peak viremia or setpoint plasma viral loads, as observed during simian immunodeficiency virus (SIV) infection of PTMs. Here, we characterized variants isolated from one of the original infected animals with CD4 depletion after nearly 4 years of infection to identify determinants of increased replication fitness. In our studies, we found that the HSIV-vif clones did not express the HIV-1 Vpr protein due to interference from the vpx open reading frame (ORF) in singly spliced vpr mRNA. To examine whether these viral genes contribute to persistent viral replication, we generated infectious HSIV-vif clones expressing either the HIV-1 Vpr or SIV Vpx protein. And then to determine viral fitness determinants of HSIV-vif, we conducted three rounds of serial in vivo passaging in PTMs, starting with an initial inoculum containing a mixture of CXCR4-tropic [Vpr-HSIV-vifNL4-3 isolated at 196 (C/196) and 200 (C/200) weeks post-infection from a PTM with depressed CD4 counts] and CCR5-tropic HSIV (Vpr+ HSIV-vif derivatives based NL-AD8 and Bru-Yu2 and a Vpx expressing HSIV-vifYu2). Interestingly, all infected PTMs showed peak plasma viremia close to or above 10⁵ copies/ml and persistent viral replication for more than 20 weeks. Infectious molecular clones (IMCs) recovered from the passage 3 PTM (HSIV-P3 IMCs) included mutations required for HIV-1 Vpr expression and those mutations encoded by the CXCR4-tropic [Vpr-HSIV-vifNL4-3 isolated at 196 (C/196) and 200 (C/200) weeks post-infection from a PTM with depressed CD4 counts] and CCR5-tropic HSIV (Vpr+ HSIV-vif derivatives based NL-AD8 and Bru-Yu2 and a Vpx expressing HSIV-vifYu2). Interestingly, all infected PTMs showed peak plasma viremia close to or above 10⁵ copies/ml and persistent viral replication for more than 20 weeks. Infectious molecular clones (IMCs) recovered from the passage 3 PTM (HSIV-P3 IMCs) included mutations required for HIV-1 Vpr expression and those mutations encoded by the CXCR4-tropic HSIV-vifNL4-3 isolate C/196. The data indicate that the viruses selected during long-term infection acquired HIV-1 Vpr expression, suggesting the importance of Vpr for in vivo pathogenesis. Further passaging of HSIV-P3...
INTRODUCTION

Several alternate animal models such as infection of macaques with simian immunodeficiency viruses (SIVs) or chimeric simian-human immunodeficiency viruses (SHIVs) have been developed to understand HIV pathogenesis and disease progression and determine the efficacy of vaccines and drugs. However, the genetic difference between HIV-1 and SIV, and the absence of other HIV-1 genes, such as gag, vif, vpr, and nef in SHIV limit the utility of these models. Therefore, there is a need to rationally and minimally modify HIV-1 such that it can replicate and cause AIDS in macaques. Such an animal model will be a valuable tool for preclinical evaluation of vaccines and the development of novel therapeutic strategies targeting HIV-1 proteins, and for understanding viral immunopathogenesis.

The important lentiviral restriction factors in macaque species such as rhesus macaques (RMs) are the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3 or A3) family of proteins, tripartite motif containing (TRIM) family of proteins, BST2/CD317/Tetherin, and sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain containing protein 1 (SAMHD1; reviewed in Thippeshappa et al., 2012; Saito and Akari, 2013). However, SIV can overcome RM TRIM5α and the APOBEC3 family of restriction factors and simian-tropic HIV-1 (stHIV-1) or macaque-tropic HIV-1 (mtHIV-1) have been developed by incorporating capsid and vif sequences from SIVmac239 (Hatzioannou et al., 2006; Saito et al., 2011; Doi et al., 2013, 2018; Nomaguchi et al., 2013; Otsuki et al., 2014). Instead of a full-length capsid substitution, an HIV-1 derivative carrying only a short 21 nucleotide segment from the SIV capsid sequence corresponding to the HIV-1 cytoplasmic A binding loop has also been constructed (Kamada et al., 2006). Additionally, variants with CCR5-tropic HIV-1 have also been developed (Otsuki et al., 2014; Doi et al., 2017). These variants of stHIV-1 or mtHIV-1 establish infection in vivo in different species of nonhuman primates (NHPs; Igarashi et al., 2007; Saito et al., 2011, 2013; Otsuki et al., 2014; Doi et al., 2018). However, none of the variants result in CD4 depletion, and there remains a need to develop a pathogenic macaque-tropic HIV-1 (reviewed in Thippeshappa et al., 2020).

Compared to other NHPs used in AIDS research, PTMs are relatively more susceptible to HIV-1 infection (Agy et al., 1992, 1997; Frumkin et al., 1993; Gartner et al., 1994a,b; Bosch et al., 1997, 2000). While PTMs can be infected with HIV-1, viral loads diminished rapidly (Agy et al., 1992). Attempts to in vivo passage HIV-1 in PTMs failed to select variants capable of persistent replication.

An explanation for the susceptibility of PTM CD4+ T cells to HIV-1 is that PTMs do not express restriction factor TRIM5α. Instead, they express novel isoforms of TRIM5 (TRIM5α and TRIM5η) and TRIM5-cyclophilin A fusion protein (TRIMcyp) that do not interfere with HIV-1 infection (Liao et al., 2007; Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008). The absence of TRIM5α suggests that other retroviral restriction factors in PTMs, such as APOBEC3 family of proteins, BST2, and SAMHD1 may limit replication of HIV-1. Since APOBEC3 family proteins can be degraded by SIVmac and HIV-2 vif, Hatzioannou et al. constructed minimally modified HIV-1 derivatives carrying either SIVmac vif or HIV-2 vif (Hatzioannou et al., 2009). PTMs infected intravenously (IV) with a mixture of these two viruses exhibited acute infection and persistent viremia for up to 25 weeks post-infection (wpi). However, CD4+ T cell depletion was not observed in the animals. To select a variant with increased fitness, serial in vivo passing of a mixture of four clonal HIV-1 Δu5l-derived viruses, each encoding CCR5-tropic gp120 env from YU2, BaL, AD8, and KB9, was conducted in PTMs transiently depleted of CD8 T cells. Viral swarm or infectious molecular clone (IMC) generated following passing caused CD4 depletion only in macaques that were transiently depleted of CD8 T cells. However, they were controlled in immunocompetent PTMs (Hatzioannou et al., 2014; Schmidt et al., 2019). Inability of passaged viruses to cause AIDS in non-CD8-depleted macaques suggests partial adaptation to the PTM host. Despite these studies, the key characteristics necessary for enhanced replication of macaque-tropic HIV-1 clones remain poorly understood.

We have constructed PTM-tropic HIV-1 viruses (HSIV-vif) by replacing the vif genes with vif from highly pathogenic PTM-adapted SIVmne027 (Kimata et al., 1998, 1999). These clones infected intravenously (IV) inoculation of PTMs with HSIV-vifΔu5l showed low viral replication during the post-acute stages of infection through 44 wpi and small rebounds in viral titer at 64 and 72 wpi in juvenile PTMs (Thippeshappa et al., 2011). Furthermore, we observed that unlike pathogenic SIVmne, HSIV-vifΔu5l replication is suppressed by type I interferon (IFN) treatment in PTM CD4+ T cells, perhaps suggesting that the IFN response during acute infection may limit virus replication in PTMs. Interestingly, we found that HSIV-vifΔu5l was resistant to interferon alpha (IFNα)-treatment in PTM CD4+ T cells in vitro, which may be due to envelope-mediated counteraction of IFNα-induced restrictions at the entry step of the viral life cycle (Thippeshappa et al., 2013). To further define important mutations in HSIV-vif that confer increased viral fitness in PTMs, we isolated and characterized variant virus isolates from peripheral blood CD4+ T cells after 196–200 wpi with HSIV-vifΔu5l infection when there was CD4+ T cell depletion, and then performed a serial in vivo passing experiment using a mixture of these virus isolates and different

IMCs in vivo may generate pathogenic variants with higher replication capacity, which will be a valuable resource as challenge virus in vaccine and cure studies.

Keywords: HIV-1, SIV, HSIV-vif, nonhuman primates, pigtailed macaques, animal model, in vivo passaging, infectious molecular clones
clones of HSIV-vif to define genetic characteristics that could contribute to persistent viral replication in vivo.

MATERIALS AND METHODS

Cell Lines
TZM-bl cells were obtained from the NIH HIV Reagent Program (Derdeyn et al., 2000; Wei et al., 2002). TZM-bl and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (p/s; DMEM complete). The immortalized PTM CD4+ T cells, obtained from Dr. Hans-Peter Kiem (Fred Hutchinson Cancer Research Center), were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% HI-FBS, 2 mM glutamine, p/s, and 100 U/ml human interleukin 2 (IL-2; Roche; Munoz et al., 2009). CEM × 174 were obtained from the American Type Culture Collection and cultured in Roswell Park Memorial Institute (RPMI) media with 10% HI-FBS, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (RPMI complete).

Isolation of Biological Clones of HSIV-vifNL4-3
Total CD4+ T cells were isolated from 1 × 107 PBMCs recovered at 196 and 200 wpi of pigtailed macaque M08009 by negative selection using the Miltenyi nonhuman primate CD4+ T cell isolation kit (Miltenyi Biotech). The cells were isolated according to the manufacturer’s protocol. The M08009 CD4+ T cells were cocultured with the human T cell-B cell hybrid cell line, CEMx174 for up to 16 days. Supernatants after 7 and 16 days were assayed for HIV-1 p24 antigen by ELISA (Advanced Bioscience Laboratories). If positive, supernatants were passed through 0.45-μm syringe filters, aliquotted, and frozen at −80°C. The infectious titers of the stocks were determined by limiting dilution infection analysis using TZM-bl reporter cells and luciferase assay as described (Misra et al., 2018). Infectious virus recovered from CD4+ T cells at 196 and 200 wpi was named C/196 and C/200, respectively.

Coreceptor Usage of HSIV-vif Biological Isolates
TZM-bl cells (1 × 104 cells per well) were plated in wells of a 96-well plate in DMEM complete with 30 μg/ml DEAE-dextran. In triplicate cultures, cells were treated with either the CXCR4 inhibitor, AMD3100, or CCR5 inhibitor, Maraviroc, such that after adding 250 infectious units of C/196, C/200, or control viruses HSIV-vifNL4-3 or HSIV-vifAD8, the final concentrations of inhibitors were 1 μM, 500 nM, or 250 nM, and the final concentration of DEAE-dextran was 20 μg/ml. After 2 days of infection, the cells were washed once with PBS and lysed with Promega Glo Lysis buffer. Lysates were assayed for luciferase activity using the Promega luciferase assay system and tube luminometer according to the manufacturer’s instructions (Promega).

Serum Neutralization
Neutralizing antibody titers in serum specimens from PTMs infected with HSIV-vifNL4-3 were determined using a TZMBL-based neutralization assay as described previously (Wu et al., 2006). Serum samples were heat-inactivated at 56°C for 30 min prior to use.

Plasmids
Construction of the HSIV-vif clones based on NL4-3, NL-AD8, and Bru-Yu2 has been reported before (Thippesappa et al., 2011). To generate Vpr+ HSIV-vifNL4-3 and HSIV-vifAD8 clones, Spih1 to Sall fragment of HSIV-vifNL4-3 encompassing HIV gag, pol, SIV vif, and HIV-1 vpr genes was cloned into pCR2.1 TOPO vector (ThermoFisher). SIV vpr start codon and two additional ATG codons upstream of the HIV-1 vpr start codon were mutated by Quickchange mutagenesis (Stratagene) and the sequence between the stop codon of vif and start codon of vpr were deleted. After mutagenesis, Spih1 and Sall fragment was cloned back into HSIV-vifNL4-3 and HSIV-vifAD8. Similarly, Spih1 to Sall fragment of HSIV-vifNL4-3 was cloned into SacI site removed pUC19 vector (ThermoFisher), ATG codons upstream of vpr were mutated, and cloned back into HSIV-vifNL4-3 (Supplementary Figure 1). HSIV-vif-VpxNL4-3 clone was generated by cloning SacI to NcoI fragment of SIVmne027 vpx gene into pUC19 vector containing Spih1 to Sall fragment of HSIV-vifNL4-3 (Supplementary Figure 2).

Virus Stocks of HSIV-vif Molecular Clones for Infection
Virus stocks were generated by transfection of 293T cells with each plasmid clone of HSIV-vif using Fugene 6 or X-tremeGENE 9 DNA transfection reagent according to the manufacturer’s protocol (Roche). Infectious titers were determined by limiting dilution infection analysis using TZM-bl indicator cells, and the amount of virus in supernatants was measured by HIV-1 p24 antigen ELISA (Advanced Bioscience Laboratories).

Western Blot
293T cells, seeded the day before, in 6-well plates were transfected with HSIV-vif plasmids using Fugene 6 or X-tremeGENE 9 DNA transfection reagent (Roche/Sigma). At 48 h post-transfection, cell culture supernatants were used to concentrate viral particles by centrifugation at 23,600 × g for 1 h at 4°C. Viral particles were mixed with 2X SDS samples buffer and separated by SDS-PAGE using Tris-HCl ready gels (Bio-Rad). Cell lysates were prepared as previously described (Thippesappa et al., 2011). Proteins were transferred to either nitrocellulose or PVDF membrane and probed with rabbit antiserum to Vpr, Nef, or Vpx. Goat anti-rabbit IgG HRP (Promega) was used as secondary antibody. Antiserum to Vpr (Catalog # ARP-11836), Nef (Catalog # ARP-2949), Vpx (Catalog # ARP-2609), and anti-HIV-1 p24 Gag monoclonal (ARP-6458) were obtained from NIH HIV reagent program.
Repllication in PTM CD4 T Cell Line, PTM PBMCs, and Human Monocyte-Derived Macrophages

For viral replication assays, PTM PBMCs and immortalized PTM CD4+ T cells were infected as previously described (Thippeshappa et al., 2011, 2013). PTM PBMCs were isolated using Ficoll hypaque gradient method. PBMCs were activated with concanavalin A (7 µg/ml) for 3 days. Cells were then washed twice and resuspended in RPMI complete media containing 40 U/ml IL-2 (Sigma) and cultured for 2 days. Approximately 1 × 10^9 activated PBMCs were infected in duplicate at a multiplicity of infection (MOI) of 0.01. To compare viral replication in PTM CD4+ T cells, approximately 2.5 × 10^5 or 5 × 10^5 cells in 100 U/ml IL-2 containing IMDM were infected in duplicate with viruses at a MOI of 0.01 or 0.05. Human monocyte-derived macrophages (MDMs) were generated from PBMCs using previously described methods (Kimata et al., 1998, 2004; Biesinger et al., 2010). PBMCs of anonymous donors were isolated from leukopacks purchased from the Gulf Coast Blood Center, Houston, TX, United States. Briefly, human monocytes were isolated from PBMCs by plate adherence methods. Approximately 4 × 10^6 PBMCs were plated into each well of a 24-well plate and monocytes were allowed to adhere to the plate for 1 h. Monocytes were then stimulated with RPMI complete containing 10 U/ml GM-CSF (Invitrogen) for 7–10 days to generate MDMs. Human MDMs were infected with the Vpr and Vpr-HSIV-vif in duplicate. Infection experiments were conducted at least 2–3 times. PBMCs from different donors were also used. After 3 h of incubation, the cells were washed twice with phosphate buffered saline (PBS) or complete medium to remove unbound virus. Infected cells were then resuspended in RPMI complete media containing IL-2. To study the effect of IFNα, 200 U/ml of Interferon-αA/D (IFN-αA/D or IFNα, Sigma) was added to the culture media. To monitor replication of HSIV-vif clones, supernatants were harvested every 2–4 days for measurement of HIV-1 p24Ag antigen using ELISA kit (Advanced Bioscience Laboratories or ExpressBio). Statistical analysis in GraphPad Prism was performed to compare groups using the Mann-Whitney test.

Serial in vivo Passaging in PTMs

Four PTMs specific pathogen free for simian T lymphotropic virus type 1, SIV, simian retrovirus type D, and herpes B virus were enrolled for the study. All animals were housed and cared for in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the Animal Care and Use Committee of the University of Washington. In passage 1: two PTMs were inoculated IV with a mixture of CXCR4 and CCR5-tropic viruses. In passage 2: pooled peripheral blood from passage 1 PTMs collected at 14 wpi was used for inoculation of 1 PTM. In passage 3, peripheral blood from passage 2 PTM at 8 wpi was used for transfusion into an additional PTM. At several time points post-inoculation, peripheral blood was drawn for CD4+ T cell count determinations and isolation of plasma, sera, and PBMC.

Plasma Viral Loads, CD4 T Cell Counts, and Antibody Response

Plasma viral load measurements were determined by using the Roche Amplicor HIV-1 monitor test, version 1.5 according to the manufacturer's protocol. CD4+ T cell counts were determined as previously described (Polacino et al., 2007). HIV-1-specific antibodies were measured by ELISA as previously described, using gradient-purified and disrupted whole HIV-1 virions as the capture antigen (Hu et al., 1989; Polacino et al., 2007).

Cloning of IMCs by Long-Range PCR

The following steps were performed to generate IMCs (Supplementary Figure 3).

Nested PCR Amplification of Near Full Length Genome

Proviral DNA was isolated from 1 × 10^8 PBMCs using Quick-DNA miniprep kit (Zymo Research). In the first round PCR, 1 to 2 µl of proviral DNA (approximately 50–100 ng) in a 25 µl reaction was amplified using the following primers: FWD-1: AAATCTCTAGCACTGGGCCCGACAG and REV-1: TGAGGGATCTCTAGTTACAGAGTC. Reaction mix contained 1X High Fidelity Buffer, 2 mM MgSO_4, 0.2 mM dNTPs, and 0.025 U/µl Platinum Taq High Fidelity (Invitrogen). PCR conditions for the first round were 94°C for 2 min, then 94°C for 30 s, 64°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 61°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 59°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 57°C for 30 s, and 68°C for 10 min for 21 cycles; and then 68°C for 10 min. About 1 µl of first-round PCR reaction product was amplified using following primers FWD-2: ACAGGGATCTGGAAAGCGAAAG and REV-2: CTAGTTACCAGTCAACACAGG. Reaction mix and PCR conditions were identical to the first round PCR. PCR products were visualized on 1% agarose gel and gel eluted using QIAquick gel extraction kit (Qiagen).

Vector PCR

About 10 ng of HSIV-vifelic; was amplified with following primers: 3LTR-V-F90: TGTGTGACTCTGGTAA AGTTTACACTGGGAC in a 50 µl reaction volume containing 1X High Fidelity Buffer, 2 mM MgSO_4, 0.2 mM dNTPs, and 0.025 U/µl Platinum Taq High Fidelity (Invitrogen). PCR conditions for the first round were 94°C for 2 min, then 94°C for 30 s, 64°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 61°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 59°C for 30 s, and 68°C for 10 min for 3 cycles; 94°C for 30 s, 57°C for 30 s, and 68°C for 10 min for 21 cycles; and then 68°C for 10 min. About 1 µl of first-round PCR reaction product was amplified using following primers FWD-2: ACAGGGATCTGGAAAGCGAAAG and REV-2: CTAGTTACCAGTCAACACAGG. Reaction mix and PCR conditions were identical to the first round PCR. PCR products were visualized on 1% agarose gel and gel eluted using QIAquick gel extraction kit (Qiagen).

NEBuilder HiFi DNA Assembly

Nested PCR amplification of near full length genome (NFLG) PCR product and vector PCR product were mixed with a minimum of 1:5 ratio in a 20 µl reaction volume containing 10 µl of HiFi assembly mix. After 1 h of incubation at 50°C, 5 µl of reaction mix was used for transformation of NEB
Stable cells (New England BioLabs). Miniprep plasmids were isolated using QIAprep spin miniprep kit (Qiagen). Plasmids containing full-length genomes were screened by restriction enzyme digestion with SalI and BamHI enzymes. To determine whether the plasmids containing full length clones generate infectious virus, transfection supernatants were generated by transfecting 293T cells with proviral clones. Infectious nature of the supernatants was determined by infecting TZM-bl cells. Sequences of IMCs were determined by using primers targeting different regions of the genome that support coverage of the entire length of the genome.

Recombination Analysis
Parental sequences for possible recombinant sequences were identified using Spits tree v4.17.1 (Huson and Bryant, 2006), and regions with evidence of recombination were confirmed by RAPR.1 Because indels are unlikely to emerge independently in the exact same position and with the same length, gaps in the alignment were also considered informative.

GenBank Accession Numbers
Sequences of HSIV-P3-114, HSIV-P3-161, and HSIV-P3-284 are deposited under accession numbers MZ146778, MZ146779, and MZ146780, respectively.

RESULTS
Isolates of HSIV-vif NL4-3 From Late-Stage Infection Evolved Resistance to Host Immune Responses
We previously reported HSIV-vif NL4-3 could persistently infect PTMs (Thippeshappa et al., 2011). We continued to monitor the viral loads in two of those HSIV-vif NL4-3-infected PTMs for nearly 4 years (Figure 1A). Although viral RNA was below the detection limit at most of the late time points measured, viral DNA could be detected in PBMCs through 200 wpi. Additionally, we recovered infectious virus from PBMCs at 196 and 200 wpi, suggesting that the virus had been replicating in the animals for nearly 4 years. Interestingly, one of the animals (M08009), despite low or undetectable viral loads, showed a gradual decline and then stably depressed CD4 T cell counts, suggesting disease progression (Figure 1B).

We recovered infectious virus by coculturing peripheral blood CD4 T cells from infected PTM (M08009, Figure 1) at 196 and 200 wpi with CEMx174 cells (biological isolates C/196 and C/200). C/196 and C/200 were susceptible to inhibition by AMD3100 but not Maraviroc, suggesting that viral isolates were only CXCR4-tropic (Supplementary Figure 4). We next determined the replication capacity of C/196 and C/200 in PTM CD4 T cells. Although the differences were not statistically significant, C/196 replicated to higher levels in PTM CD4 T cells in both the presence (22-fold) and absence (17-fold) of IFNα relative to the parental clone, HSIV-vif NL4-3 (Figure 2).

While C/200 displayed more limited replication, it was not affected by the addition of IFNα (Supplementary Figure 5). Partial genome sequencing analysis of PCR amplified fragments of C/196 and C/200 indicated that both isolates were clonal (Supplementary Figure 6). We also observed that both C/196 and C/200 were neutralization resistant to sera from M08009, the animal in which it evolved, as well as sera from the other PTM, F08003 that had been infected with HSIV-vif NL4-3 (Table 1). The emergence of immune escape variants of HSIV-vif NL4-3 suggest that it had persistently replicated in PTMs.

Characterization of HSIV-vif Clones Expressing Vpr and Vpx
An explanation for the low but persistent replication of HSIV-vif NL4-3 in vivo is that the virus is attenuated because it does not express accessory proteins necessary for more robust viral replication. Two possible proteins that could enhance replication of HSIV-vif NL4-3 are the HIV-1 Vpr or SIV Vpx. Since we introduced SIV vif, which includes a partial open reading frame (ORF) for vpx into HIV-1 backbone, we determined whether it had affected the expression of HIV-1 vpr.
HIV-1 Vpr protein was not observed in virions of HSIV-vif clones, HSIV-vifNL4-3 or HSIV-vifΔvpx (Figure 3). We determined that this is because singly spliced HIV-1 vpr RNA from HSIV-vif is generated using the splice acceptor site within the SIV vif gene (Supplementary Figure 1). The transcript, therefore, includes the partial ORF for the SIV Vpx protein upstream of the translational initiation site for HIV-1 Vpr, which may interfere with its expression (Supplementary Figure 1).

Vpr is a small 96 amino acid (14 kDa) protein that is not required for HIV-1 replication in vitro. However, it is conserved among all primate lentiviruses, indicating its importance for pathogenesis (Tristem et al., 1992, 1998). Therefore, we modified HSIV-vif clones to express Vpr. We deleted the sequence between the stop codon of vif and start codon of vpr and disrupted the vpx translational start codon and other ATG codons upstream of the vpr initiation site by site directed mutagenesis (Supplementary Figures 1 and 8). Mutation of these three ATG codons, one of which results in M181L in SIV Vif, resulted in expression of Vpr, which is incorporated into progeny virions (Vpr+ HSIV-vifNL4-3 and Vpr+ HSIV-vifΔvpx, Figure 3). Antibody to Nef was used as a control for incorporation of a virion-associated protein in HIV-vif clones. Additionally, since Vpx counteracts the function of SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011) and is essential for replication of SIV in macaques (Hirsch et al., 1998; Belshan et al., 2012; Shingai et al., 2015) and because HSIV-vif already has a partial ORF for vpx, we also generated an HSIV-vif derivative carrying the full-length vpx gene (Supplementary Figure 2). We used HSIV-vifΔvpx, which is IFN-resistant (Thippeshappa et al., 2013), to generate HSIV-vif-vpx carrying the full-length vpx gene (named HSIV-vif-vpxNL4). By Western blot using rabbit anti-serum to HIV-2 Vpx protein, Vpx could be detected in the cell lysates of SIV, HIV-2, and HSIV-vif-vpxNL4, but not SIVΔvpx. However, it was not detected in virion lysates of HSIV-vif-vpxNL4 (Supplementary Figure 2) as HIV-1 does not have determinants in p6 Gag required for virion incorporation of Vpx (Sunseri et al., 2011).

We tested the effect of Vpr and Vpx expression on HSIV-vif replication in an immortalized PTM CD4+ T cell line (Munoz et al., 2009). PTM CD4+ T cells were infected with Vpr- (HSIV-vifNL4-3, HSIV-vifΔvpx, HSIV-vifΔΔvpx), Vpr+ (Vpr+HSIV-vifNL4-3, Vpr+HSIV-vifΔvpx, and Vpr+HSIV-vifΔΔvpx), HSIV-vif-vpxNL4 or wild type HIV-1 (NL4-3, NL-AD8, and Bru-Yu2) viruses at an MOI of 0.01. Vpr+ HSIV-vif viruses, and HSIV-vif-vpxNL4 replicated in PTM CD4+ T cells to similar levels as Vpr- viruses (Figure 4). Expectedly, wild type HIV-1 (NL-AD8 and Bru-Yu2) failed to replicate (Figure 4).

We also determined the replication capacity of vpr and vpx carrying HSIV clones in human MDMs. Human MDMs were generated using previously described methods (Biesinger et al., 2010) and infected with the Vpx+, Vpr+, and Vpr- HSIV-vif clones at an MOI of 0.01. Vpr+ HSIV- vifΔvpx (Figure 5A) and Vpr+ HSIV-vif-vpxNL4 (Figure 5B) replicated to similar levels as Vpr- HSIV-vifΔvpx and Vpr+ HSIV-vifNL4, but slightly less than wild type HIV-1 NL-AD8 and Bru-Yu2 (Figures 5A,B), although it was not statistically significant. Additionally, HSIV-vif-vpxNL4 replicated as well as the parental HIV-1 Bru-Yu2 (Figure 5B).

**Serial in vivo PASSAGING OF HSIV-VIF**

Because HSIV-vifNL4-3 replication in the initial PTM experiment was low with peak viremia <10⁶ copies/ml (Figure 1; Thippeshappa et al., 2011), we conducted animal to animal transfer of infected PTM peripheral blood to adapt HSIV-vif to PTMs. For this experiment, the initial inoculum contained a mixture of CXCR4- (C/196 and C/200) and CCR5-tropic viruses (Vpr+ HSIV-vifΔvpx, Vpr+ HSIV-vifΔΔvpx, and HSIV-vif-vpxNL4). At 14 wpi, pooled blood from infected PTMs (Z09080 and Z09067) was used to inoculate a naïve macaque (Z13086). At 8 wpi, peripheral blood from Z13086 was transferred into an additional PTM (Z13098). Interestingly, all the macaques showed a peak viremia close to or above 10⁶ copies/ml and the viral loads persisted for at least 20 wpi (Figure 6A). Furthermore, increases in antibody titer over time suggest that all PTMs were persistently infected with HSIV-vif (Figure 6B). However, CD4+ T cell decline was not observed in the infected PTMs (Figure 6C).

**CLONING AND CHARACTERIZATION OF HSIV IMCs**

We generated IMCs from proviral DNA isolated from PBMCs of the passage 3 macaque (Z13098). Near full-length proviral
insertion of 737 bp region spanning the 3' end of vif to 5' end of vpu (nucleotides 5,447–6,171 according to HXB2 reference sequence) from Vpr-AD8HSIV-vifNL4-3. We confirmed the expression of Vpr from HSIV-P3 IMCs by western blot using rabbit antiserum against Vpr protein (Figure 7). Interestingly, virion-associated Vpr was higher than that for the VPR+ HSIV-vifNL4-3 clone.

We determined whether HSIV-P3 IMCs (HSIV-P3-114, HSIV-P3-161, and HSIV-P3-284) replicate in PTM PBMCs. PBMCs isolated from different donor PTMs were activated with concanavalin A for 3 days and maintained in IL-2 containing media for 2 days. Activated PBMCs were infected with HSIV-P3 IMCs and Vpr-AD8HSIV-vifNL4-3 at an MOI of 0.01. Viral supernatants were collected at various days post-infection to assay for p24 levels. We observed that HSIV-P3 IMCs replicated with different efficiency in PBMCs from different donor PTMs (Figure 8). Among the three HSIV-P3 IMCs, HSIV-P3-284 replicated to similar levels as Vpr+ HSIV-vifNL4-3 in activated PBMCs.

**DISCUSSION**

Since first-generation SHIV constructs replicated poorly in macaques, serial *in vivo* passages were conducted to enhance their infectivity or replicative capacity (Luciw et al., 1995; Joag et al., 1996; Reimann et al., 1996; Igarashi et al., 1999; Chen et al., 2000; Song et al., 2006). We previously reported the construction of HSIV-vifNL4-3 and replication trend in juvenile and newborn PTMs (Thippeshappa et al., 2011). Although HSIVvifNL4-3 persisted for nearly 4 years, the peak viremia was below 10^5 copies/ml, rapidly declined, and was intermittently detectable thereafter in juvenile PTMs. Therefore, we conducted serial *in vivo* passaging of HSIV-vif in PTMs using a mixture of different molecular clones and variants as an initial inoculum. We report consistent replication of HSIV with peak plasma viral RNA levels close to or greater than 1 × 10^5 viral RNA copies/ml and continuously detectable for 20–30 wpi in the passaged macaques. Additionally, we performed passaging at 14 (passage 2) and 8 wpi (passage 3) when the viral loads were low in the donor monkey. Therefore, peak viremia of 10^5 copies/ml in the recipient monkey suggests consistent replication of HSIV in PTMs. To further characterize HSIV-vif selected in PTMs, we PCR cloned and generated IMCs from the passage 3 macaque using DNA isolated from PBMCs from passage 3 PTM at 4 wpi. Our characterization indicated that the selected variants appear to be Vpr expressing recombinants of the HSIV-vifNL4-3 biological isolates from the long-term infected PTM with depressed CD4+ T cell counts. These data suggest that neutralization resistant variants which had evolved in association with CD4+ T cell decline and acquired the ability to express the HIV-1 Vpr had a fitness advantage for replication in PTMs (Overview of the experiments and data is provided in Supplementary Figure 9).

We have previously reported potential reasons for the attenuated replication of HSIV-vifNL4-3 in PTMs. In those studies, we noticed that HSIV-vifNL4-3 was inefficient in degrading PTM APOBEC3 family restriction factors compared to highly pathogenic SIVmne027 (Thippeshappa et al., 2011). We also observed that replication of HSIV-vifNL4-3 is inhibited in the
presence of IFNα in PTM CD4+ T cells (Thippeshappa et al., 2013). This perhaps suggests that HSIV-vifNL4-3 may not be able to overcome type I IFN response induced during acute stage of infection. Here, we also show that HSIV-vif clones do not express Vpr due to interference by the partial vpx ORF in the singly spliced vpr mRNA. We speculate that the absence of Vpr expression may have affected viral replication of HSIV-vifNL4-3 in the initial inoculation of PTMs (Figure 1).

HIV-1 Vpr incorporates into virions through an interaction with p6 of Gag (Bachand et al., 1999; Selig et al., 1999). In vitro studies have attributed several biological function to Vpr, which include: (i) cell cycle arrest and apoptosis (Di Marzio et al., 1995; He et al., 1995; Jowett et al., 1995; Panelles et al., 1995; Re et al., 1995; Bartz et al., 1996; Goh et al., 1998; Stewart et al., 1999, 2000; Zhang and Bieniasz, 2020); (ii) nuclear import of viral DNA (Popov et al., 1998a, b; Le Rouzic et al., 2002; Riviere et al., 2010); (iii) regulation of viral gene expression (Forget et al., 1998; Subbramanian et al., 1998; Vanitharani et al., 2001; Yurkovetskiy et al., 2018); (iv) infection of non-dividing cells (Balliet et al., 1994; Connor et al., 1995; Campbell and Hirsch, 1997; Miller et al., 2017); (v) modulation of immune responses (Ayyavoo et al., 2002; Muthumani et al., 2004, 2005; Majumder et al., 2005, 2008; Okumura et al., 2008; Doehle et al., 2009; Khan et al., 2020); and (vi) interaction with uracil DNA glycosylase (UNG2), a DNA repair enzyme that specifically removes uracil from DNA, and reduction of G to A mutations during reverse transcription (Mansky et al., 2000; Chen et al., 2004; Ahn et al., 2010). Since Vpr performs multiple functions during HIV replication, we hypothesized that absence of HIV-1 Vpr expression in HSIV-vifNL4-3 may affect persistent viral replication in PTMs.

It is interesting that even without Vpr expression, HSIV-vifNL4-3 persisted for nearly 4 years (Figure 1). While the functions of Vpr have not been clearly defined in vivo, the potential importance of Vpr for HSIV-vif replication in pigtailed monkeys is supported by pathogenesis studies of SIVmac, which demonstrate deletion of either Vpr or Vpx alone or together attenuates viral replication and ability to cause disease (Lang et al., 1993; Gibbs et al., 1995), and Vpx is necessary for SIV infection of CD4+ T cells.
in vivo (Belshan et al., 2012). Interestingly, Vpr null viruses reverted to Vpr expressing virus in SIV-infected macaques (Lang et al., 1993; Hoch et al., 1995), suggesting the importance of Vpr for in vivo pathogenesis. We speculated that the HIV-1 Vpr expressing HSIV may replicate better than the parental Vpr- HSIV-vif NL4-3 in vivo. Therefore, we generated HSIV derivatives expressing HIV-1 Vpr by introducing mutations in ATG codons upstream of the Vpr start codon. Since Vpx performs similar roles as Vpr and SIV vif gene already has partial ORF for vpx, we also generated an HSIV-vif derivative expressing the full length vpx gene. We cloned full-length vpx gene into HSIV-vif Yu2 backbone, as we have previously shown that this clone resists IFN treatment in PTM CD4 T cells. HSIV-vif clones expressing either the HIV-1 vpr or SIV vpx were replication competent in vitro. However, accessory proteins such as Vpr and Vpx are not necessary for HIV-1 or SIV replication in vitro. Therefore, it is difficult to show the impact of Vpr or Vpx expression for HSIV-vif replication using in vitro studies. Infecting PTMs with different clones would be a better method to define the significance of the HIV-1 vpr for HSIV-vif replication. Indeed, in our passage studies, we used both HIV-1 Vpr- and Vpr+ HSIV to determine the importance of HIV-1 Vpr for in vivo pathogenesis in PTMs. Recovery of Vpr+ HSIV IMCs from the passage 3 macaque again suggests a role for Vpr in pathogenesis in vivo.

In our studies to characterize persistent HSIV-vif variants, we have developed and standardized a rapid and robust approach to generate IMCs from proviral DNA. We screened 54 plasmids for their ability to generate infectious virus. Out of which, only three generated infectious virus, which roughly correspond to 5% of total clones. This is not surprising as 90–95% of the proviral DNA is noninfectious (Ho et al., 2013; Bruner et al., 2016; Hiener et al., 2017). Interestingly, recovered IMCs from passage 3 macaque were Vpr+ HSIV-vif NL4-3. Although PCR-mediated recombination event is possible (Liu et al., 2014), this suggested a possible recombination between Vpr+ HSIV-vif clones (either HSIV-vif AD8 or HSIV-vif Yu2) with biological isolates C/196 and C/200 recovered from M08009. Three observations suggest a recombination event: First, the three recovered IMCs had deletion of bases between SIV vif stop codon and HIV-1 vpr start codon. Second, most mutations observed in env and nef were already
present in the HSIV-vif<sub>i</sub> biological clones (C/196 and C/200) from M08009. Third, an analysis using RAPR program indicated that recovered IMCs may have resulted from recombination between biological clone C/196 and Vpr<sup>+</sup> HSIV-vif<sub>L</sub>, with a segment spanning from within vif to vpu of Vpr<sup>+</sup> HSIV-vif<sub>L</sub>, which includes the mutation necessary for Vpr expression inserted into the biological clone C/196. Therefore, the recombination event to generate Vpr<sup>+</sup> HSIV-P3 IMCs demonstrates the importance of HIV-1 Vpr for HSIV-vif pathogenesis in vivo.

**TABLE 2** Nonsynonymous mutations observed in HSIV-P3 infectious molecular clones (IMCs).

| Gene | IMCs 114 | IMCs 161 | IMCs 284 |
|------|----------|----------|----------|
| Gag  | K15R, E344G*, and T469* | K15R | K15R, R387G* |
| Pol  | F8L, T27A, G113R, C317S, and G705K | F8L, T27A, G113R, C317S, and G705K | F8L, T27A, G113R, C317S, and G705K |
| Vif  | E20G*, M181** | M181** | M181** |
| Vpu  | I42T | I42T | I42T |
| Env  | L22p*, H105Q, K117R, G145E*, R146G*, I448T, R166K, R166K, K229E*, A279V, Q308H*, K341R, T371M*, E427K, I489L*, C530A, C530A, T531A, D545G, A576T, E732G, D756N, V780G, N814Y*, and V827I* | H105Q, K117R, G145E*, R146G*, I448T, R166K, R166K, K229E*, A279V, Q308H*, K341R, T371M*, E427K, I489L*, C530A, C530A, T531A, D545G, A576T, E732G, D756N, V780G, N814Y*, and V827I* | H105Q, K117R, G145E*, R146G*, I448T, R166K, R166K, K229E*, A279V, Q308H*, K341R, T371M*, E427K, I489L*, C530A, C530A, T531A, D545G, A576T, E732G, D756N, V780G, N814Y*, and V827I* |
| Rev  | G65E, C89R, and G96R | G65E, C89R, and G96R | G65E, C89R, and G96R |
| Nef  | R105K, S163N*, M173G, V180M*, R184K, and E201D | R105K, S163N*, M173G, V180M*, R184K, and E201D | R105K, S163N*, M173G, V180M*, R184K, and E201D |
| *unique mutations to each of the IMCs. |
| *mutations were observed in HSIV-vif biological isolates recovered from M08009. |
| **M181L mutation introduced in SIV Vif changed to isoleucine in the recovered IMCs. |

Although we used mixture of CXCR4- and CCR5-tropic viruses for inoculation into passage 1 PTMs, it is interesting that CXCR4-tropic HSIV-vif<sub>L</sub> persisted through three passages. We have previously reported that SIV variants emerging during late-stage disease have a higher replicative capacity and increased pathogenicity (Kimata et al., 1999). Similar observations have also been made with SHIV-1157ipd (Song et al., 2006). Therefore, recovered virus (C/196 or C/200) isolated during the late stage in our study may have greater fitness for replication in PTMs. Since recovered virus was also neutralization resistant, it may have helped the virus overcome antibody responses during additional passages. We also observed several nonsynonymous mutations throughout the genome of HSIV-P3-IMCs. Most of the mutations were shared among the three HSIV-P3 IMCs. However, HSIV-P3 IMCs also had mutations unique to each of the clones. We speculate that these mutations could help the virus overcome restriction factors, better utilize host dependency factors, or they could help the virus escape adaptive immune responses.

In conclusions, our results suggest that serial in vivo passaging improves HSIV replication and persistence in PTMs. Identification of several nonsynonymous mutations in IMCs recovered from...
passage 3 macaque also indicated that serial in vivo passaging helps in acquisition of mutations. Since these mutations are in the context of replication competent virus, they may play a significant role in the replication and pathogenesis in vivo. However, a shortcoming of the studies is the limited duration of the passage experiment and limited number of animals used for the study. While variants of HSIV-vif\textsubscript{NL4-3} that acquired the ability to express the HIV-1 Vpr appear to have a selective advantage, the short duration of the experiments was insufficient to determine if the selected variants had increased pathogenicity. However, the in vivo data show consistent replication of HSIV-vif to \(10^5\) viral RNA copies/ml in four PTMs. Further in vivo passaging of HSIV-P3 IMCs with longer follow-up periods will be necessary to verify their increased replication fitness, and to generate pathogenic variants with enhanced replication capacity. Development of such pathogenic variants will be valuable as challenge viruses for preclinical evaluation of novel vaccines and therapeutics, as these HSIV clones have all the HIV immunologic and vaccine targets, such as Gag, Pol, Env, Tat, Rev, and Nef. Furthermore, establishment of HIV reservoirs in this model also provides an avenue for developing therapeutic vaccination approaches targeting HIV Gag, Pol, and Env, apart from testing latency reversal agents and cure strategies.

**DATA AVAILABILITY STATEMENT**

Sequences of HSIV-P3-114, HSIV-P3-161, and HSIV-P3-284 are deposited under accession numbers MZ146778, MZ146779, and MZ146780, respectively.

**ETHICS STATEMENT**

The animal study was reviewed and approved by University of Washington.

**AUTHOR CONTRIBUTIONS**

RT designed studies, performed experiments, and wrote manuscript. PP coordinated and analyzed data from animal studies and edited manuscript. SSC designed and performed experiments and edited manuscript. KT and AM performed experiments and edited manuscript. S-LH obtained funding, coordinated animal studies, and edited manuscript. PCA performed experiments and edited manuscript. JTK obtained funding, designed studies, performed experiments, and wrote manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.779460/full#supplementary-material

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