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Pathogenic Consequences of Vaginal Infection with CCR5-Tropic Simian-Human Immunodeficiency Virus $SHIV_{SF162P3N}$

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We previously reported efficient transmission of the pathogenic R5 simian-human immunodeficiency virus $SHIV_{SF162P3N}$ isolate in Indian rhesus macaques by intravenous and intrarectal inoculations, with a switch to CXCR4 coreceptor usage in ~50% of infected animals that progressed rapidly to disease. Since women continue to be disproportionately affected by HIV, we developed an animal model based on the intravaginal challenge of female rhesus monkeys with $SHIV_{SF162P3N}$ and sought to validate the utility of this model to study relevant aspects of HIV transmission and pathogenesis. The effect of viral dose on infection outcome was evaluated to determine the optimal conditions for the evaluation of HIV-1 preventive and therapeutic strategies. We found that the virus can successfully cross the vaginal mucosal surface to establish infection and induce disease with coreceptor switch, but with lower efficiencies compared to intravenous and rectal transmissions. In contrast to intrarectal infection, peak and cumulative viral load over a 1 year-infection period were significantly greater in macaques exposed intravaginally to lower rather than higher inoculum doses. Moreover, low and transient viremia was observed only in macaques that were challenged intravaginally twice within the same day with a high dose of virus, which can be seen as doubling the dose. Taken together, these results show that $SHIV_{SF162P3N}$ can successfully transmit across the genital mucosa, undergo coreceptor switch, and induce disease. However, the administered dose appears to impact $SHIV_{SF162P3N}$ vaginal infection outcome in an unexpected manner.

Over 80% of all HIV transmissions occur across mucosal surfaces, with women accounting for 60% of adults infected with HIV in sub-Saharan Africa (18, 56, 60). Close to 80% of heterosexual transmissions with HIV-1 clades A, B, C, and D are established by a single viral variant from the donor, with higher numbers of transmitted/founder viruses detected in men who have sex with men and wider variations in variant numbers in intravenous drug users (2, 4, 15, 23, 24, 31, 48, 49). The majority of the transmitted/founder viruses are CCR5-tropic, with neutralization susceptibility profiles that are typical of primary viruses (24, 31, 49).

Nonhuman primate (NHP) models play a key role in providing a clearer understanding of the very early events in mucosal HIV transmission. Studies in the SIV/macaque model of vaginal transmission showed that within a few days of virus transmission, replication converges on the gut-associated lymphoid tissues (32, 63, 64), with loss of memory $CD4^+$ T cells in this as well as in multiple lymphoid tissue compartments (40, 58). Furthermore, similar to the human situation, transmission of a very small number of variants was observed in rhesus macaques (RMs) exposed intrarectally (i.r.) or intravaginally (i.v.g.) to low-dose SIV and chimeric simian-human immunodeficiency virus (SHIV) (25, 36, 51, 57). Of note, macaques treated by depo-provera to thin the vaginal mucosa and exposed to a single supraphysiological dose of R5 SHIV also harbored few SHIV variants, indicating a particularly strong genetic bottleneck in vaginal transmission (7). NHP mucosal infection models, therefore, are relevant for the evaluation of intervention strategies such as vaccines, topical microbicides, and preexposure prophylaxis to prevent HIV-1 sexual transmission, providing information that allows clinical researchers to make informed decisions in the choice of intervention concepts or approaches with the greatest potential for success in humans.

Since HIV-1 strains transmitted between humans use primarily CCR5 as their coreceptor, prevention strategies must protect against R5 virus challenges. We recently documented successful intravenous (i.v.) and i.r. transmission of the late (time of overt immunodeficiency) R5 $SHIV_{SF162P3N}$ isolate in Indian RMs (20, 47), with pathogenic sequelae that consistently recapitulate key features of HIV-1 infection in humans. These include acute $CD4^+$ T cell depletion in the gut, uncontrolled replication, and progression to AIDS with a switch in coreceptor preference toward CXCR4 in ~50% of infected animals. Efficient transmission, consistency of high viral set points, and disease in RMs infected i.v. or i.r. with $SHIV_{SF162P3N}$ suggest that the virus will be useful not only for testing HIV-1 envelope-based prophylactic or early postinfection interventions but also for the discovery of intervention strategies that rely on reduction in steady-state viral loads and prevention or delay of disease as indicators of protection. However, because the risk of HIV-1 infection varies with different viral doses and exposure routes (19) and because vaginal transmission is responsible for a large number of new HIV-1 infections (18, 56, 60), it is necessary to demonstrate that R5 $SHIV_{SF162P3N}$ can successfully breach the vaginal mucosa and induce disease to establish the full utility of this model. An understanding of the biological consequences of infection with R5 $SHIV_{SF162P3N}$ via different routes and doses will also be important. We document here transmission, persistent infection, and coreceptor switching in macaques exposed intravaginally to R5 $SHIV_{SF162P3N}$ and examine the im-

Received 4 April 2012 **Accepted** 14 June 2012

Published ahead of print 27 June 2012

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Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.00852-12

pact of viral dose on disease severity in the vaginally infected monkeys.

MATERIALS AND METHODS

Animal inoculation and clinical assessments. All inoculations were carried out in adult rhesus monkeys (*Macaca mulatta*) of Indian origin housed at the Tulane National Primate Research Center in compliance with the *Guide for the Care and use of Laboratory Animals*. Animals were confirmed to be serologically negative for simian type D retrovirus, simian immunodeficiency virus (SIV), and simian T-cell lymphotropic virus prior to infection and screened for the presence of the Mamu-A*01, Mamu-B*17, and Mamu-B*08 class I alleles previously shown to be associated with the control of pathogenic SIVmac239 replication using standard PCR with allele-specific primers (13). They were also genotyped for TRIM5α expression, since this innate host defense mediator has been reported to contribute to SIV control as well (26, 35). Macaques received a single intrarectal (i.r.) or intravaginal (i.v.g.) inoculation with 10^4 or lower 50% tissue culture infectious dose ($TCID_{50}$) of the cell-free challenge stock SHIV$_{SF162P3N}$ or two i.v.g. inoculations with 10^4 $TCID_{50}$ virus, 4 h apart. The challenge virus was propagated and titered in RM peripheral blood mononuclear cells and was generated through successive rapid transfer in RMs of the CCR5 molecular clone SHIV$_{SF162}$, recovered from passage 3 in animal T353 at end-stage disease (17, 20). Whole blood from the inoculated animals was collected weekly for the first 8 weeks, biweekly for another 16 weeks, and monthly thereafter. Surgery was performed at peak (2 to 3 weeks postinfection [wpi]) and postacute (~12 wpi) infection for collection of tissues from one external and one internal lymph node and from internal organs such as the gut, bone marrow, thymus, and spleen. Animals were euthanized at the end of the study period by intramuscular administration of telazol and buprenorphine, followed by an overdose of sodium pentobarbital. Euthanasia was considered to be AIDS related if the animal exhibited peripheral blood $CD4^+$ T cell depletion (<200/mm^3), >25% loss of body weight, and combinations of the following conditions: diarrhea unresponsive to treatment, opportunistic infections, peripheral lymph node atrophy, and abnormal hematology (e.g., anemia, thrombocytopenia, or leukopenia). Tissues from multiple sites were collected. Plasma viremia was quantified by branched DNA analysis (Siemens Medical Solutions Diagnostic Clinical Lab, Emeryville, CA), and absolute $CD4^+$ and $CD8^+$ cell counts were monitored in TruCount tubes (BD Biosciences, Palo Alto, CA). The percentages of $CD4^+$ T cells in the tissue cells were analyzed by flow cytometry (FACSCalibur) using CD3-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), and CD8-peridinin chlorophyll protein (PerCP) antibodies. Except for CD3-FITC (BioSource, Camarillo, CA), all antibodies were obtained from BD Biosciences.

Viral RNA isolation, *env* amplification, and sequencing. Viral RNA was prepared from 300 to 500 μl of plasma using a commercially available RNA extraction kit (Qiagen, Chatsworth, CA,) followed by reverse transcription (RT) with Superscript III RT (Invitrogen, Carlsbad, CA) and random hexamer primers (Amersham Pharmacia, Piscataway, NJ). The V1 to V5 region of gp120 was amplified from the RT products using *Taq* DNA polymerase (Qiagen) with the primers ED5 and ED12 or ES7 and ES8 as previously described (9), and full-length gp160 was amplified using the outer primers SH50 (5′-TAGAGCCCTGGAAGCATCCAGGAAGTC AGCCTA and SH51 (5′- TCCAGTCCCCCCTTTTCTTTTATAAAA) and the inner primers SH43 (5′-AAGACAGAATTCATGAGAGTGAAG GGGATCAGGAAG-3′) and SH44 (5′-AGAGAGGGATCCTTATAGCA AAGCCCTTTCAAAGCCCT-3′) (20). For single-genome amplification (SGA) of full-length *env*, limited endpoint dilution PCR was performed with primers SH50/51 and SH43/44 to identify the DNA dilution that gives <30% positive reactions in the total number of reactions (48). SGA amplicons were subjected to direct automated sequencing while standard PCR products were cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions, followed by automated sequencing of cloned amplicons (Genewiz, South Plainfield, NJ). Nucleotide sequences were aligned with CLUSTAL X (29) and edited manually using BioEdit v7.0.9. A phylogenetic tree was constructed using a maximum-composite-like model in the MEGA, version 5.03 software, and bootstrap values were generated with 1,000 repetitions.

Plasmid constructs and pseudotyped virus production. For the expression of envelope glycoproteins, full-length gp160 coding sequence amplified from RT products was subcloned into the pCAGGS vector (20). To generate luciferase reporter viruses capable of only a single round of replication, envelope *trans*-complementation assay was used. Briefly, Env expression plasmid and the NL4.3LucE-R+ vector were cotransfected by polyethyleneimine (Polyscience, Warrington, PA) into 2.5×10^6 293T cells plated in a 100-mm plate. Cell culture supernatants were harvested 72 h later, filtered through 0.45-μm-pore-size filters, and stored at −70°C in 1-ml aliquots. Pseudotyped viruses were quantified for p24 Gag content by enzyme-linked immunosorbent assay (Beckman Coulter, Fullerton, CA).

Determination of coreceptor usage. Coreceptor usage of pseudotyped reporter viruses was determined by infection of U87.CD4 indicator cell lines. Briefly, 7×10^3 U87.CD4.CCR5 or U87.CD4.CXCR4 cells were seeded in 96-well plates 24 h before use and infected, in triplicates, with 5 ng of p24 Gag equivalent of the indicated pseudotyped viruses, followed by incubation for 72 h at 37°C. At the end of the incubation period, the cells were harvested, lysed, and processed for luciferase activity according to the manufacturer's instructions (luciferase assay system; Promega, Madison, WI). Entry, as quantified by luciferase activity, was measured with an MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, VA).

Statistical analysis. Disease-free survival curves for the i.v.-, i.r.-, and i.v.g.-infected macaques were estimated using the Kaplan-Meier method. Differences in time to AIDS between groups were assessed using log-rank tests, while differences in peak and cumulative viral loads (log-transformed RNA copies/ml plasma) were examined using Mann-Whitney U tests. The cumulative viral load was computed as an integration of the area under the curve for a 1-year infection duration. A P value of <0.05 was considered statistically significant.

RESULTS

Persistent infection and disease induction in macaques exposed intravaginally to a single high dose R5 SHIV$_{SF162P3N}$ challenge. To determine whether R5 SHIV$_{SF162P3N}$ can transmit across the vaginal mucosa, we inoculated 12 female Indian RMs once with a high inoculum dose (10^4 $TCID_{50}$ virus). To develop a more relevant model of vaginal transmission, the animals were not treated with Depo-provera, a procedure that thins the vaginal epithelium to achieve consistent rates of SIV/SHIV infection (39). Eight of the twelve depo-naive macaques were systemically infected, with an infection rate (66.7%) that is comparable to the rate we observed for single high dose i.v.g. transmission of the lineage-related early (20 wpi) R5 SHIV$_{SF162P3}$ virus in depo-naive Indian rhesus monkeys (16). Peak viremia of 6 to 8 $\log_{10}$ RNA copies/ml of plasma was seen at 2 wpi (Fig. 1A). This was followed by three different patterns of postacute viremia. Two of the eight infected animals (EL48 and EI77) continued to replicate virus at high levels, with progression to disease and euthanasia within 30 wpi. Three (FR25, FV44, and GH62) had a viral set-point of 4 to 5 $\log_{10}$ RNA copies/ml of plasma, while the remaining three (CA33, CC50, and GR56) replicated only to levels of 3 $\log_{10}$ RNA copies/ml or lower. In contrast, macaques BK49, CR18, EA19, and EK83 remained aviremic over 30 weeks of observation, suggesting that they resisted high dose SHIV i.v.g. infection. However, substantial amount of mucus was noted in the vaginal vault of EK83 at the time of challenge, providing an impediment which could have limited virus transmission (27). Transient peripheral $CD4^+$ T cell

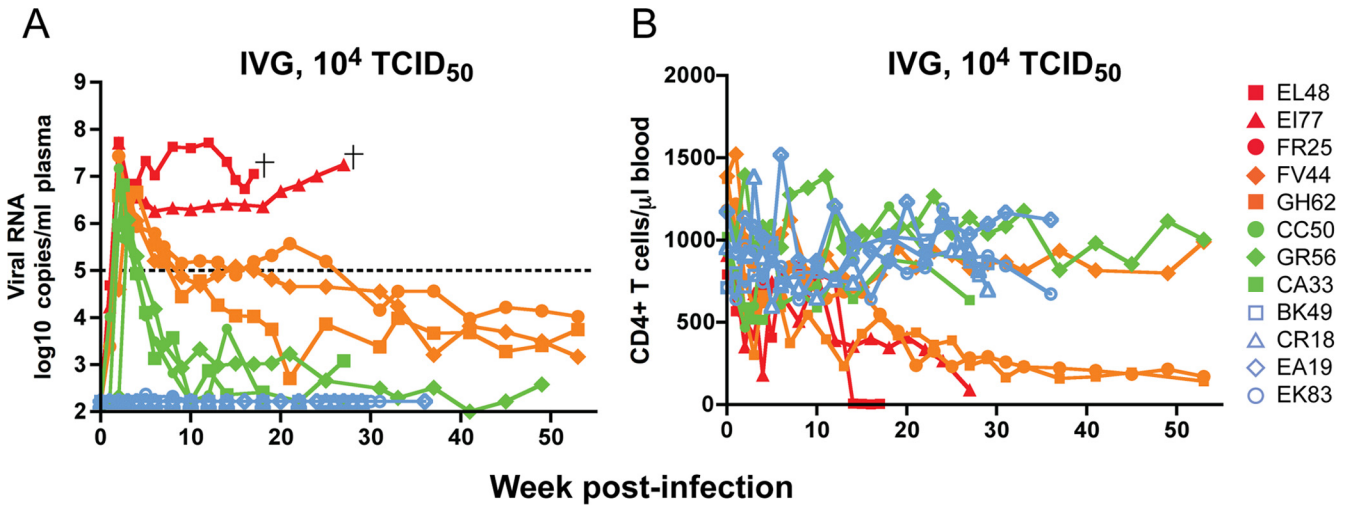

FIG 1 Virologic (A) and immunologic (B) measurements in RMs ($n = 12$) receiving a single i.v.g. inoculum of 10^4 $TCID_{50}$ R5 $SHIV_{SF162P3N}$. A "+" sign indicates euthanasia with clinical symptoms of AIDS, open symbols denote those animals that resisted challenge, and the dotted line in panel A marks a set point of 5 log_{10} RNA copies/ml of plasma.

loss accompanied peak viremia in half of the infected macaques. The exceptions were the highly viremic macaques EL48, EI77, FR25, and GH62 (Fig. 1B), where blood $CD4^+$ T cell count continued to decline, with precipitous drop in EL48 and EI77 toward end-stage disease. In contrast to the other infected monkeys, these two latter animals failed to sustain an anti-SHIV antibody response (data not shown) and were euthanized at 17 and 27 wpi, respectively, with clinical symptoms of AIDS.

Compared to macaques challenged i.v. or i.r. with a similar high virus dose, the transmission efficiency of R5 $SHIV_{SF162P3N}$ i.v.g. exposure was lower (66.7% versus 100% for the i.v. and i.r. routes) (Table 1). Moreover, the kinetics of early virus dissemination (Fig. 2A) and the rate of disease progression over a 1-year infection period (Fig. 2B) were slower in high dose R5 $SHIV_{SF162P3N}$ i.v.g.-infected macaques than in the i.v.- or i.r.-infected macaques. The percentages of animals AIDS-free at 1 year were 22.2 and 27.3%, respectively, for the i.v.- and i.r.-infected macaques, compared to 75% for animals infected i.v.g. The difference in the rate of disease progression between the i.v.- and i.v.g.-infected macaques is statistically significant ($P = 0.03$), with the difference between the i.r.- and i.v.g.-infected monkeys approaching significance ($P = 0.07$).

Coreceptor switching in R5 $SHIV_{SF162P3N}$ vaginally infected monkeys that progressed rapidly to disease. The precipitous decline in peripheral $CD4^+$ T lymphocytes toward end-stage disease in the two i.v.g. rapid progressor (RP) macaques (EL48 and EI77) prompted us to investigate the emergence of X4 viruses. Analyses of the percentages of $CD4^+$ T cells in tissue compartments during peak viremia (2 wpi) revealed minimal $CD4^+$ T cell loss (<20%) in peripheral lymph nodes (LN), with a modest drop in $CD4^+$ T lymphocytes at effector sites such as the lamina propria (LP) of the gut seen only in EI77 (~40%; Fig. 3A). Depletion of $CD4^+$ T lymphocytes increases in lymphoid tissues during chronic infection, with close to 90% loss in the gut of both animals at 12 wpi. At the time of death, >99% of $CD4^+$ T lymphocytes in the gut and LN of EL48 were depleted. Massive depletion of $CD4^+$ T cells was also seen in the gut of EI77 at the time of euthanasia, but 20 to 35% of this T cell subset was preserved in secondary lymphoid tissues. Sequence analysis showed that 2 of 19 envelope (*env*) clones amplified from plasma of EL48 at the time of necropsy had four amino acid deletions in the C terminus of the V3 loop (Δ22-25) that increased the net charge of this domain to +6 (Fig. 3B). This Δ22-25 V3 deletion sequence had previously been shown to confer CXCR4 usage to variants that retained and preferred CCR5 for entry in R5 $SHIV_{SF162P3N}$ i.v.- and i.r.-infected macaques (50). Indeed, V3 Δ22-25-bearing Envs from EL48 were found to be dualtropic, infecting U87.CD4 cells that expressed either the CCR5 or CXCR4 coreceptor (Fig. 3C). In comparison, 5 of 20 *env* amplicons from plasma of EI77 at the time of necropsy harbored three amino acid insertions (GHI) immediately upstream of the

TABLE 1 Summary of single-dose i.v., i.r., and i.v.g. challenges with R5 $SHIV_{SF162P3N}$

Route	Dose ($TCID_{50}$)	No. of animals	No. (%) of infected animals	No. (%) of infected animals with:				Source or reference
				AIDS	RP phenotype	Controlled infection	CoR switch	
i.v.	3,000	9	9 (100)	8 (89)	6 (66.7)	0	4 (44.4)	20, 21
i.r.	10,000	11	11 (100)	10 (91)	4 (36.4)	0	5 (45.4)	47; this study
	≤1,000	6	6 (100)	3 (50)	1 (16.7)	2 (33.3)	0	This study
i.v.g.	10,000	12	8 (66.7)	2 (25)	2 (25)	2 (25)	2 (25)	This study
	1,000	6	4 (66.7)	2 (50)	2 (50)	2 (50)	1 (25)	This study

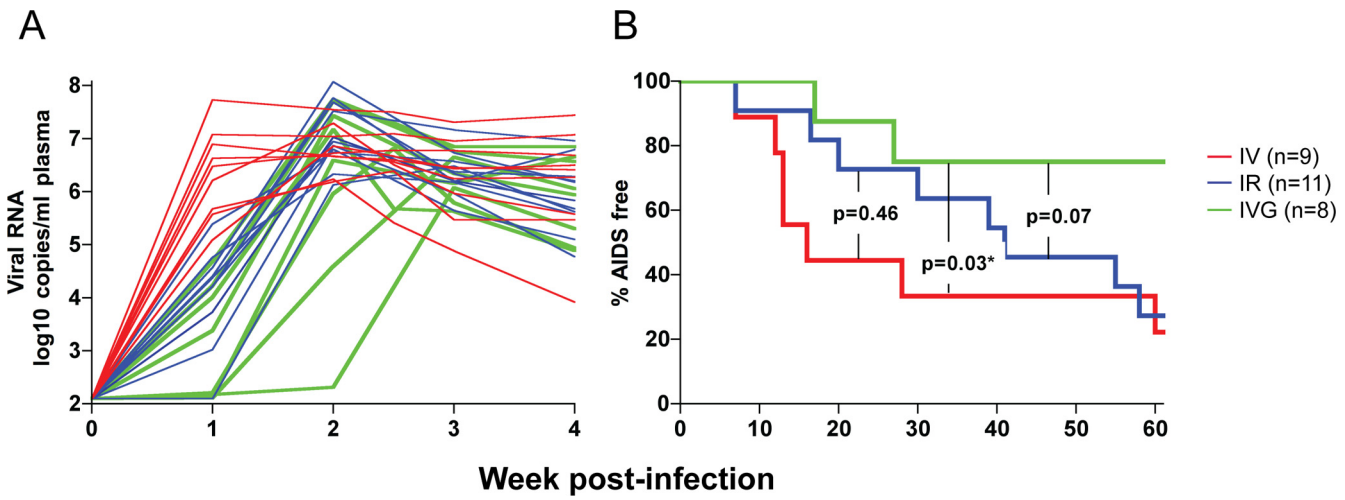

FIG 2 Kinetics of early virus dissemination (A) and disease-free survival curves (B) for macaques infected i.v. ($n = 9$), i.r. ($n = 11$), or i.v.g. ($n = 8$) with a single high dose of R5 SHIV$_{SF162P3N}$. Plasma RNA levels within the first 4 weeks of infection (A) and AIDS development over a 1-year infection period (B) are shown. A P value of <0.05 is considered statistically significant.

GPGR crown in the V3 loop. This V3 insertion was also found in a dualtropic variant recovered from a RP macaque infected i.v. with R5 SHIV$_{SF162P3N}$, and GHI-bearing viruses in EI77 infected CCR5, as well as CXCR4-expressing cells (Fig. 3C). Furthermore, and consistent with the findings in i.v.- and i.r.-infected macaques (50, 53), the dualtropic variants in the i.v.g.-infected animals entered TZM-bl less efficiently than the coexisting R5 viruses. These results show coreceptor switching in the two i.v.g. R5 SHIV$_{SF162P3N}$-infected RP monkeys, supporting the notion of similar pathogenic sequela of R5 SHIV$_{SF162P3N}$ i.v., i.r., and i.v.g. infections.

Effect of dose on virus replication in R5 SHIV$_{SF162P3N}$ i.v.g.-infected macaques. Because low-dose challenge models are believed to more closely reflect the viral concentrations found in semen (44), we investigated the effect of challenge dose on i.v.g. R5 SHIV$_{SF162P3N}$ transmission and used a 10-fold-lower inoculum than in the first series of animals studied. We found that four of six female RMs exposed once i.v.g. to 1,000 TCID$_{50}$ of SHIV$_{SF162P3N}$ were systemically infected (66.7% transmission rate), with a high peak viremia of 7 to 8 log$_{10}$ RNA copies/ml of plasma at 2 wpi (Fig. 4A). Viremia remained high ($\geq$6 log$_{10}$ RNA copies/ml of plasma) in two (DG17 and GC70) of the four infected macaques, with progression to disease at 22 to 24 wpi in the absence of sustained seroconversion. This contrasts with the other two infected animals, CG45 and CG63, where infection leveled off at 3 to 4 log$_{10}$ RNA copies/ml of plasma, with seroconversion at 4 to 6 wpi. The peripheral CD4$^+$ T cell count remained relatively stable throughout the course of infection in the two i.v.g.-infected animals that had low chronic viremia, but with protracted decline in DG17 and dramatic loss in GC70, the two highly viremic monkeys. At the time of euthanasia, the gut CD4$^+$ T cells were severely depleted in both animals, with drastic LN CD4$^+$ T cell loss only in GC70 (data not shown). Sequence analysis of GC70 plasma viruses showed the presence of variants harboring the Δ22-25 V3 deletion sequence (data not shown), suggesting that it represented another i.v.g.-infected RP with a coreceptor switch.

The apparent lack of a dose effect on R5 SHIV$_{SF162P3N}$ i.v.g. transmission efficiency and virus replication contrasts with prior observations using the lineage-related early R5 SHIV$_{SF162P3}$ (55), the dualtropic SHIVDH12R (10), and SIVmac251 (43), where animals infected i.v.g. with low inoculum doses often developed transient viremia. Accordingly, we examined whether the lack of a dose effect on R5 SHIV$_{SF162P3N}$ i.v.g. transmission and replication was a feature unique for this route of mucosal challenge by comparing the infection outcome in 11 RMs challenged i.r. with high doses (10^4 TCID$_{50}$) to six RMs that were exposed to 1- to 2-log-lower inoculum sizes (Fig. 4B). All i.r.-exposed macaques were infected, irrespective of the challenge dose. Nine of eleven macaques infected i.r. with a high inoculum dose sustained a viremia of $>$5 log$_{10}$ RNA copies/ml of plasma and developed AIDS, with a rapid progressor phenotype in four monkeys and coreceptor switching in ~50% of the infected animals (Table 1). Of the two animals that had a 1-log-lower steady-state viremia, one (DP85) developed clinical symptoms of AIDS and was euthanized at 80 wpi. Peak viremia was of the same order of magnitude (7 to 8 log$_{10}$ RNA copies/ml) among macaques infected i.r. with lower inoculum doses, but only three of six macaques infected with a lower dose progressed to disease, with one displaying a rapid progressor phenotype (K420). Of the remaining three animals, two controlled their infection to undetectable levels at 40 to 50 wpi ($<$2 log$_{10}$ RNA copies/ml of plasma). Comparison of the peak and cumulative viral load (area under the curve) over a 1-year infection period for macaques infected i.v.g. or i.r. with various R5 SHIV$_{SF162P3N}$ doses showed that the peak ($P = 0.016$) as well as the overall ($P = 0.049$) viral burden was significantly lower in macaques inoculated i.v.g. with high inoculum doses (10^4 TCID$_{50}$) than in macaques inoculated i.v.g. with lower inoculum doses (10^3 TCID$_{50}$), and this was not observed in the i.r.-infected animals (Fig. 4C). These findings suggest that an inverse association between challenge dose and R5 SHIV$_{SF162P3N}$ replication and pathogenesis exists but that it is route dependent, i.e., it is true for i.v.g. challenge but not for i.r. challenge.

Atypical pattern of virus replication in macaques exposed intravaginally twice within the same day to high dose R5 SHIV$_{SF162P3N}$. In several studies, two high doses of SIV administered i.v.g. 4 h apart were shown to infect a higher proportion of

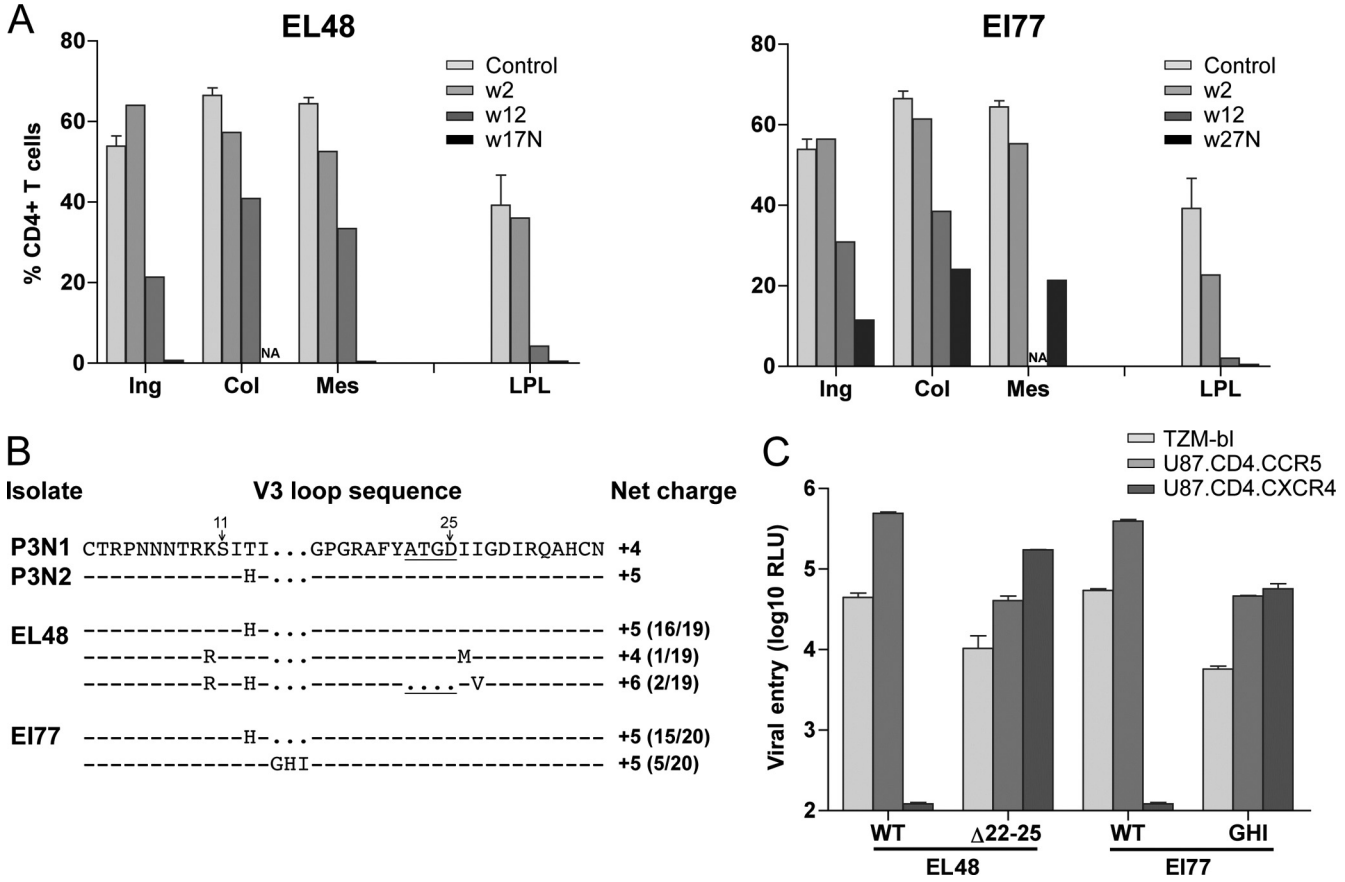

FIG 3 Tissue CD4$^+$ T cell frequency (A), V3 loop sequence (B), and coreceptor usage (C) of viral variants in macaques EL48 and EI77. (A) Percentages of CD4$^+$ T cells in the inguinal (Ing), colonic (Col), and mesenteric (Mes) lymph nodes and lamina propria lymphocyte (LPL) from the jejunum during peak (w2) and chronic (w12) stage of infection and at time of necropsy (N) are reported. Baseline values generated from three uninfected macaques (control) are shown for reference. NA, not available. (B) V3 loop sequence comparison of representative SHIV$_{SF162P3N}$ clones (P3N1 and P3N2) and plasma viruses in macaques EL48 and EI77 at time of necropsy. Dots indicate gaps, and dashes stand for identity in sequences, with the net positive charge of the V3 region shown on the right. Positions 11 and 25 within the V3 loop are indicated by arrows, and the 4-amino-acid deletion is underlined. The numbers in parentheses represent the numbers of clones matching the indicated sequence per total number of clones sequenced. (C) Relative entry of pseudoviruses bearing EL48 and EI77 Envs into TZM-bl, U87.CD4.CCR5, and U87.CD4.CXCR4 indicator cells. RLU, relative light units. The data are means and standard deviations from triplicate wells and are representative of at least two independent experiments.

animals than single-dose exposures (37, 38, 41). We therefore investigated whether this exposure regimen would increase the vaginal transmission rate of R5 SHIV$_{SF162P3N}$ and abrogate the seemingly inverse effect of high inoculum dose on i.v.g. infection. A wide variation in peak viremia, ranging from 2 to 8 $\log_{10}$ RNA copies/ml of plasma, was detected at 2 to 3wpi in five of seven depo-naive macaques exposed twice to 10^4 TCID$_{50}$ SHIV$_{SF162P3N}$ within a 4-h period (Fig. 5A). We observed a 71.4% transmission rate for R5 SHIV$_{SF162P3N}$ using two high doses administered within the same day (five of seven exposed monkeys), which was comparable to that observed using a single high dose (66.7%). However, viremia was transient in three of the five infected monkeys (AH94, DE37, and FE47), with intermittent viral blips of $\leq$3 $\log_{10}$ RNA copies/ml of plasma detected in DE37 and FE47 over a 1-year infection period. The two remaining infected macaques (CF18 and FH84) established a set-point of 4 to 5 $\log_{10}$ RNA copies/ml of plasma, with a 1-log increase in virus replication seen in CF18 at 40 wpi. This macaque developed clinical signs of AIDS and was euthanized at 52 wpi.

Peripheral CD4$^+$ T cell counts remained relatively stable in three of the five macaques infected i.v.g. following two virus exposures, with a gradual decline in the two animals (CF18 and FH84) with chronic viremia. CF18, FH84, and AH94 seroconverted at 4 to 6 wpi, but two of the animals with transient viremia, DE37 and FE47, remained seronegative at the end of a 1-year study period. This phenotype of infection without seroconversion, or occult infection, was seen only in the 2×10^4 TCID$_{50}$ i.v.g. challenge group, suggesting very rapid control of viral spread (Table 2). A comparison of the peak and cumulative viral load in macaques infected i.v.g. with R5 SHIV$_{SF162P3N}$ using various doses showed an inverse trend between the i.v.g. R5 SHIV$_{SF162P3N}$ inoculum and virus replication (Fig. 5B). Peak and cumulative viral load were significantly lower in animals infected with 10^4 and 2×10^4 TCID$_{50}$ of virus than in those receiving 10-fold-lower doses ($P < 0.05$), with a difference in peak viremia that approaches statistical significance ($P = 0.065$) between the 10^4 and 2×10^4 TCID$_{50}$ challenge conditions. Furthermore, the kinetics of virus dissemination was slower in macaques exposed to 10^4 TCID$_{50}$ R5 SHIV$_{SF162P3N}$ twice than in those challenged once, even with 10-fold-lower inoculum sizes (Fig. 5C). The time of peak viremia was

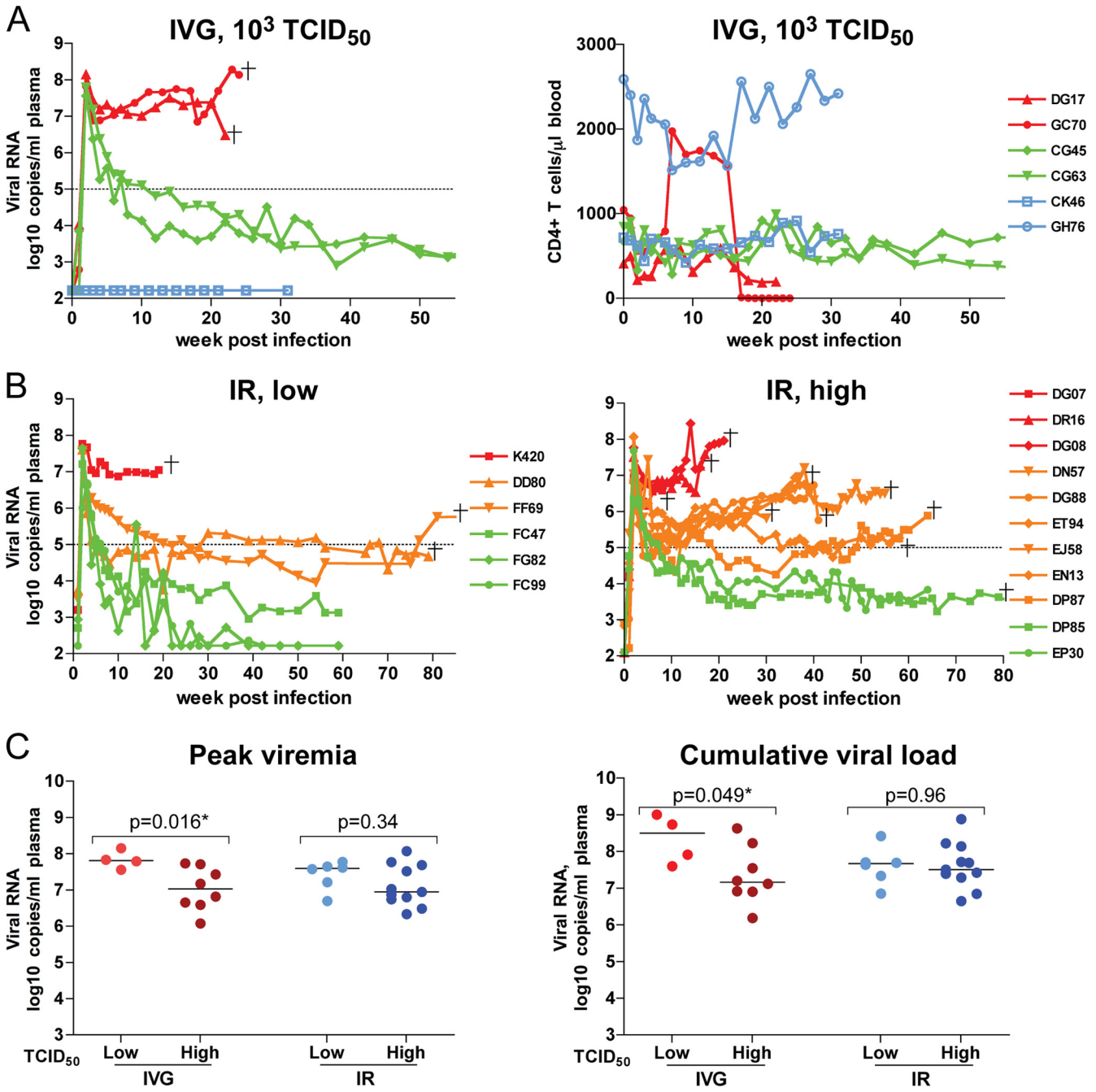

FIG 4 Comparison of virologic and immunologic measurements in RMs inoculated i.v.g. or i.r. with various doses R5 SHIV$_{SF162P3N}$. (A) Viral load and peripheral CD4$^+$ T cell counts in macaques receiving a single low intravaginal inoculum of 10^3 TCID$_{50}$ R5 SHIV$_{SF162P3N}$. A "+" indicates death due to euthanasia, and open symbols designate those macaques that resisted challenge. (B) Viral load in macaques inoculated i.r. with a single low (10^2 to 10^3 TCID$_{50}$) or high (10^4 TCID$_{50}$) dose of R5 SHIV$_{SF162P3N}$. A "+" indicates euthanasia with clinical symptoms of AIDS. Dotted line in panels A and B mark a set-point of 5 log$_{10}$ RNA copies/ml of plasma. (C) Peak and cumulative viral load (area under the curve over a 1-year infection period) comparison of R5 SHIV$_{SF162P3N}$ i.v.g.- and i.r.-infected macaques. The line represents the median viral RNA copies for each group. An asterisk (*) indicates statistical significance ($P < 0.05$).

week 2 in all four macaques infected with 10^3 TCID$_{50}$ and in five of the eight animals inoculated with 10^4 TCID$_{50}$ virus, whereas it took 3 weeks to reach peak viremia in the majority (four of five) of monkeys receiving 10^4 TCID$_{50}$ virus twice within the same day.

The effect of challenge dose on i.v.g. R5 SHIV$_{SF162P3N}$ infection cannot be explained by differences in host genetics or in the nature of the transmitted viruses. Since the presence of the Mamu-A*01, Mamu-B*17, and Mamu-B*08 class I haplotypes and that of certain TRIM5α alleles has previously been shown to be associated with control of pathogenic SIV replication (13, 26, 34), we examined whether these host restriction factors played a role in R5 SHIV$_{SF162P3N}$ i.v.g. infection. None of the animals that resisted i.v.g. challenge expressed the three restrictive Mamu alleles (Table 3), and there was no clear association between restric-

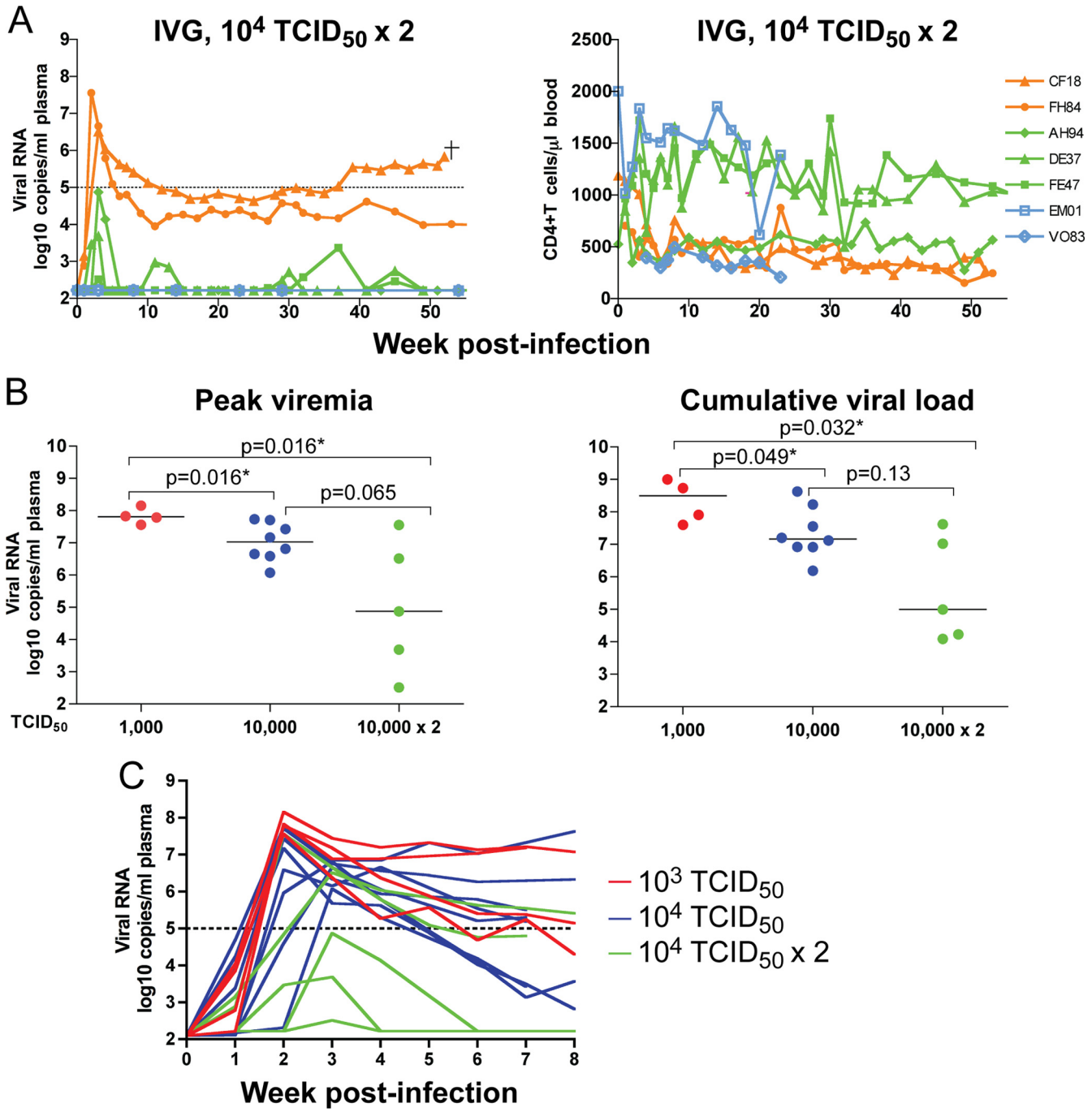

FIG 5 (A) Virologic and immunologic measurements in RMs ($n = 7$) receiving two doses of 10^4 $TCID_{50}$ R5 $SHIV_{SF162P3N}$ administered i.v.g. twice within the day. A "+" indicates euthanasia with clinical symptoms of AIDS, open symbols designate the macaques that resisted challenge, and dotted line marks a set-point of 5 log_{10} RNA copies/ml of plasma. (B) Peak viremia and cumulative viral load comparison of macaques infected i.v.g. with various doses. The line represents the median viral RNA copies for each group. An asterisk (*) indicates statistical significance ($P < 0.05$). (C) Virus dissemination in macaques infected i.v.g. with different doses of $SHIV_{SF162P3N}$. Plasma RNA levels within the first 8 weeks of infection are shown.

tive major histocompatibility complex (MHC) class I expression and SHIV control. For example, one of the three macaques that expressed Mamu-B*08 showed an RP phenotype (GC70), with the other two sustaining (FV44) or controlling (CC50) viremia (Fig. 1). The only monkey that tested positive for Mamu-B*17 (CG45; Fig. 4A) also remained viremic over a 1-year study period. As for TRIM5, four of the eight resistant macaques expressed the restrictive homozygous TFP/TFP allele, while the remaining four had the permissive heterozygous TFP/Q genotype (Table 3), with similar representation of these two alleles among the animals in the three dosage groups. There was also no apparent association between TRIM5 allelic polymorphisms and SHIV virus replication levels (see Fig. S1 in the supplemental material). The one macaque with TRIMcyp had low and transient viremia (FE47).

TABLE 2 Effects of dose on i.v.g. infection with R5 SHIV$_{SF162P3}$

Dose (TCID$_{50}$)	No. of exposed animals	No. (%) infected animals	No. (%) of infected animals with transient viremia	No. (%) of infected animals with an RP phenotype	No. of strong seroconverters[a]/no. of infected animals (%)
10,000 × 2	7	5 (71.4)	3 (60)	0	3/5 (60)
10,000	12	8 (66.7)	0	2 (25)	6/8 (75)
1000	6	4 (66.7)	0	2 (50)	2/4 (50)

[a] RPs that mounted a transient anti-SHIV antibody response are not considered strong seroconverters.

However, low and transient viremia were also seen in macaque AH94 with the permissive heterozygous TFP/Q genotype. The small cohort of animals studied may not have revealed subtle TRIM effects. However, because SHIV$_{SF162P3N}$ was derived from a SIVmac239 backbone into which an HIV$_{SF162}$ *env* gene was inserted, our findings are consistent with reports of a much lower effect of TRIM5α on SIVmac239 and SIVmac251 infection, compared to its effect on SIVsmE660 (11, 26, 35).

To investigate whether the differences in virus replication in the macaques infected intravaginally following two high-dose exposures were related to differences in the transmitted/founder virus, we performed a pilot study to analyze *env* sequences in the first viral RNA positive plasma samples from a macaque with high (FH84) or transient (DE37) viremia (Fig. 5A). Because of the low levels of virus replication in DE37, the envelope sequences were obtained by standard nested PCR/cloning and not by SGA. Nonetheless, increasing evidence suggests that with an adequate number of PCR templates analyzed, bulk sequencing captures a measure of population diversity similar to that determined by SGA (22, 30). Phylogenetic tree analysis of the early replicating viruses in the two monkeys showed clustering of DE37 (transient/low VL) *env* sequences with those of FH84 (high VL) (Fig. 6), suggesting that differences in the transmitted/founder virus were not the underlying reason for the variable infection outcome in the group of animals exposed twice within the same day to high doses of R5 SHIV$_{SF162P3N}$.

DISCUSSION

In this report, we document that R5 SHIV$_{SF162P3N}$ can breach the vaginal mucosa of RMs to establish persistent infection. Consistent with previous findings in macaques infected i.v. or i.r. with this virus, coreceptor switching was seen primarily in i.v.g.-infected animals that progressed rapidly to disease in the absence of a strong antiviral antibody response. Moreover, the V3 genetic sequence requirement for coreceptor switch in the i.v.g.-infected macaques overlapped with those found in i.v.- and i.r.-infected monkeys. Compared to the lineage-related early isolate R5 SHIV$_{SF162P3}$, (16, 17, 52, 62), infection with R5 SHIV$_{SF162P3N}$ re-

TABLE 3 Effect of host factors on R5 SHIV$_{SF162P3N}$ i.v.g. challenge

Dose (TCID$_{50}$)	Animal	Age (yrs)	Wt (kg)	No. of births	Infection outcome[a]	MHC (Mamu) allele			TRIM5 allele
						A*01	B*08	B*17	
1,000	DG17	8.98	5.83	3	RP	–	–	–	TFP/TFP
	GC70	5.76	5.6	0	RP	–	+	–	TFP/Q
	CG45	11.47	9.4	4	CP	–	–	+	TFP/Q
	CG63	11.55	8.0	4	CP	–	–	–	TFP/TFP
	CK46	12.03	6.5	5	Resistant	–	–	–	TFP/TFP
	GH76	6.05	6.75	0	Resistant	–	–	–	TFP/Q
10,000	EL48	7.3	6.05	1	RP	–	–	–	TFP/Q
	EI77	7.57	4.3	0	RP	–	–	–	TFP/Q
	FR25	6.84	8.85	0	CP	–	–	–	TFP/TFP
	FV44	6.53	8.3	0	CP	–	+	–	TFP/Q
	GH62	5.66	6.5	0	CP	–	–	–	Q/Q
	CC50	7.16	9.3	1	CP	–	+	–	TFP/Q
	GR56	5.48	7.45	0	CP	–	–	–	TFP/TFP
	CA33	7.25	7.8	1	CP	–	–	–	TFP/TFP
	BK49	8.56	7.05	1	Resistant	–	–	–	TFP/TFP
	CR18	6.59	6.8	0	Resistant	–	–	–	TFP/TFP
	EA19	8.65	8.55	0	Resistant	–	–	–	TFP/Q
	EK83	7.69	9.0	0	Resistant	–	–	–	TFP/Q
10,000 × 2	CF18	9.98	5.2	5	CP	–	–	–	Q/Q
	FH84	7.44	4.7	0	CP	–	–	–	TFP/Q
	AH94	12.27	11.1	6	Transient	–	–	–	TFP/Q
	DE37	9.73	5.6	3	Transient	–	–	–	TFP/TFP
	FE47	6.7	10.14	1	Transient	+	–	–	TFP/Cyp
	EM01	7.68	8.9	1	Resistant	–	–	–	TFP/Q
	V083	14.84	14.6	8	Resistant	–	–	–	TFP/TFP

[a] RP, rapid progressor; CP, chronic progressor.

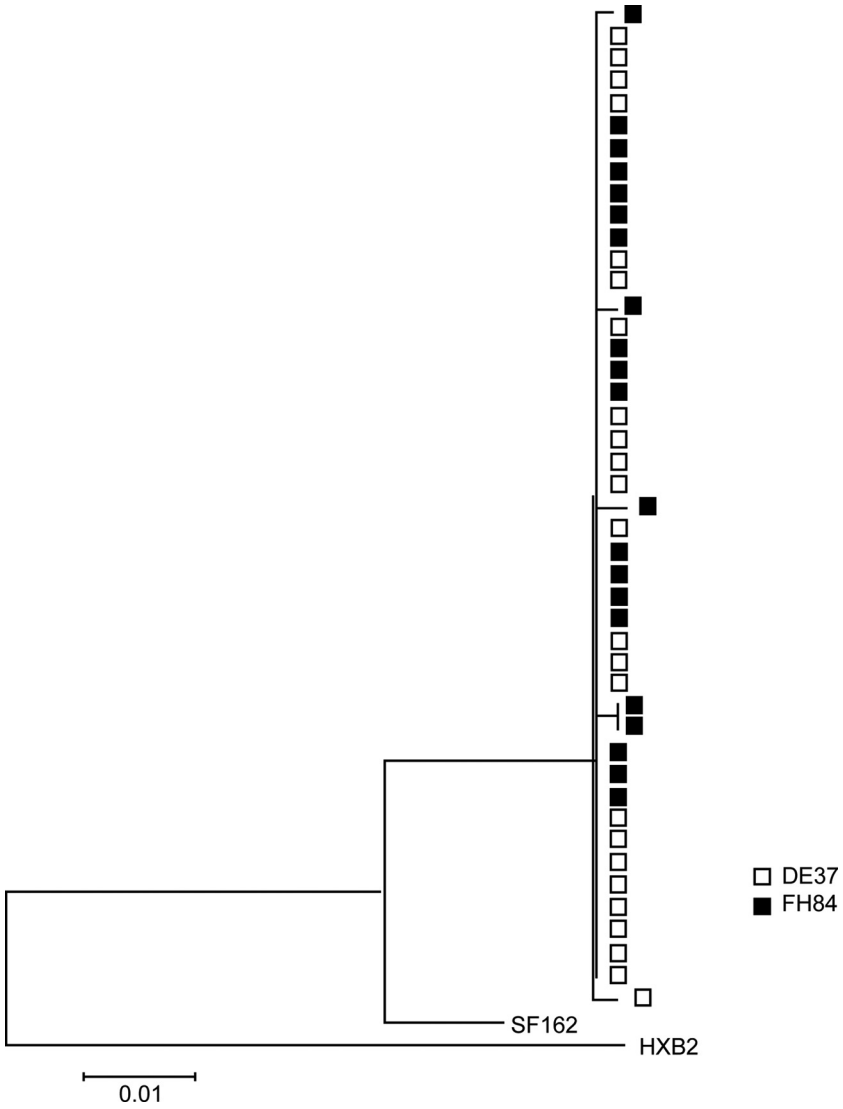

FIG 6 Early replicating viruses in viremic (FH84) and aviremic (DE37) R5 $SHIV_{SF162P3N}$-infected macaques. A phylogenetic tree shows the relationship between Env variant sequences (V3 to V5) in plasma of macaques FH84 and DE37 at the first viral RNA-positive time point (2 wpi). A neighbor-joining tree rooted on the sequences of HxB2 and SF162 was generated. The scale bar indicates the genetic distance along the branches in nucleotides.

sulted in less variability in chronic viremia and disease outcome. The increase in replicative capacity and pathogenicity of R5 $SHIV_{SF162P3N}$ may be related to the fact that it is a late-stage AIDS-associated isolate that has a longer period of adaptation in rhesus monkeys and is more diverse and divergent than $SHIV_{SF162P3}$ (20). R5 $SHIV_{SF162P3N}$ infection of RMs is therefore a useful model to study all modes of HIV-1 transmission and aspects of pathogenesis and for the testing of the efficacy of antiviral strategies during established infection.

Early studies with nonphysiological high doses of SIVs and SHIVs showed that there was no significant difference in the infection rates between macaques inoculated i.v. or i.r., but that this rate was lower after inoculation by the i.v.g. route (6, 8, 14, 38, 42, 54). Moreover, the kinetics of virus dissemination was slower, and the RNA levels were more variable in monkeys infected by the i.v.g. route than in monkeys infected by the i.v. or i.r. routes (3, 14, 43, 45). Our findings of reduced risk of infection (Table 1), slower dissemination (Fig. 2A), and greater variability in set-point viremia (Fig. 1A) with single high-dose i.v.g. R5 $SHIV_{SF162P3N}$ challenge in comparison to i.v. or i.r. infection with the same inoculum are in agreement with these earlier studies, further documenting the restrictions on virus transmission and spread imposed by the vaginal mucosa. Despite this barrier, we showed sufficient pathogenic sequela in female Indian RMs exposed i.v.g. Moreover, while the lower vaginal transmission efficiency could be a potential limitation in studies by this route, infection was achieved without the need for depo-provera treatment, which could perturb specific susceptibility factors such as mucosal antibodies and the levels of mucus and mucins, as well as innate antimicrobial mediators. Since the virus is CCR5-tropic, $SHIV_{SF162P3N}$ RM challenge provides a good model for studying the early stages of HIV-1 vaginal transmission and host response.

In this regard, although the number of animals studied was limited and the viral concentrations used could still be considered

high compared to inoculum doses likely to be encountered in humans, an unexpected inverse association was seen between virus replication and the i.v.g.-administered dose of $SHIV_{SF162P3N}$ that produced a systemic infection. Virus replication was significantly lower in macaques inoculated with a single high inoculum dose (10^4 $TCID_{50}$) than in those receiving a 1-log-lower dose challenge (Fig. 4C), with a higher proportion of the infected animals in the lower dose challenge group developing a RP phenotype (Table 2). Moreover, transient and low viremia was observed only in macaques exposed i.v.g. twice within the same day to high-dose inoculum, which can be seen as doubling the dose (Fig. 5A). These differences in $SHIV_{SF162P3N}$ i.v.g. infection could not be explained by MHC class I and TRIM5α genetic polymorphisms (Table 3 and see Fig. S1 in the supplemental material) or a selection for particular transmitted/founder viruses (Fig. 6), raising the possibility of modulation as a result of dose-dependent and exposure-induced immune factors. Because the virus stocks were propagated in activated macaque lymphocytes, the supernatants are bound to contain a range of secreted immune factors, which could have induced a protective effect. However, the inverse association was only seen with i.v.g. and not with i.r. challenge (Table 1), suggesting a differential host response to virus/supernatant dose at these two mucosal sites with structurally distinguishable epithelium barriers. A very-high-dose vaginal $SHIV_{SF162P3N}$ challenge could have tipped the balance between infection-promoting and inhibitory responses in favor of antiviral defense mechanisms that dampen SHIV expression or susceptibility (28, 46). Indeed, very early involvement of innate defenses, such as initiation of the interferon cascade to produce virus restriction factors and release of virus-inhibiting chemokines by pDCs that could blunt the initial wave of viral replication had been reported after i.v.g. SIV inoculation (1, 33). Nonetheless, other host factors such as age, preexisting genital inflammation and menstrual cycle status are also known to effect vaginal HIV/SHIV transmission efficiency (5, 12, 59, 61). $SHIV_{SF162P3N}$ i.v.g. challenge in additional animals will be required to draw firm conclusion on the effect of high inoculum dose. These studies might help to elucidate the nature of the factors that confer protection against vaginal transmission and systemic infection to guide microbicide and mucosal vaccine design.

In conclusion, we show that R5 $SHIV_{SF162P3N}$ can transmit across the female genital tract, with unexpected biological consequences at a very high inoculum dose. Whether decreased virus replication and disease modulation are the results of *de novo* innate immune factor synthesis triggered by the SHIV virion, the result of immune factors secreted into the cell culture supernatants along with virus or incorporated into the virion membrane and other host factors remains to be determined. Regardless, because mucosal infection with R5 $SHIV_{SF162P3N}$ recapitulates key effects of HIV-1 infection in humans and shows much more consistent pathogenicity than the lineage-related early $SHIV_{SF162P3}$ isolate, this virus holds promise in studies to understand the impact of R5 virus infection on biological responses in the mucosa and for the development of HIV biomedical prevention methods.

ACKNOWLEDGMENTS

We thank Lisa Chakrabarti for helpful discussion and critique of this work and Hiroshi Mohri for help with statistical analysis.

The following were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: reagents TAK779 (catalog no. 4983 from Takeda Chemical Industries, Ltd.), AMD3100 (8128 from AnorMed, Inc.), TZM-bl (catalog no. 8129 from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc.), and U87.CD4 indicator cell lines (catalog no. 4035 and 4036 from HongKui Deng and Dan Littman). This study was supported by the National Institutes of Health grants RO1AI046980 and AI084765 and by Primate Center Base grant P51-OD011104-51.

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