Relative Transmissibility of an R5 Clade C Simian-Human Immunodeficiency Virus Across Different Mucosae in Macaques Parallels the Relative Risks of Sexual HIV-1 Transmission in Humans via Different Routes

Agnès L. Chenine,2,3 Nagadenahalli B. Siddappa,2,3 Victor G. Kramer,2 Gaia Sciaranghella,2,3 Robert A. Rasmussen,2,3 Sandra J. Lee,1 Michael Santosuosso,1,4 Mark C. Poznansky,2,4 Vijayakumar Velu,4 Rama R. Amara,4 Chris Souder,4 Daniel C. Anderson,1 François Villinger,4 James G. Else,4 Francis J. Novembre,4 Elizabeth Strobert,6 Shawn P. O’Neil,3,5 W. Evan Secor,7 and Ruth M. Ruprecht2,3

1Department of Biostatistics and Computational Biology, 2Dana-Farber Cancer Institute, 3Harvard Medical School, 4Partners AIDS Research Center and Infectious Diseases Medicine, Massachusetts General Hospital, Boston, 5New England Primate Research Center, Southborough, Massachusetts; 6Yerkes National Primate Research Center, Emory University, and 7Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Background. Worldwide, ∼90% of all human immunodeficiency virus (HIV) transmissions occur mucosally; almost all involve R5 strains. Risks of sexual HIV acquisition are highest for rectal, then vaginal, and finally oral exposures.

Methods. Mucosal lacerations may affect the rank order of susceptibility to HIV but cannot be assessed in humans. We measured relative virus transmissibility across intact mucosae in macaques using a single stock of SHIV-1157ipd3N4, a simian-human immunodeficiency virus encoding a primary R5 HIV clade C env (SHIV-C).

Results. The penetrability of rhesus macaque mucosae differed significantly, with rectal challenge requiring the least virus, followed by vaginal and then oral routes (P = .031, oral vs vaginal; P < .001 rectal vs vaginal). These findings imply that intrinsic mucosal properties are responsible for the differential mucosal permeability. The latter paralleled the rank order reported for humans, with relative risk estimates within the range of epidemiological human studies. To test whether inflammation facilitates virus transmission—as predicted from human studies—we established a macaque model of localized buccal inflammation. Systemic infection occurred across inflamed but not normal buccal mucosa.

Conclusion. Our primate data recapitulate virus transmission risks observed in humans, thus establishing R5 SHIV-1157ipd3N4 in macaques as a robust model system to study cofactors involved in human mucosal HIV transmission and its prevention.

Most human immunodeficiency virus type 1 (HIV-1) infections worldwide occur mucosally, involving unprotected sexual acts or mother-to-child transmission. Viruses with exclusive R5 tropism cause most mucosal HIV-1 acquisitions, which occur rectally, vaginally, and orally (reviewed in [1–6]). Oral transmission is seen in breastfeeding infants and has been described for oral-genital contact in adults (reviewed in [1, 7]). Epidemiological studies, including studies of serodiscordant couples, have yielded relative risk estimates for the 3 routes and identified unprotected rectal intercourse as the riskiest behavior, followed by vaginal exposure, and...
finally orogenital contact [3, 8–11]. Multiple cofactors have been linked to HIV-1 transmission, including coinfections and mucosal tears, but it is difficult to measure the impact of these factors on the rank order of mucosal HIV-1 acquisition in humans. Thus, to evaluate relative virus transmissibility across intact mucosal surfaces, we tested different exposure routes in nonhuman primates free of known pathogens.

Mucosal lentiviral transmission studies have been performed for rectal, vaginal, and oral acquisition in macaques, which are susceptible to simian immunodeficiency virus (SIV) and chimeric simian-human immunodeficiency virus (SHIV) strains encoding HIV-1 

env [7, 12–19]. Previously, we compared the relative transmissibility of SIV, using an identical SIVDelta B670 stock, by using the intravenous, intrarectal, and oral routes in macaques that underwent nontraumatic mucosal inoculations [14]. Unexpectedly, oral challenge required 60 times less virus to achieve systemic infection than did intrarectal challenge. This puzzling finding contradicted the relative risks of HIV-1 acquisition through anogenital versus orogenital sexual exposure in humans. Multiple factors may account for the discrepancy between the primate model data and human epidemiological observations, including the fact that SIVDeltaB670 is dual-tropic. Because almost all mucosal HIV-1 infections are caused by R5 viruses, we sought to address the question of relative lentiviral transmissibility with a clade C SHIV (SHIV-C) that encodes an HIV-1 envelope with exclusive CCR5 tropism.

Most SHIVs encode the env sequence of HIV-1 clade B, which causes <10% of all global infections. In contrast, HIV-1 clade C (HIV-C) causes 56% of all infections worldwide and predominates in Sub-Saharan Africa and India [20]. Here, we report the relative mucosal transmissibility and pathogenesis of an R5 SHIV-C, SHIV-1157ipd3N4 [21, 22].

METHODS

Rhesus monkeys. Macaques of Indian and Chinese origin were used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals at the Yerkes National Primate Research Center (Emory University) and the Centers for Disease Control and Prevention (CDC), both fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal experiments were approved by Institutional Animal Care and Use Committees of Emory University, the CDC, and the Dana-Farber Cancer Institute. All procedures were performed on anesthetized macaques. Chinese-origin macaques used for intrarectal titration served as normal controls elsewhere [23].

Major histocompatibility complex class I typing of macaques. Indian-origin macaques were Mamu-A*01-negative, except RB0-6, RDL-9, and RQv-8 (by vaginal titration), and RAf-10 (by oral titration). Major histocompatibility complex (MHC) alleles for Chinese-origin macaques were unavailable.

Virus stock. The exclusive R5-tropic SHIV-1157ipd3N4 [21] was grown in pooled peripheral blood mononuclear cells (PBMCs) from 2 Indian macaques (capsid protein 27, 227 ng/mL; 50% tissue culture infectious doses [TCID_{50}] by TZM-bl cell assay [24], 4 \times 10^4/mL).

Mucosal SHIV-1157ipd3N4 challenges. All inoculations were performed atraumatically; anesthetized macaques received various virus stock dilutions in a total volume of 3 mL (oral route) or 1 mL (intrarectal and intravaginal routes). For details, see the Appendix, which is not available in the print edition of the Journal. For standard oral inoculations, virus was applied to the back of the tongue, in contrast to challenge with buccal mucosa.

Plasma viral RNA levels. RNA was isolated with QiaAmp Viral RNA Mini-Kits (Qiagen); viral RNA levels were measured by quantitative reverse-transcription polymerase chain reaction (assay sensitivity, 50 copies/mL) [25].

Staining of cells from blood, lymph node, and rectal biopsies. PBMCs and lymph node cells were isolated using standard procedures; lymphocytes from rectal pinch biopsies were obtained by collagenase digestion followed by Percoll gradient centrifugation; \( \sim 1 \times 10^4 \) lymphocytes were surface stained as described elsewhere [21, 26].

Induction of local inflammation in buccal mucosa. A circle was drawn inside the right cheek of anesthetized macaques with a surgical pen and a template. Multiple 30-\( \mu \)L aliquots of 10% acetic acid were administered submucosally into the circle with a microsyringe. To study the inflammation time course, 4 macaques had 10% acetic acid (6 \times 30 \( \mu \)L submucosally) applied inside the circle. Biopsy specimens were obtained from 1 of the macaques on days 0, 3, 4, or 7 (for details, see the Appendix, which appears only in the electronic edition of the Journal.)

SHIV-1157ipd3N4 challenge across normal or inflamed buccal mucosa. Four macaques received 6 \times 30 \( \mu \)L of 10% acetic acid. On day 4 after this, while the macaque was lying with the right cheek down, a plastic ring was placed over the inked circle, into which a total volume of 300 mL of virus was placed, left for 5 min, then adsorbed with gauze, and the ring removed. Four acetic acid–naive macaques were challenged likewise.

Immunohistochemical analysis. CD4+ cells were identified in formalin-fixed, paraffin-embedded buccal mucosa sections using reagents from Vector Labs (Burlingame): anti-human CD4 monoclonal antibody (clone 1F6), biotinylated horse antimouse immunoglobulin G, immunoperoxidase kits (Vectastain ABC Elite), and the chromogenic substrate 3,3′-diaminobenzidine [27].

Statistical analyses. The distribution of 50% animal infectious dose (AID_{50}) values for all titrations was determined by the Spouge method [28], which uses log-transformed data
and provides AID_{50} mean values and standard deviations. To assess associations between AID_{50} values and the different titration groups, 2-sample t tests were performed with mean values and standard deviations that were estimated according to Spouge approximation [28]. Viral RNA loads and flow cytometry data were evaluated using Prism software (version 4; GraphPad) and Mann-Whitney tests.

**RESULTS**

**SHIV-C titrations by different mucosal routes.** Indian-origin macaques were given different dilutions of the identical SHIV-1157ipd3N4 stock orally (n = 6), intravaginally (n = 7), and intrarectally (n = 6) and followed up prospectively for plasma viral RNA loads; systemic infection was confirmed by Western blot analysis at week 12. Although macaques were exposed to different viral doses, peak viremia levels were similar for all 3 routes of transmission, and no differences were seen in viral setpoints (Figure 1). For single-virus challenges, including those reported here, a lack of correlation between inoculum size, peak viremia, viral setpoints, and disease progression has been described elsewhere [29, 30].

**Determination of SHIV-1157ipd3N4 minimal infectious dose and AID_{50}.** The minimal infectious dose needed to obtain systemic infection was lowest for the intrarectal route (dilution, 1:100), followed by the intravaginal route (1:20) and finally, the oral route (1:10) (Table 1). The statistical method of Spouge [28] was used to determine AID_{50} values for each route which again yielded the rank order: rectal (5.5 μL), followed by vaginal (23.9 μL), followed by oral (181 μL) (Table 1). Thus, without mucosal lesions, the rectal route was most permissive, followed by the intravaginal route, and finally the oral route. The relative rank order for mucosal R5 SHIV-C transmission in our primate model paralleled that observed for humans sexually exposed to HIV-1 via different risk behaviors.

**Relative mucosal permeability and sexual transmission.** We reasoned that SHIV-C AID_{50} values, expressed as volumes of undiluted virus stock required to achieve systemic infection in half of all exposed macaques, are indirectly proportional to the ease with which virus crossed a given mucosal surface. Thus,

![Figure 1](image-url). Viremia in Indian-origin rhesus macaques inoculated with SHIV-1157ipd3N4 orally (A), vaginally (B), or intrarectally (C) and in Chinese-origin rhesus inoculated intrarectally (D). Animals were exposed to different dilutions of the same stock. *RQ3911 had been exposed to virus once (at 1:1000) without infection. Dashed line, lower limit of detection (<50 copies/mL).
Table 1. Relative Mucosal Permeability to SHIV-C in Rhesus Monkeys and Relative Risks of Sexual Human Immunodeficiency Virus (HIV) Acquisition

<table>
<thead>
<tr>
<th>Monkey origin</th>
<th>Route of exposure</th>
<th>Virus dilution yielding systemic infectiona</th>
<th>AID50, µL</th>
<th>Relative rhinogenous mucosal permeability (1/AID50 normalized)</th>
<th>Probability of HIV-1 acquisition in humans, no. of infection per 1000 exposures (normalized)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indian</td>
<td>Oral</td>
<td>1:10 (3 of 4)</td>
<td>181</td>
<td>.031 (oral vs vaginal)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Vaginal</td>
<td>1:20</td>
<td>23.9</td>
<td>&lt;.001 (rectal vs vaginal)</td>
<td>7.6</td>
<td>1.3–12.5</td>
</tr>
<tr>
<td></td>
<td>Rectal</td>
<td>1:100 (2 of 3)</td>
<td>5.5</td>
<td>33</td>
<td>8.3–125</td>
<td>[9–11, 31–35] (reviewed in [2, 3])</td>
</tr>
<tr>
<td>Chinese</td>
<td>Rectal</td>
<td>1:50 (2 of 3)</td>
<td>24.6</td>
<td>.0015 (vs Indian monkey, rectal)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Defined by viral RNA load >10⁴ copies/mL and confirmed by Western blot analysis at week 12 after inoculation.
b 2-sided \( P \) value based on the \( t \) test.

the AID50 values for the oral (181 µL), vaginal (23.9 µL), and rectal routes (5.5 µL) were converted to 1/AID50 and normalized according to their ratios, after assigning a value of 1 to the oral route (Table 1). The ratios of 1:7.6:33 (oral:vaginal:rectal exposure) reflect the relative mucosal permeability to our R5 SHIV in experimental settings, where mucosal trauma was avoided.

Next, we searched the literature for estimates of relative risks of HIV-1 acquisition for different routes of sexual transmission (unprotected oral sex, male-to-female transmission by vaginal intercourse, and rectal intercourse), which are generally expressed as numbers of new HIV infections per 1000 exposures [9–11, 31–35] (reviewed in [1–3, 37]). Given the complexity of human sexual practices, the possible influence of cofactors (including coinfections and mucosal tears), and the inability to determine exact times of HIV exposure and inoculum size, it is not surprising that these estimates vary widely. Expressed as the number of new infections per 1000 unprotected exposures, the incidence of acquisition ranged from 3.3 to 50 for rectal exposure, 0.5 to 5 for vaginal exposure [9–11, 31–35] (reviewed in [2, 3]), and 0.4 for orogenital exposure [36] (reviewed in [1, 37]). As we sought to compare the relative risks of HIV-1 acquisition by various mucosal routes with our primate data, we again set the value for oral-genital HIV-1 exposure at 1. Proportionally, the ratios of oral:vaginal:rectal HIV-1 acquisition were 1:((1.3–12.5):(8.3–125) (Table 1). Remarkably, the ratios measured experimentally for relative mucosal permeability by our nontraumatic mucosal R5 SHIV inoculations in macaques fell within the ranges extrapolated from epidemiological studies in HIV-1-exposed humans.

Susceptibility of Chinese-origin macaques to intrarectal SHIV-C exposure. Because Indian-origin rhesus macaques have been widely used for AIDS research, their supply has become limited. Chinese-origin rhesus macaques are more readily available and have been used for SIVmac infection [38, 39]. To evaluate whether Chinese-origin macaques would yield similar results to those of Indian-origin macaques, we compared the susceptibility of the 2 subspecies to rectal SHIV-1157ipd3N4 exposure.

Nine Chinese-origin macaques were used for intrarectal titration; the minimal infectious dose was higher than that used for Indian macaques (Figure 1C, 1D). The AID50 extrapolated according to Spouge approximation [28] for Chinese macaques was 4.5 times higher than that for Indian macaques (\( P = .015; 2\)-sided \( t \) test), which probably reflects the previous SHIV-1157ipd3N4 adaptation in Indian macaques [21, 22].

Figure 2. Induction of local inflammation of buccal mucosa (black rectangle) 4 days after acetic acid administration.
both infected macaque subspecies showed similar peak viral RNA loads \((7.1 \times 10^6 \text{ copies/mL})\) for Indian macaques vs \((3.8 \times 10^6 \text{ copies/mL})\) for Chinese macaques; \(P = .22\); Mann-Whitney test). Interestingly, only 40\% of the infected Indian macaques were still viremic at week 12, in contrast to all infected Chinese macaques, in which viremia was significantly higher than that found in their Indian counterparts \((P = .008\); Mann-Whitney test; Figure 1D). Together, these data indicate that intrarectal SHIV-1157ipd3N4 transmission is reproducible in both macaque subspecies. Furthermore, mucosal SHIV-1157ipd3N4 challenge and patterns of viremia in Chinese macaques indicate that this subspecies is well suited for lentiviral pathogenesis and vaccine development studies.

Establishment of a macaque model for local mucosal inflammation. To test whether our SHIV-C mucosal challenge model would yield an increased rate of transmission in the presence of inflammation, as would be expected from human epidemiologic studies, we induced localized inflammation in buccal mucosa, in which viremia was significantly higher than that found in their Indian counterparts \((P = .008\); Mann-Whitney test; Figure 1D). Together, these data indicate that intrarectal SHIV-1157ipd3N4 transmission is reproducible in both macaque subspecies. Furthermore, mucosal SHIV-1157ipd3N4 challenge and patterns of viremia in Chinese macaques indicate that this subspecies is well suited for lentiviral pathogenesis and vaccine development studies.

Transmission across inflamed and across normal buccal mucosa. Macaques \((n = 8)\) were entered into the study pair-wise: one with inflammation and one control. The macaque exposed to acetic acid was monitored daily for signs of inflammation. Once the latter was confirmed, both macaques were exposed to SHIV-1157ipd3N4 using either undiluted virus \((3 \text{ pairs})\) or virus diluted 1:10 \((1 \text{ pair})\), for a total volume of 300 \(\mu\text{L}\). To prevent virus dispersion throughout the oral cavity, a small plastic ring was placed over the site of buccal inflammation or normal buccal mucosa, and virus was applied inside the ring. This procedure restricts virus exposure to buccal mucosa, in contrast to our standard oral inoculation protocol. Two of 4 macaques with buccal inflammation given undiluted virus developed systemic infection, whereas all animals given dilut-
Figure 4. SHIV-1157/ipd3N4 pathogenicity. (A–C) Percentage of CD4+ T cells (vs total T cells) of macaques with systemic SHIV-1157/ipd3N4 infection or naive controls; lymph node and rectal biopsies were performed at weeks 5 and 12; *P* < .05 for both comparisons (Mann-Whitney test). Horizontal bars, median values. (D) Percentage of memory CD4+CD29+ T cells (dotted line, lower limit of normal) and (E) viral loads and absolute CD4+ T cell counts for Indian-origin macaque RJs-10, and Chinese-origin macaque RQ3911; both developed AIDS (CD4+ T cells <200 cells/µL according to the CDC-defined threshold for human AIDS [50]; dotted line).

ed virus and all controls remained uninfected, suggesting that healthy buccal mucosa is relatively impervious to SHIV-C transmission. The increased presence of viral target cells probably accounts for enhanced transmission across inflamed mucosa. We conclude that our SHIV-C model recapitulates the increased mucosal HIV transmission noted in humans with oral lesions [41, 42].

**CD4+ lymphocyte depletion during acute SHIV-C infection.** To assess SHIV-1157/ipd3N4 pathogenicity, blood, lymph node, and rectal biopsies were performed on Indian macaques at weeks 5 and 12 after inoculation. Significant depletion of CD4+ T cells was noted in all 3 compartments in infected macaques, compared with uninfected macaques (*n* = 8; *P* < .05; Mann-Whitney test; Figure 4A–4C). Similar degrees of gut CD4 T cell depletion have been noted during acute infection with another R5 SHIV-C [26].

**Progression to AIDS in mucosally infected macaques.** During acute infection (within 12 weeks after inoculation), gradual depletion of peripheral blood CD4+ memory T cells (assessed by CD4+CD29+ double-staining) was noted in 2 of the 21 systemically infected macaques in different titration groups. Rapid disease progression did not occur. Of 5 chronically infected Indian-origin macaques followed up long term to estimate SHIV-1157/ipd3N4 pathogenicity, monkey RJs-10 developed AIDS at week 102, and 1 of five Chinese-origin macaques (RQ3911) developed AIDS at week 142 (Figure 4D, 4E). This animal also had severe thrombocytopenia. At necropsy 20 weeks later, streptococcal endocarditis and multiple focal hemorrhages were found. Two additional Chinese macaques had CD4 cell counts <500 cells/µL, which indicates progressive disease in 3 of the 5 macaques. These pilot data suggest that SHIV-1157/ipd3N4 is pathogenic in macaques, with gradual disease progression, which is similar to progression of HIV-1 infection in humans.

**DISCUSSION**

Our primate model data show that (1) SHIV-1157/ipd3N4 is transmissible across all mucosal routes tested; (2) relative mucosal permeability was rectal, followed by vaginal, followed by oral, mirroring the risk order of sexual HIV acquisition among humans; (3) SHIV-1157/ipd3N4 was transmissible across inflamed buccal mucosa but not across normal buccal mucosa; and (4) SHIV-1157/ipd3N4 showed signs of pathogenicity during acute infection and caused gradual progression to AIDS. Our R5 SHIV-C mucosal transmission data contrast with our earlier rhesus macaque study involving SIVDeltaB670, where the oral route was 60 times more permissive than the rectal route [14], an unexpected result we ascribe to expanded SIVDeltaB670 coreceptor usage. In contrast, SHIV-1157/ipd3N4...
soley uses CCR5. Accordingly, this R5 SHIV-C better models the HIV-1 strains typically transmitted sexually among humans. Indeed, the permeability of intact macaque mucosae to SHIV-1157ipd3N4 was rectal, followed by vaginal, followed by oral, a pattern that not only followed the rank order but also fell within the ranges extrapolated from HIV-1-exposed humans [9–11, 31–36] (reviewed in [1–3, 37]). These findings attest to the biological relevance of our new R5 SHIV-C primate model.

Estimating the relative risks of HIV-1 acquisition resulting from exclusive orogenital contact among humans is difficult; not surprisingly, a recent survey [1] was unable to perform a metaanalysis of earlier reports. The complexity of human sexual practices makes it difficult to study sufficiently large numbers of individuals whose only risk of sexual HIV-1 acquisition is orogenital exposure. Assessing the route of HIV-1 acquisition depends on recall, which may be inaccurate and underestimate the influence of sexual practices known to be high risk, such as lack of condom use for rectal intercourse. Several human cohort studies reported no cases of HIV-1 seroconversion attributable solely to orogenital contact (reviewed in [1]); the only quantitative risk-per-exposure estimate we could locate in the literature was a risk of 0.4 infections per 1000 exposures [36]. Consequently, human epidemiological studies would have to enroll very large cohorts to more accurately estimate the relative risks of oral exposure relative to vaginal and rectal HIV-1 exposure. In contrast, primate model studies allow stringent control of virus dose, strain and tropism, timing, mucosal route and status of mucosal tissues. Our R5 SHIV-C primate model system can address basic questions of mucosal permeability to a virus encoding HIV-1 env with the tropism typical of that of sexually transmitted HIV-1. As such, our data confirmed that the oral route carried the lowest risk, but the difference between oral exposure and vaginal exposure was <10-fold in the absence of mucosal trauma or inflammation.

Possible sites of virus entry after oral challenge and subsequent viral dissemination have been evaluated elsewhere in SIV/macaque models [17, 43]. When concentrated SIV was swabbed directly onto tonsils, rapid infection ensued at this site, followed by spread to local and regional lymph nodes [43]. A subsequent study assessed initial virus target tissues and the rapidity of virus dissemination in infant and juvenile macaques upon repeated high-dose SIV challenge with buccal mucosa, gingiva, and tonsils, followed by swallowing of the inoculum [17]. Postmortem analysis 1 day after this method of virus exposure revealed high SIV DNA copy numbers in gingival tissues, esophagus, submandibular and peripheral lymph nodes, and Peyer’s patches. Given the number of SIV-positive tissues, the initial portal(s) of viral entry remain unclear. To test whether local inflammation enhanced transmission, our study limited R5 SHIV exposure to buccal mucosa. The increased transmission with inflamed buccal mucosa we observed is compatible with clinical observations that the presence of oral ulcers is associated with seroconversion after oral HIV exposure [41, 42]. Given the infiltration of CD4+ cells into inflamed buccal mucosa in our macaques, the increased presence of viral target cells probably accounted for enhanced SHIV-C transmission.

Several MHC class I alleles have been linked to control of chronic SIV/HIV infection. Previous studies have shown that Mamu-A*01+ Indian rhesus macaques better controlled chronic SIVmac infection and survived longer than animals without this allele (reviewed in [44]). In our study, 4 of 27 Indian macaques were Mamu-A*01+. We found no correlation between Mamu-A*01 status and susceptibility to de novo infection or viral set points. In fact, some Mamu-A*01+ animals had high viral RNA levels during acute infection. To our knowledge, no link has been established between MHC class I alleles and primate susceptibility to lentiviral acquisition. A priori, we would not expect to find any correlation, because MHC class I presentation of viral antigenic peptides can only occur after productive infection of the host has taken place.

Acute infection is associated with marked depletion of CD4+ memory T cells, primarily in mucosae in SIV-infected macaques and HIV-infected humans [45, 46]. SHIV89.6P and X4 SHIVs predominantly target and destroy naive CD4+ T cells, leading to their rapid loss in peripheral blood [47]. In contrast, during R5 SHIV-1157ipd3N4 infection, an initial decline in peripheral blood memory T cells was followed by a gradual loss in the absolute number of CD4+ T cells, a pattern described for other R5 viruses [26, 47]. AIDS has occurred in 2 SHIV-1157ipd3N4-infected macaques to date. Gut CD4+ cell depletion, together with the gradual T cell depletion in blood, mimic HIV disease progression in humans. Of note, another R5 SHIV, SHIVSF162P3, also gradually induced AIDS in some but not all infected macaques [48].

In summary, SHIV-1157ipd3N4 exhibits biological characteristics that parallel many aspects of HIV-1 transmission and pathogenesis in humans. This R5 SHIV-C could be a biologically relevant tool to assess mechanisms of mucosal transmission, including the role played by local inflammation and coinfection with other pathogens [23], and it could also be used to assess the protective potential of microbicides or vaccines in macaques of either Indian [49] or Chinese origin against mucosal challenge.

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**References**