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Detection of Simian Immunodeficiency Virus in Semen, Urethra, and Male Reproductive Organs during Efficient Highly Active Antiretroviral Therapy

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ABSTRACT

A number of men receiving prolonged suppressive highly active antiretroviral therapy (HAART) still shed human immunodeficiency virus (HIV) in semen. To investigate whether this seminal shedding may be due to poor drug penetration and/or viral production by long-lived cells within male genital tissues, we analyzed semen and reproductive tissues from macaques chronically infected with simian immunodeficiency virus mac251 (SIVmac251) who were treated for 4 months with HAART, which was intensified over the last 7 weeks with an integrase inhibitor. We showed that a subset of treated animals continued shedding SIV in semen despite efficient HAART. This shedding was not associated with low antiretroviral drug concentrations in semen or in testis, epididymis, seminal vesicles, and prostate. HAART had no significant impact on SIV RNA in the urethra, whereas it drastically reduced SIV RNA levels in the prostate and vas deferens and to a lesser extent in the epididymis and seminal vesicle. The only detectable SIV RNA-positive cells within the male genital tract after HAART were urethral macrophages. SIV DNA levels in genital tissues were not decreased by HAART, suggesting the presence throughout the male genital tract of nonproductively infected cells. In conclusion, our results demonstrate that 4 months of HAART induced variable and limited control of viral infection in the male reproductive organs, particularly in the urethra, and suggest that infected long-lived cells in the male genital tract may be involved in persistent seminal shedding during HAART. These results pave the way for further investigations of male genital organ infection in long-term-treated infected individuals.

IMPORTANCE

A substantial subset of men receiving prolonged HAART suppressing viral loads in the blood still harbor HIV in semen, and cases of sexual transmission have been reported. To understand the origin of this persistence, we analyzed the semen and male reproductive tissues from SIV-infected macaques treated with HAART. We demonstrated that persistent seminal shedding was not linked to poor drug penetration in semen or semen-producing prostate, seminal vesicle, epididymis, and testis. We revealed that HAART decreased SIV RNA to various extents in all male genital organs, with the exception of the urethra, in which SIV RNA$^+$ macrophages were observed despite HAART. Importantly, HAART did not impact SIV DNA levels in the male genital organs. These results suggest that infection of male genital organs, and particularly the urethra, could be involved in the release of virus in semen during HAART.

Approximately 35.3 million people worldwide are living with human immunodeficiency virus (HIV)/AIDS, and 2.3 million new infections occur annually (1). Over 80% of these new infections are caused by unprotected sexual intercourse. The risk of sexual transmission of HIV is strongly correlated with HIV RNA levels in genital secretions (2). Because semen is the most common vector of HIV dissemination worldwide (3–5), preventing HIV transmission by semen is a public health priority. Highly active antiretroviral (ARV) therapy (HAART), although not eradicating the virus, does greatly and rapidly reduce blood and genital secretion viral loads (usually within 1 month) in a majority of patients, leading to undetectable levels in standard assays (6–8). However, a subset of individuals receiving HAART continue to have detectable virions and infected cells in their semen for months to years after therapy initiation. This persistent virus release in semen occurs despite an undetectable blood HIV RNA load and an absence of detectable sexually transmitted infections (STIs), the latter being a well-identified factor favoring HIV shedding in semen irrespective of HAART (9–11). HIV RNA has been

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evidenced in the semen of 3% to 48% of chronically infected men under suppressive HAART for over 6 months with no detected STIs (6, 12–19) (see additional references in reference 20). Levels of HIV RNA detected during HAART are often low; nevertheless, high levels of shedding (i.e., >1,000 RNA copies/ml) have been described in a substantial subset of individuals (6, 14, 15, 17–19). This level has been associated with heterosexual transmission events (2) and increased risk of HIV transmission *in vitro* (21). Furthermore, rectal transmission in macaques has been shown to occur with a virus level much lower than that needed for vaginal transmission (22), suggesting that even low levels of HIV in semen may lead to transmission through the rectum. In addition to viral RNA (vRNA) in the cell-free fraction of semen, infected leukocytes harboring HIV DNA and capable of productive infection have been detected in the semen of men receiving HAART (7, 23–26). Recent evidence indicates that HIV-infected cells in genital secretions could play a role in the sexual transmission of HIV (27–29). Although HAART in the infected partner very significantly reduces heterosexual transmission at the population level, a risk for residual transmission remains (30–32). Importantly, the transmission probabilities are higher in men having sex with men (MSM) than in heterosexual couples (33, 34). The risk of HIV transmission in MSM despite HAART in the infected partner has never been assessed in large cohorts and is currently under study (35). As a matter of fact, cases of sexual transmission from men under suppressive HAART have been reported (36, 37), and a resurgence of the HIV-1 epidemic among MSM is occurring in countries where potent HAART is available (38).

In this context, understanding the origins of persistent HIV shedding in semen despite efficient HAART is of prime importance. Sequences derived from HIV RNA recovered from the semen of men under suppressive HAART were found to be distinct from those from blood (6). This is similar to the compartmentalization of viral strains between blood and semen described in a subset of therapy-naive men (39–41) and macaques (42). Such a compartmentalization indicates that HIV/simian immunodeficiency virus (SIV) contaminating the semen of those individuals arises from the production of viral strains within the male genital tract (MGT) with distinct evolution from blood strains. We and others have revealed that macrophages and $CD4^+$ T cells present in semen-producing organs (testis, epididymis, prostate, seminal vesicle) of therapy-naive men and macaques during the chronic stage are infected by HIV/SIV and may efficiently seed the semen with free viral particles and infected cells (43, 45–48, 100). Therefore, viral persistence in the semen may be attributable to viral production within the MGT organs, either because of incomplete penetration of antiretroviral drugs and/or because of the infection of long-lived cells in those tissues. Because of the great difficulty in accessing MGT organs from men shedding HIV in semen despite HAART, this hypothesis has never been explored. Our study is the first to analyze both semen and MGT tissue infection during HAART, taking advantage of the SIVmac251-infected cynomolgus macaques. This animal model not only offers the unique opportunity to access semen and MGT organs but also to control drug intakes and tissue distribution. Importantly we and others recently validated its relevance to HIV pathogenesis in the male genital tract (29, 42, 45).

We focused on the measurement of viral loads and drug concentrations in semen and MGT organs after 4 months of standard HAART intensified over the last 7 weeks with raltegravir, as well as on the characterization of SIV target and infected cells in selected MGT organs from untreated and HAART-treated animals. We showed persistent shedding of SIV RNA in the semen of a subset of animals, despite HAART efficiency in blood. The persistent shedding in semen did not correlate to drug concentrations in semen and testis, epididymis, seminal vesicle, and prostate. Using quantitative reverse transcription (RT)-PCR and *in situ* hybridization combined with immunofluorescence, we found that HAART decreased SIV RNA levels to various extents in all MGT organs with the exception of the urethra. The urethra was the only MGT organ encompassing SIV RNA^+ cells after HAART, which we identified to be macrophages. We also discovered that SIV DNA levels in MGT organs are not affected by HAART. Our results suggest that long-lived infected cells in MGT organs, in particular the urethra, could be involved in persistent viral shedding during HAART.

MATERIALS AND METHODS

Ethics statement. Adult cynomolgus macaques (*Macaca fascicularis*) imported from Mauritius were housed in the facilities of the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA; Fontenay-aux-Roses, France). Nonhuman primates (NHP) are used at the CEA in accordance with French national regulation and under national veterinary inspectors (CEA permit number A 92-032-02). The CEA is in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals of the Office for Laboratory Animal Welfare (OLAW; United States) (49) under OLAW assurance number A5826-01. The use of NHP at CEA is in accordance with the recommendation of the newly published European directive (2010/63, recommendation Nu9) (50). Animals were housed in adjoining individual cages allowing social interactions, under controlled conditions of humidity, temperature, and light (12-hour light/12-hour dark cycles). Water was available *ad libitum*. Animals were monitored and fed 1 or 2 times daily with commercial monkey chow and fruits by trained personnel. Macaques were provided with environmental enrichment, including toys, novel foodstuffs, and music under the supervision of the CEA Animal Welfare Body. The protocols employed were approved under statement number 10-060 (13 November 2012) by the ethical committee of the CEA Comité d'Ethique en Expérimentation Animale, registered with the French Research Ministry under number 44. The animals were used under the supervision of the veterinarians in charge of the animal facility. Experimental procedures were conducted after animal sedation with ketamine chlorhydrate (Rhone-Merieux, Lyon, France; 10 mg/kg of body weight) as previously described (51). Tissues from the MGT were collected during animal necropsy at the end of the treatment after sedation of animals (ketamine chlorhydrate, 10 mk/kg) followed by euthanasia (sodium pentobarbital, 180 mg/kg).

Animals, infection, and treatment. Fourteen adult male cynomolgus macaques (3 to 4 years old, body weight of 5 kg, all mature as attested by the presence of full spermatogenesis) were intravenously inoculated with 5,000 50% animal infectious doses (AID_{50}) of pathogenic cell-free SIVmac251 as previously described (51). Six macaques (OKU6, OBCY5, OBRG6, OBCA5, 30235, and 23014) were given two reverse-transcriptase inhibitors, (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA; i.e., tenofovir [TFV]; 20 mg/kg) and emtricitabine (FTC; 50 mg/kg), once a day subcutaneously, as well as the protease inhibitor lopinavir (LPV; 36 mg/kg) boosted with ritonavir (RTV; 12 mg/kg) orally. This treatment was initiated 47 weeks postinoculation, administered for 17 weeks, and intensified during the last 7 weeks with an integrase inhibitor, raltegravir (RAL; 200 mg/animal) orally. The others eight macaques (20351, 30675, 19554, 30855, 30569, 29965, 30717, and 30838) were untreated.

Specimen collections and blood and seminal viral load measurement. Blood and semen were periodically collected throughout the infection, and at the time of euthanasia, blood plasma viral loads (PVL) and semen viral loads (SVL) were assessed as previously described (29). The detection limit was estimated at 12 copies/ml in both blood and semen

samples. Tissues (testes, epididymides, vas deferens, seminal vesicles, prostate, penile urethra, lymph nodes, and spleen) were collected immediately after euthanasia and exsanguination of the animals, extensively washed, and cut into fragments weighing about 300 mg each. The fragments were either stored at −80°C or fixed in 4% formaldehyde.

Protocol for SIV DNA and RNA quantification in tissues. Total DNA and RNA were extracted from frozen tissues using the QIAamp DNA tissue minikit and the RNeasy isolation kit (both Qiagen), respectively. Tissue DNA and cDNA from MGT organs and spleen were subjected to a preamplification step followed by a quantitative real-time (TaqMan) PCR step, as previously described (52, 53), with the following modifications. The two primer sets for *gag* and *actin* were the same for the preamplification and real-time PCR steps (SIV*gag*-F, GCCAGGATTTCAGGCACTGT; SIV*gag*-R, GCTTGATGGTCTCCCACACAA; *Act*-F, TACAGCTTCACC ACCACGG; *Act*-R, TGCTCGAAGTCTAGGGCGA). The preamplification PCR mixture contained both pairs of primers (multiplex PCR), and the reaction mixture consisted of 5 μM deoxynucleoside triphosphates (dNTPs), 1 U of *Taq* polymerase, 10 μl of Qiagen 10× buffer, and 25 nM each primer in a total volume of 100 μl. The PCR settings were 94°C for 3 min, followed by 20 cycles of 94°C for 45 s, 56°C for 2 min, and 72°C for 1 min 30 s. A total of 10 μl of the product of the first PCR was subsequently used as a template in the second real-time PCR amplification, performed on ABI 7500 using commercially available master mix, 500 nM the specific primers indicated above, and 200 nM target probe, MGB (AAGGTTGCACCCCCTATGACATTAATCAGATGTTA [SIV*gag*]) or 6-carboxytetramethylrhodamine (TAMRA; AATCGTGCGTGACATTA AGGAGAAGCTGTG [*actin*]). For RNA quantitation, the eluted RNA samples were first subjected to DNase treatment (Promega) to remove contaminating DNA and subsequently submitted to reverse transcriptase reactions using the high-capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer's instructions.

The protocol for real-time PCR quantification and the preamplification step was validated as follows. Standard curves for SIV*gag* and *actin* copy numbers were built with serial dilutions of a pCRII-TOPO plasmid DNA (Invitrogen) in which the SIV*gag* and the *actin* regions of interest were inserted (45). The same efficiency of amplification and a linear dynamic range of 10 to 10^{10} copies were observed for the two regions of interest (see Fig. S1A in the supplemental material). SIV*gag* and *actin* copy numbers in unknown samples were systematically inferred by plotting the threshold cycle (C_T) value against this calibration curve (see Fig. S1A). The amount of input DNA or cDNA was normalized through *actin* quantification for each PCR. We assessed that the preamplification efficiencies for SIV*gag* and cellular *actin* were nearly identical, by comparing the slopes of the C_T values to cycles of preamplification curves (10, 15, 20, and 25 cycles of preamplification tested), the ΔC_T ($\Delta C_T = C_T$SIV*gag* − C_T*actin*), and the $\Delta\Delta C_T$ values ($\Delta\Delta C_T = \Delta C_T$xcycles − ΔC_T0cycle) (see Fig. S1B). The efficiencies between two targets are considered equal when the $\Delta\Delta C_T$ is in the range between −1.5 and +1.5 (61); the $\Delta\Delta C_T$ values obtained with our samples were all in this validating range. The preamplification factor was determined by quantifying *actin* and *gag* by real-time PCR performed before and after the preamplification step. The same preamplification factor was observed for the two targets.

We chose to run 20 cycles of preamplification in order to enhance the possibility of detecting SIV infection in low-infected samples from treated animals, without affecting the efficiency of the real-time PCR step with a too high input of template. We assessed that no PCR inhibition occurred with the amount of template input by comparing the results for *actin* and SIV*gag* for a range of preamplification cycles (see Fig. S1B) and of template dilutions (see Fig. S1C). For *actin* quantification, the first PCR product was diluted 1/1,000 to obtain C_T in the middle of the curve (see Fig. S1C). The reproducibility of the 2-step protocol was assessed by performing 4 preamplifications followed by quantitative PCR on DNA and RNA (see Fig. S1D). To increase the chances of detection of focal infection of the testis, epididymis, prostate, and seminal vesicle, as we and others have previously described (45, 52, 54), two independent fragments of each tissue were assayed in 4 preamplification PCRs, each performed on 500 ng of extracted DNA or on 200 ng of reverse-transcribed RNA. Regarding penile urethra, one fragment was tested, as only a limited quantity of tissue was available because of the small size of this duct in macaques. The lack of contamination in PCR assays was systematically ensured by concurrently running a minimum of 4 negative controls. For all assays, no positive signals were detected in the negative controls or in the non-reverse-transcribed (–RT) controls for RNA assays. Quantification of SIV DNA in lymph nodes was performed as previously described (55). Results were expressed as SIV*gag* copy numbers per copy of *actin* for both vRNA and viral DNA (vDNA). The presence of a mix of haploid and diploid cells in the testis and epididymis prevented extrapolation of actin copy number to cell number for vDNA quantification. We validated *actin* as a reference gene for RNA quantification among MGT organs (see Fig. S1E) as follows. Equivalent volumes of reverse-transcribed product corresponding to 5 ng equivalent RNA extracted from MGT organs from 3 to 15 animals were subjected to real-time PCR amplification performed in a 25-μl total volume as described above. The PCR settings were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The specific primers and TAMRA probes for actin were as described above. Those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and peptidylpropyl isomerase A (PPIA) were as previously described (56). The relative expression of each mRNA was calculated by the $2^{-\Delta\Delta CT}$ method (61), where ΔC_T is obtained for each sample by subtracting the C_T value for each gene from a reference gene C_T value, and the $-\Delta\Delta C_T$ is obtained by subtraction of the ΔC_t from one sample from the ΔC_t of a reference sample. The prostate from one macaque was used for all assays as the reference C_T.

SIV DNA detection in semen. Quantification of DNA in semen (seminal cell fraction) performed as described above was not possible due to the too low DNA quantity rescued from the cell pellet tested. DNA extracted from the seminal cell pellet was thus subjected to a qualitative nested PCR amplifying SIVmac251 *env*, with primer pairs previously described by and conditions adapted from Keele et al. (57). Briefly the first round of PCR was performed on 30 ng of template in 25 μl of PCR mix containing 0.2 mM deoxynucleoside triphosphates (Promega), 1 U of Phusion *Taq* (New England Biolabs), and 300 nM each primer in 1× Phusion *Taq* buffer (New England Biolabs). The PCR settings were as follows: 98°C for 45 s, followed by 35 cycles of 98°C for 15 s, 55°C for 30 s, and 72°C for 6 min. A total of 1 μl of the product of the first PCR was subsequently used as a template in the second nested PCR performed in 25 μl of PCR mix as described above and with the same PCR settings but for 40 cycles. The product of the second PCR was then run on a 1.2% agarose gel and analyzed.

ISH for SIV RNA and phenotyping of infected cells. Formalin-fixed, paraffin-embedded tissues were assayed for SIV RNA expression using a digoxigenin-labeling technique (58, 59) modified as we previously described (54). The digoxigenin-UTP-labeled riboprobe that was used spans the whole genome of SIVmac239 (Lofstrand Labs, Gaithersburg, MD, USA). Nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate toluidinium (NBT-BCIP) was used to visualize infected cells in the tissues of treated and untreated animals. The specificity of the hybridization signal was systematically checked by hybridizing sense probes with parallel sections and antisense probes on uninfected genital tissues. Positive controls (MGT tissue from a highly infected macaque and lymphoid tissue from an untreated animal) were run concomitantly. *In situ* hybridization (ISH)-stained tissues were visualized and photographed with an Olympus Proxis microscope and a Zeiss AxioCam ICc1 camera. A minimum of 30 sections/organ/animal were analyzed.

Characterization of infected cells in genital tissues was performed using ISH combined with immunostaining for cell markers, as described before (54, 59). Briefly, sections were treated with methanol-hydrogen peroxide, and the hybridized tissues were incubated with sheep anti-digoxigenin peroxidase (SAD-POD; Roche Molecular Biochemicals) for 1 h at room temperature. SAD-POD was detected by a fluorescent tyramide signal amplification (TSA) technique (TSA plus fluorescein isothiocya-

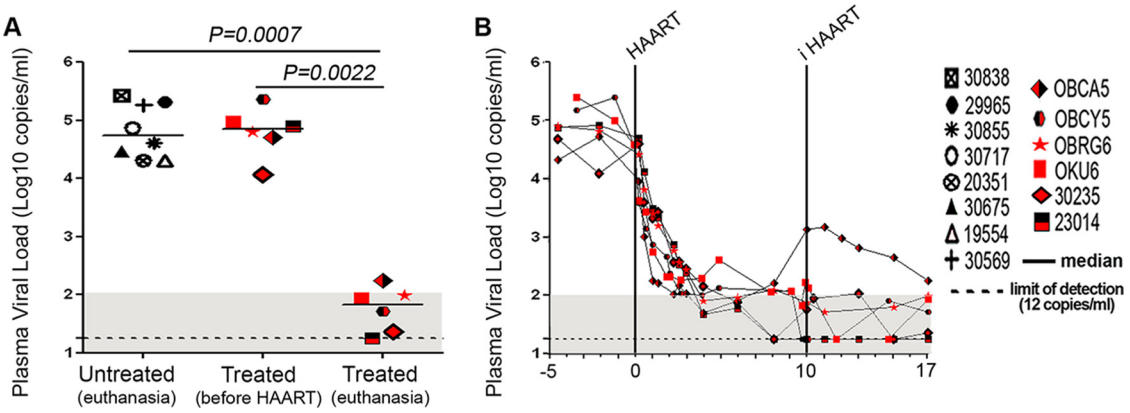


FIG 1 Impact of treatment on viral load and viral dynamics in the blood. (A) Comparison of blood viral loads in untreated ($n = 8$) and treated ($n = 6$) macaques before and at the end of treatment; (B) longitudinal follow-up of blood viral loads in treated animals ($n = 6$) before and over the course of antiretroviral treatment. Vertical lines indicate the initiation of therapy (HAART) and of intensification (iHAART). Each symbol represents one macaque. Statistical analysis with nonparametric test: Mann-Whitney test (untreated versus treated) and Wilcoxon signed-rank test (treated before HAART versus treated at euthanasia). Dotted lines represent the detection limit of the assay for vRNA (12 copies/ml). The gray areas delineate plasma viral loads of ≤100 copies/ml.

nate [FITC], NEL741; PerkinElmer). Following ISH assay, the sections were incubated with a combination of two mouse monoclonal antibodies against CD68 (clone KP1, 4 μg/ml; Dako) and CD163 (clone 10D6, 0.5 μg/ml; Novocastra) and with a monoclonal rabbit anti-human CD3 antibody (SP7, 1:100; Thermo Scientific). Sections were subsequently stained with a goat Alexa-594 anti-mouse IgG antibody (4 μg/ml; Invitrogen BP) and a goat Alexa-633 anti-rabbit IgG antibody (4 μg/ml; Invitrogen BP). Positive (MGT tissue from a highly infected animal) and negative (hybridization with a sense probe on parallel sections of infected animals and hybridization with an antisense probe on MGT tissue from an uninfected animal) controls were systematically run. Additional controls for TSA amplification included mock hybridization (no probe) and hybridization in the absence of primary antibodies. The double-stained sections were mounted in Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole; Vectashield, Vector Laboratories, Ltd.). Images were acquired with a Zeiss AxioImager M1 microscope equipped with 4 filters and analyzed with AxioVision Rel 4.7.1 and ImageJ software. The final images were generated with Adobe Photoshop CS2. A minimum of 30 sections/organ/animal were analyzed.

Immunohistochemistry. The following antibodies and matching isotype controls were used at the indicated concentrations: mouse monoclonal anti-HLA-DR (clone TAL.1B5, 0.6 μg/ml; Dako), anti-CD68 (clone KP1, 4 μg/ml; Dako), anti-CD163 (clone10D6, 0.5 μg/ml; Novocastra), anti-CD4 (clone 1F6, 2.5 μg/ml; Novocastra), anti-TIA-1 (clone 2G9, 1 μg/ml; Immunotech), anti-MAC387 (1.42 μg/ml; Dako), anti-DC-SIGN (2 μg/ml; R&D Systems), anti-CD20 (clone F7.238, 0.44 μg/ml; Dako), rabbit monoclonal anti-CD3 (SP7, 1:100; Thermo Scientific), mouse IgG1 isotype control (Dako), and rabbit IgG isotype control (Jackson ImmunoResearch). Alexa-633, Alexa-594, or biotin-conjugated secondary antibodies were purchased from Invitrogen and Dako. Immunohistochemistry was performed as previously described (43). No staining was ever observed with isotype control antibodies. For quantitative and semiquantitative measurement, slides were scanned with Nanozoomer NDP (Hamamatsu), and cell counts were performed using the ImageJ software. For semiquantitative analysis of HLA-DR$^+$ cell foci, 8 untreated and 6 treated animals were included and 4 sections/organ/animal were screened. For quantitative analysis of T lymphocytes and macrophages, counting was undertaken on 3 animals/group, and 2 sections/organ/animal were screened.

Determination of antiretroviral drug concentrations in blood, semen, and tissues. TFV, FTC, LPV, RTV, and RAL were quantified in the treated macaques' seminal fluids. TFV-diphosphate (TFV-DP) and FTC-triphosphate (FTC-TP), the intracellular active metabolites of TFV and FTC, respectively, and LPV, RTV, and RAL concentrations were determined in the testis, the epididymis, the prostate, and the seminal vesicles. All analyses were performed using 2-chloroadenosine and 2-chloroadenosine-5′triphosphate, LPV D8, RTV D6, and RAL D4 as internal standards. A simple protein precipitation was used for seminal fluid and blood plasma sample preparation. Tissue samples were homogenized using a Precellys tissue homogenizer (Bertin Technologies). TFV, FTC, TFV-DP, FTC-TP, LPV, RTV, and RAL from extract samples were quantified using a Waters Acquity ultraperformance liquid chromatography (UPLC) system with a 2.1- by 100-mm, 1.7-μm Acquity UPLC BEH RP18 shield column coupled to a Waters Xevo TQ-MS mass spectrometer operated in positive ion electrospray multiple-reaction-monitoring (MRM) mode. Two chromatographic conditions were used, one for FTC and TFV and another one for RTV, LPV, RAL, TFV-DP, and FTC-TP, both adapted from previously published methods (105, 106).

Data analysis. All data visualization and statistical analyses were carried out with GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA, USA). Nonparametric Spearman's rank correlation tests were used to investigate the relationships between parameters. The nonparametric Mann-Whitney test was used to compare groups of macaques, and the nonparametric Wilcoxon signed-rank test was used to compare data from the same macaques at different time points, before and after HAART treatment. The nonparametric Kruskal-Wallis test, followed by Dunn's test, was used to compare viral loads, drug concentrations, and reference gene expression levels between different tissues or body fluids. The Fisher exact test was used to compare the percentages of SIV-positive animals in the untreated and treated groups. *P* values of 0.05 or less in two-tailed tests were considered significant.

RESULTS

SIV RNA persists in the semen of a subset of animals despite a rapid decrease of viremia following HAART. The chronically infected animals ($n = 14$) included in the study displayed a plasma viral load (PVL) of between 4 and 5.5 $\log_{10}$ copies/ml (11,866 to 263,027 copies/ml) in the absence of treatment (median of 54,954 copies/ml for the untreated group and 70,958 for the treated group before HAART) (Fig. 1A). The treated group was subjected to TFV (PMPA)/FTC/LPV/RTV for 4 months, with intensification with RAL over the last 7 weeks. RAL intensification was designed to study its impact on persistent seminal shedding, as this drug was reported to (i) have good penetration in semen in humans (70), (ii) rapidly decrease viral load in semen as well as HIV

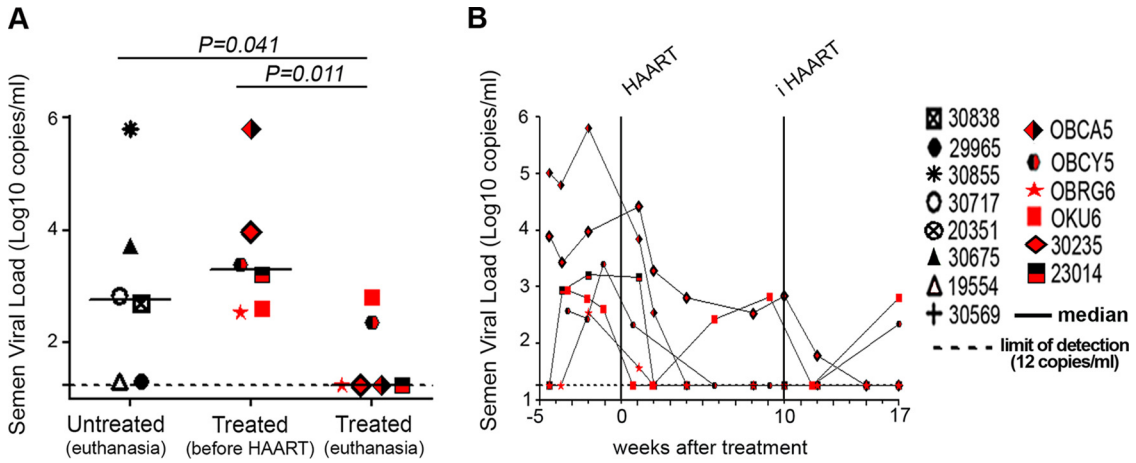


FIG 2 Impact of treatment on viral load and viral dynamics in semen. (A) Comparison of semen viral loads in untreated ($n = 6$) and treated ($n = 6$) macaques before and at the end of treatment; (B) longitudinal follow-up of semen viral loads in treated animals ($n = 6$) before and over the course of antiretroviral treatment. Vertical lines indicate the initiation of therapy (HAART) and of intensification (iHAART). Each symbol represents one macaque. Statistical analysis with nonparametric test: Mann-Whitney test (untreated versus treated) and Wilcoxon signed-rank test (treated before HAART versus treated at euthanasia). Dotted lines represent the detection limit of the assay for vRNA (12 copies/ml).

residual replication (14, 62, 63), and (iii) be effective against SIVmac (64). The initiation of HAART quickly and drastically decreased viremia. All the treated animals went down to a PVL measurement of ≤100 copies/ml at weeks 3 to 8 after the initiation of HAART (threshold indicative of efficient HAART [52, 65–69], delineated in gray on the graph in Fig. 1B). For 5 out of 6 animals, the PVL remained under this threshold with only occasional blips until euthanasia at week 17, going down to below the detection limit of 12 copies/ml in several measurements for 4 animals. One animal (OBCA5) presented a rebound of viremia at week 10. This rebound was neither associated with a lower intake of oral LPV/RTV nor with a lower concentration of antiretrovirals (ARVs) in plasma (see Fig. S2 in the supplemental material). The viremia of this animal declined after RAL intensification (Fig. 1B), to reach 184 copies/ml at euthanasia. At the end of the treatment, the median PVL of macaques subjected to HAART was 67 copies/ml (Fig. 1A).

The median seminal viral loads (SVL) of the treated animals before HAART and of the untreated animals were similar (respectively, 3.3 $\log_{10}$ and 2.8 $\log_{10}$ copies/ml, i.e., 2,056 and 575 copies/ml, $P = 0.47$), whereas SVL significantly decreased after HAART to a median below the limit of detection of the assay (<12 copies/ml) ($P = 0.011$) (Fig. 2A). For half of the animals, 2 to 4 weeks of HAART was sufficient to achieve and maintain an undetectable SVL throughout the treatment, even when high SVL were measured before treatment (Fig. 2B). However, viral shedding was still observed during HAART in the semen of 3 animals displaying PVL below 12 copies/ml in several measurements. In animal 30235, continuous SIV shedding was observed during the nonintensified treatment period but decreased to undetectable levels during intensification with raltegravir (Fig. 2B). Animal OKU6 displayed intermittent seminal shedding and OBCY5 rebounded. For these two macaques, the SVL measured at the end of treatment was discordant with PVL and in the same range as that before treatment (Fig. 2A and B).

HAART has no impact on SIV DNA in MGT organs. We developed a highly sensitive two-step quantitative PCR capable of detecting SIV nucleic acids in tissues with low levels of infection, as described in Materials and Methods. To assess the efficiency of HAART in reducing infection at the tissue level, SIV DNA was first measured by quantitative PCR in axillary lymph node biopsy specimens collected in the treated group of animals before and at the end of the HAART treatment. In all the animals tested, a drastic decrease of axillary lymph node viral DNA (vDNA) level was observed after HAART (Fig. 3A). Using the two-step quantitative PCR, we similarly found significantly lower levels of vDNA in the spleens from treated than from untreated animals, further confirming HAART efficiency within deep tissues (Fig. 3B). In contrast, HAART had no significant impact on the median vDNA levels in the testes, epididymides, seminal vesicles, prostates, vas deferens, and urethras of treated compared to untreated animals (Fig. 3C). However, two treated animals consistently tested negative for SIV DNA in the prostate and one animal in the vas deferens, whereas all untreated animals tested positive in these tissues (Fig. 3C; see Table S1 in the supplemental material). Regarding vDNA in semen, seminal cells from all treated and untreated animals were positive in PCR (see Table S1).

HAART significantly decreases SIV RNA in several MGT organs but has no significant impact in the urethra. We used two-step quantitative PCR to measure viral RNA (vRNA) in the spleen and in the MGT organs, in the presence ($n = 6$) or absence ($n = 8$) of HAART. In the spleen, HAART drastically reduced the median vRNA level by 4.2 $\log_{10}$ ($P = 0.0007$) (Fig. 4A). In the MGT from untreated animals, vRNA levels in the epididymides, seminal vesicles, prostates, and vas deferens were within the same range (Fig. 4B). The testis was among the least productively infected MGT organ, as we previously described (45), whereas the urethra was among the most infected (Fig. 4B). HAART significantly reduced vRNA in the epididymis, seminal vesicle, prostate, and vas deferens by, respectively, 1.4, 1.9, 5, and 4.8 $\log_{10}$ (Fig. 4B). The most profound effect was found in the prostate and vas deferens, with median levels after HAART below the detection threshold (Fig. 4B) and only 33% of animals testing positive for SIV RNA after HAART, as opposed to 100% without HAART ($P = 0.015$) (see Table S1). Although HAART decreased SIV RNA in the testis to a very low median level, it did not reach significance ($P = 0.08$) (Fig.

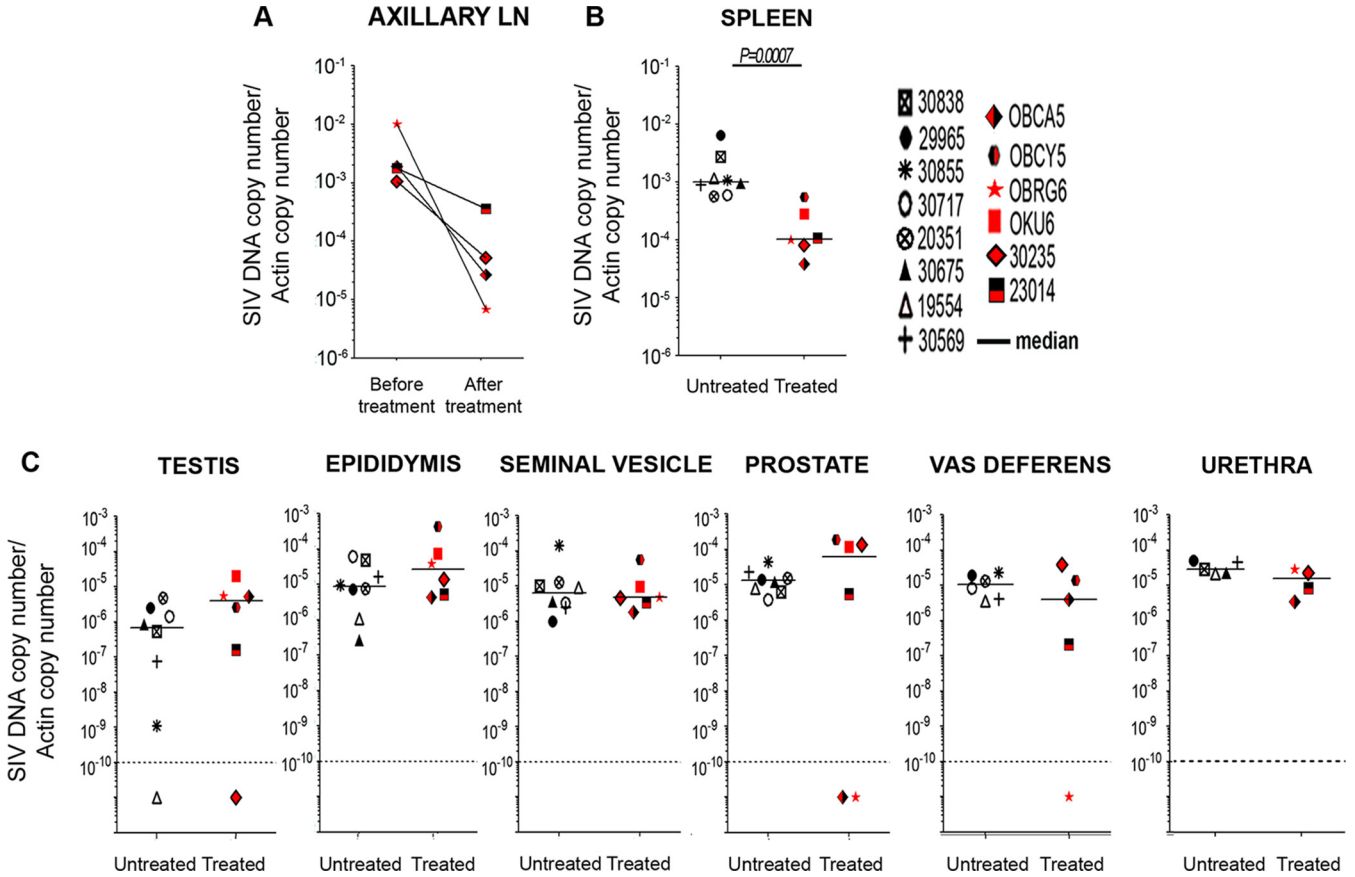


FIG 3 Impact of treatment on SIV DNA levels in lymphoid and MGT tissues. (A) Axillary lymph node biopsy specimens were collected from treated animals before and after 4 months of HAART for SIV DNA quantification. SIV DNA was measured in the spleen (B) and MGT tissues (C) from treated and untreated animals, using a two-step quantitative PCR. Each symbol in the graph represents the mean from two tissue fragments from one animal. Dotted lines represent the detection limit of the SIV DNA quantitative assay. Statistical analysis with a Mann-Whitney nonparametric test.

4B). HAART had no significant impact on vRNA level in the urethra ($P = 0.12$) when comparing the 6 untreated and 4 treated animals for which this tissue was available (Fig. 4B). Of note is that when the other MGT organs from the same animals were compared, vRNA levels were still significantly decreased in the prostate ($P = 0.013$), vas deferens ($P = 0.018$), and seminal vesicle ($P = 0.04$) and became borderline for the epididymis ($P = 0.05$). The decrease remained nonsignificant in the testis ($P = 0.087$).

In situ hybridization experiments demonstrated the presence of SIV RNA$^+$ cells in the lamina propria of the urethra of both treated and untreated animals ($n = 3$ tested in each group), including animal 23014 with viremia consistently below 12 copies/ml (Fig. 5C and D). Further comparative quantification could not be undertaken due to the small tissue surface area of the urethra (the macaque's penile urethra being a small duct) and the rarity of material available. Furthermore, the patchy distribution of the infected cells was another additional intrinsic obstacle to quantification. In contrast to the urethra, we failed to detect SIV RNA$^+$ cells in the testis, epididymis, prostate, and seminal vesicles following HAART despite extensive screening of the animals testing positive in RT-PCR for SIV RNA (minimum of 30 sections/organ/animal) and even though larger tissue surface areas than that of urethral ducts were screened, whereas these organs displayed infected cells in the absence of HAART (as shown for prostate in Fig. 5F), in agreement with our published findings (45, 54). The very low level of vRNA observed through quantitative RT-PCR in these organs after treatment, together with the scattered distribution of infected cells in therapy-naive animals, is likely to account for the lack of detection using *in situ* hybridization.

Antiretroviral drug concentrations in semen, testis, epididymis, prostate, and seminal vesicle do not correlate with viral shedding in semen. Since suboptimal antiretroviral concentrations in semen and/or in some MGT tissues may be responsible for persistent viral production and seminal shedding, we investigated the concentrations of the antiretroviral drugs in semen and four MGT organs using validated ultrahigh-performance liquid chromatography (ultra-HPLC) with mass spectrometry detection. Prior to this, the drug pharmacokinetics and concentrations in the blood of the treated macaques were assessed at different time points, in order to confirm the good systemic levels of the drugs (see Fig. S2 in the supplemental material). In semen, concentrations of all the antiretrovirals measured 24 h after drug administration (C24h) were in the same range as those measured in HIV-1-infected patients under suppressive HAART (70–74) and, as described in other body fluids (75, 76), were well above C24h in blood, suggesting better stability (Fig. 6A and B). Interestingly, the highest drug concentrations in semen were measured for a persistent shedder (OBCY5) (Fig. 6B), indicating that seminal shedding

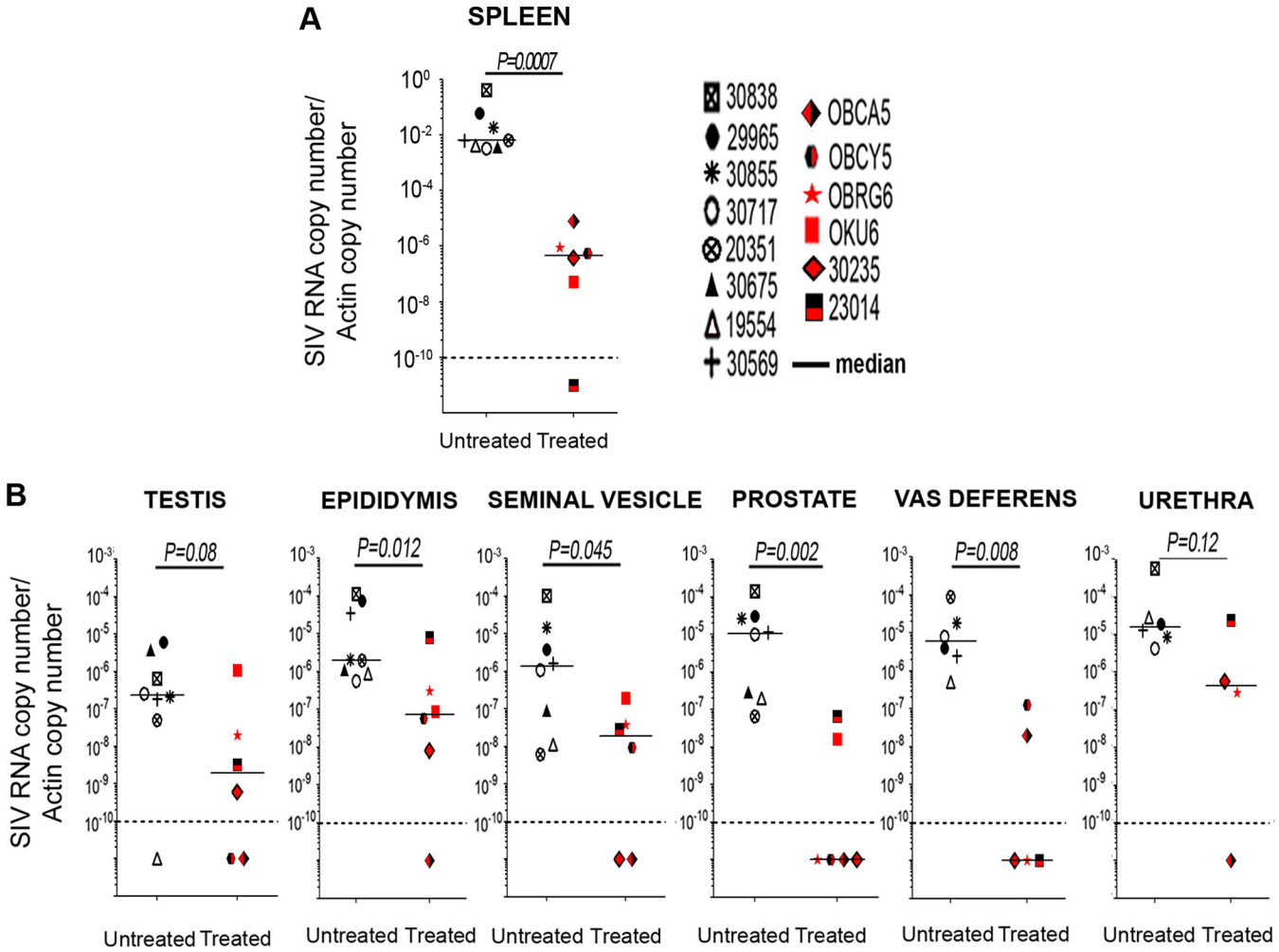


FIG 4 Impact of treatment on SIV RNA levels in spleen and MGT tissues. SIV RNA was measured in the spleen (A) and MGT tissues (B) from treated and untreated animals using a two-step quantitative RT-PCR. Each symbol in the graph represents the mean from two tissue fragments from one animal. Dotted lines represent the detection limit of the SIV RNA quantitative assay. Statistical analysis with a Mann-Whitney nonparametric test.

despite HAART in this animal was not due to lower drug concentrations in semen than in nonshedders. No correlation was found between vRNA levels and individual drug concentrations in semen at the end of HAART ($P = 0.33$, Spearman test; see Table S2 in the supplemental material). This is similar to what was observed in men receiving HAART (73, 77).

We then measured drug concentrations in MGT tissues (TFV-DP, FTC-TP, LPV, RTV, RAL) to determine whether they correlated with seminal shedding and whether differences in drug penetration may explain the differential effect of HAART on MGT tissue infection. We found that the median concentrations of the active phosphorylated form of reverse transcriptase inhibitors (TFV-DP and FTC-TP) were the lowest in the testis and epididymis and the highest in the prostate and seminal vesicles (Fig. 6C). LPV and RAL concentrations were the lowest in testis (Fig. 6C).

For all inhibitors, the prostate was the organ for which the most heterogeneous drug levels were observed among animals (Fig. 6C). However, there was no significant difference among MGT organs for any drug concentration (Kruskal-Wallis–Dunn's test). The small size of the excurrent ducts (i.e., vas deferens and urethra) precluded pharmacological measurements. Importantly, no correlations were observed between drug concentrations in epididymis, testis, and prostate and their tissue vRNA or vDNA levels at the end of 4 months of HAART (Spearman test; see Table S2 in the supplemental material for vRNA). Statistically significant negative correlations between antiretroviral concentrations and tissue SIV RNA level were found only for ritonavir in the seminal vesicles ($P = 0.033$, Spearman $r = -0.89$; see Table S2). No negative correlations were found between the level of ARVs measured in MGT organs and SIV RNA loads in semen (Spearman test; see Table S3 in the supplemental material).

Nature and quantification of SIV target cells in MGT organs with and without HAART. We investigated SIV potential target cells in 3 MGT organs (urethra, epididymis, and prostate) in which HAART had a different effect on SIV RNA levels. The urethra clearly showed numerous macrophages as identified using a combination of antibodies against CD68 and CD163, located in the lamina propria and to a lesser extent in the epithelium (Fig. 7A). $CD4^+$ T lymphocytes were 3- to 6-fold less numerous than macrophages (Fig. 7A) and represented on average 39% of $CD3^+$ cells in the urethra (see Fig. S3 in the supplemental material). Similar numbers of macrophages and $CD4^+$ T lymphocytes were

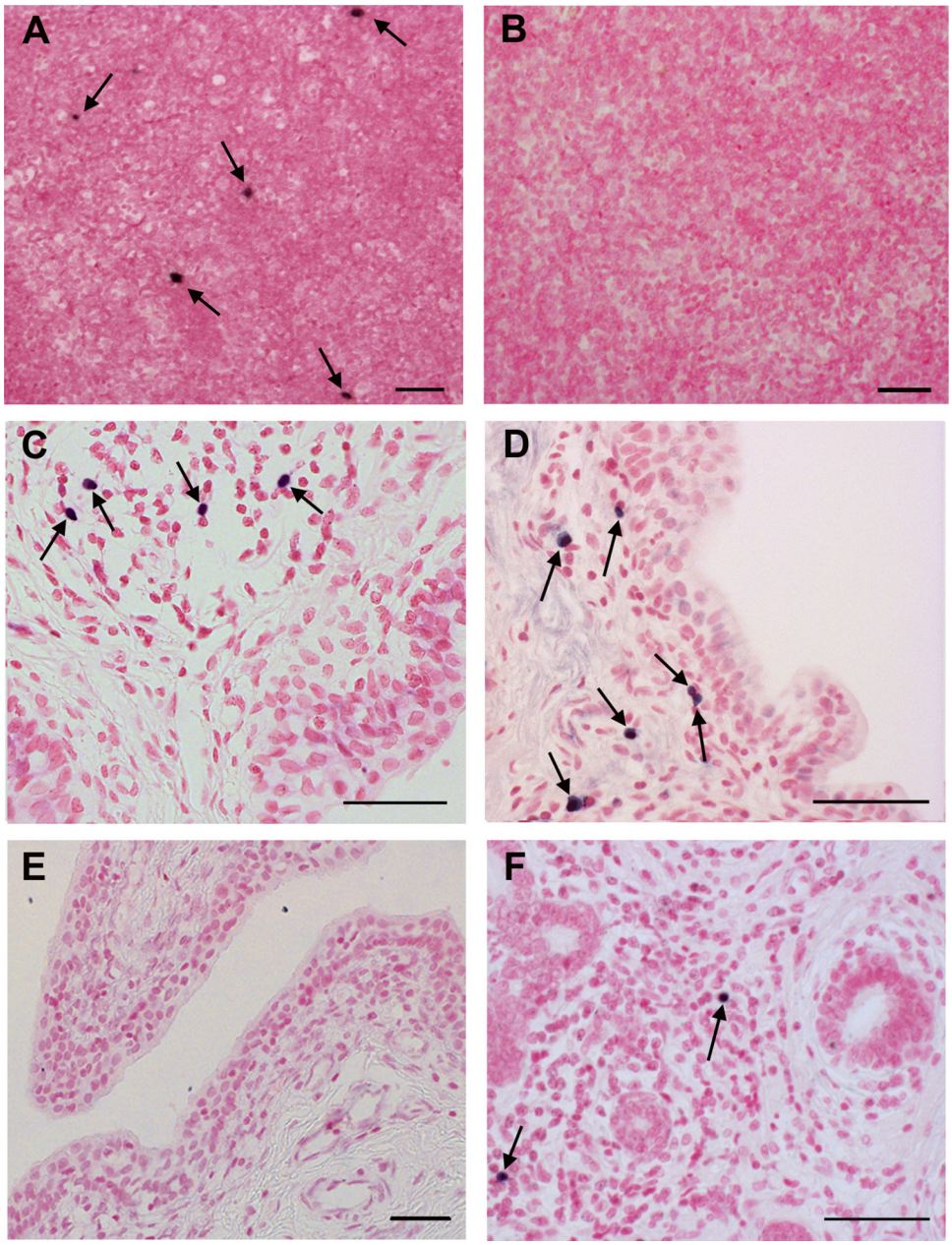


FIG 5 Detection and localization of SIV-infected cells in the MGT tissues by *in situ* hybridization. (A) Positive control, sections of mesenteric lymph node from an untreated animal hybridized with SIV antisense probe showing infected cells (arrows); (B) negative control, parallel sections of mesenteric lymph node hybridized with SIV sense probe showing no signal; urethra from untreated (C) and treated (D) macaques displaying infected cells (arrows); (E) hybridization of parallel sections of urethra with SIV sense probe showing no signal for SIV RNA (negative control); (F) prostate from untreated macaque displaying infected cells (arrows). Scale bar = 50 μm.

found in the urethral tissue from treated and untreated animals (Fig. 7A). In the epididymis, scattered tissue macrophages, located in both stroma and epithelium, were 30- to 60-fold more abundant than $CD4^+$ T lymphocytes (Fig. 7A), which represented on average 60% of $CD3^+$ T lymphocytes (see Fig. S3). In the prostate, similar numbers of macrophages and $CD4^+$ T lymphocytes (50% of $CD3^+$ T lymphocytes [see Fig. S3]) were observed (Fig. 7A). These numbers were unchanged by HAART (Fig. 7A). In addition to these scattered leukocytes and as we previously described (45, 54), $HLA\text{-}DR^+$ immune cell infiltrates composed of potential SIV target cells (i.e., $CD4^+$ T lymphocytes and $CD68^+$ myeloid cells) and other immune cell types (cytotoxic T lymphocytes and B cells) were observed in the epididymis, prostate, and seminal vesicle from infected macaques (see Fig. S4 in the supplemental material). Such cell foci were absent from the urethra and testis, where isolated $HLA\text{-}DR^+$ cells were more homogenously distributed and barely present, respectively (see Fig. S4). To take into account the heterogeneous distribution and size of the infiltrates, the large surface areas of the epididymis and prostate were screened for semiquantitative analysis of $HLA\text{-}DR^+$ cell foci. A lower number

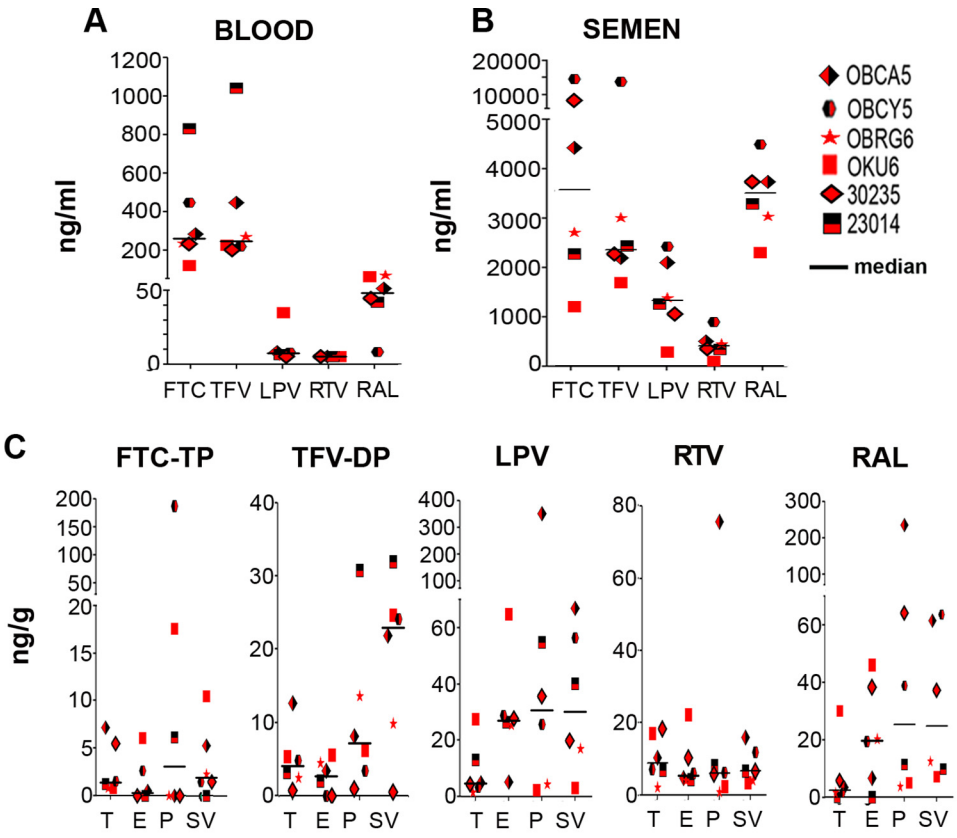


FIG 6 Concentrations of antiretroviral drugs in blood, semen, and MGT tissues. Blood plasma (A) and seminal plasma (B) concentrations of emtricitabine (FTC), tenofovir (TFV), lopinavir (LPV), ritonavir (RTV), and raltegravir (RAL) measured 24 h postadministration (C24h) 120 days post-HAART at the time of euthanasia. (C) MGT tissue concentrations (ng/g) of emtricitabine-triphosphate (FTC-TP), tenofovir-diphosphate (TFV-DP), lopinavir (LPV), ritonavir (RTV), and raltegravir (RAL) in the MGT tissues (T, testis; E, epididymis; P, prostate; SV, seminal vesicles). Each symbol in the graphs represents one macaque.

of immune cell infiltrates, and thus a reduced number of potential SIV target cells, was observed in treated animals than in untreated animals, indicating lower local inflammation (Fig. 7B). Overall, the urethras from treated and untreated animals consistently displayed a much higher proportion of SIV target cells than the epididymides and prostates, even when considering immune cell infiltrates in the latter.

SIV RNA$^+$ macrophages are detected in the urethra post-HAART. We analyzed the nature of SIV RNA$^+$ cells in the MGT tissues using *in situ* hybridization for SIV RNA combined with cell markers. In agreement with our published findings (45, 54), we evidenced the presence of infected T lymphocytes and macrophages in MGT organs from therapy-naive animals (as shown for prostate in Fig. 8G). In contrast to untreated animals, infected CD3$^+$ T lymphocytes were never encountered in MGT organs after treatment. However, we revealed the presence of SIV RNA within CD68/CD163$^+$ macrophages localized in the lamina propria of the urethra from treated animals, including animal 23014 with suppressed viremia (Fig. 8C, E, and F).

DISCUSSION

A growing number of studies are reporting the persistence of HIV RNA and DNA in the semen of a subset of HIV-infected men receiving suppressive HAART in the blood for an extended period of time (reviewed in reference 20). This persistent shedding is believed to arise from local sources within the MGT, but the nature of these sources remains unknown. With the resurgence of the HIV-1 epidemic in countries where HAART is widely available (38), understanding the origin and causes of this viral shedding is an essential step on the path to better treatment. To this end, this study provides the first analysis of semen and a wide range of MGT tissues (testis, epididymis, seminal vesicle, prostate, vas deferens, and urethra) in macaques treated by HAART. SIV RNA and DNA were quantified in semen and MGT tissues and drug concentrations measured. In addition, the nature and impact of the treatment on SIV target cells in male reproductive tissues were analyzed.

Our results reveal that although HAART can lead to undetectable viral load in macaque semen, seminal SIV shedding occurs in a subset of animals despite drastically reduced viremia in the blood, as is seen in humans (reviewed in reference 20). Where the macaque model offers a unique advantage over human patient studies is that it avoids the effects of medication nonadherence and acquisition of other STIs during the course of HAART, which are known to increase HIV release in semen (10, 80). We showed that shedding in semen during HAART is not linked to reduced ARV concentrations in semen. This corroborates the observation of persistent HIV shedding in the semen of men receiving drugs usually achieving good concentrations in semen (6, 9, 17, 18). The intensification of HAART with raltegravir over the last 7 weeks of

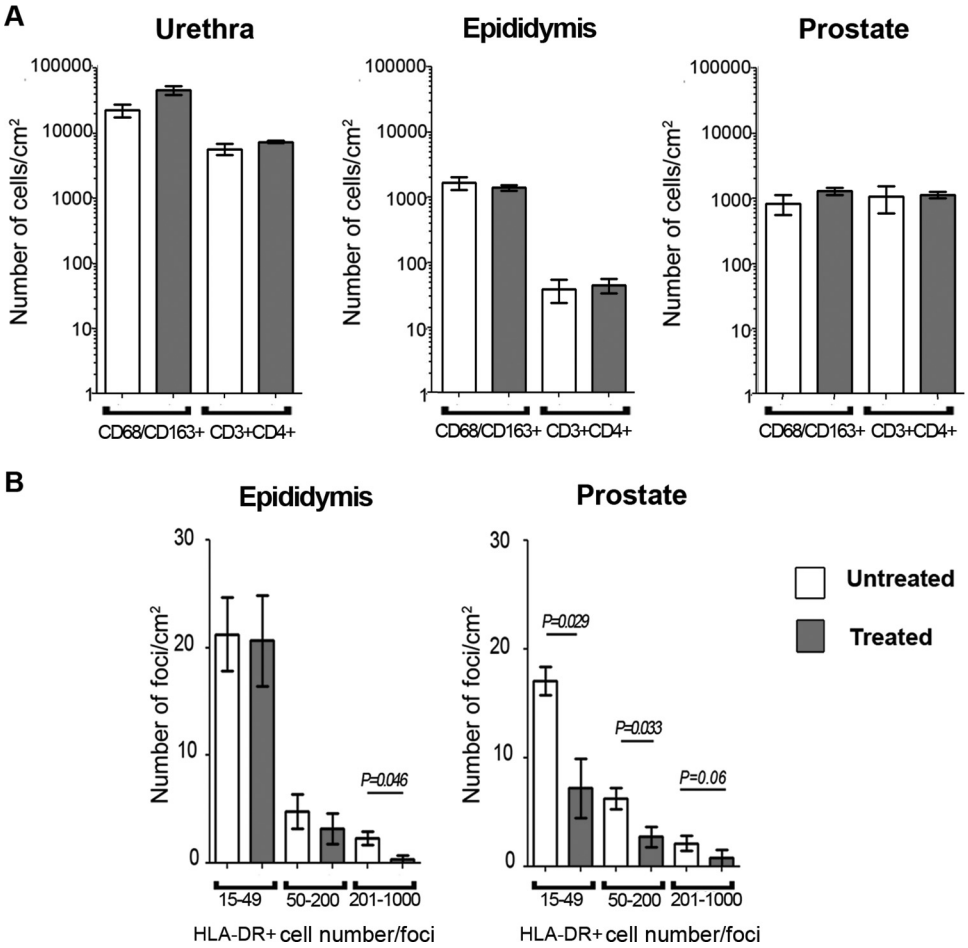
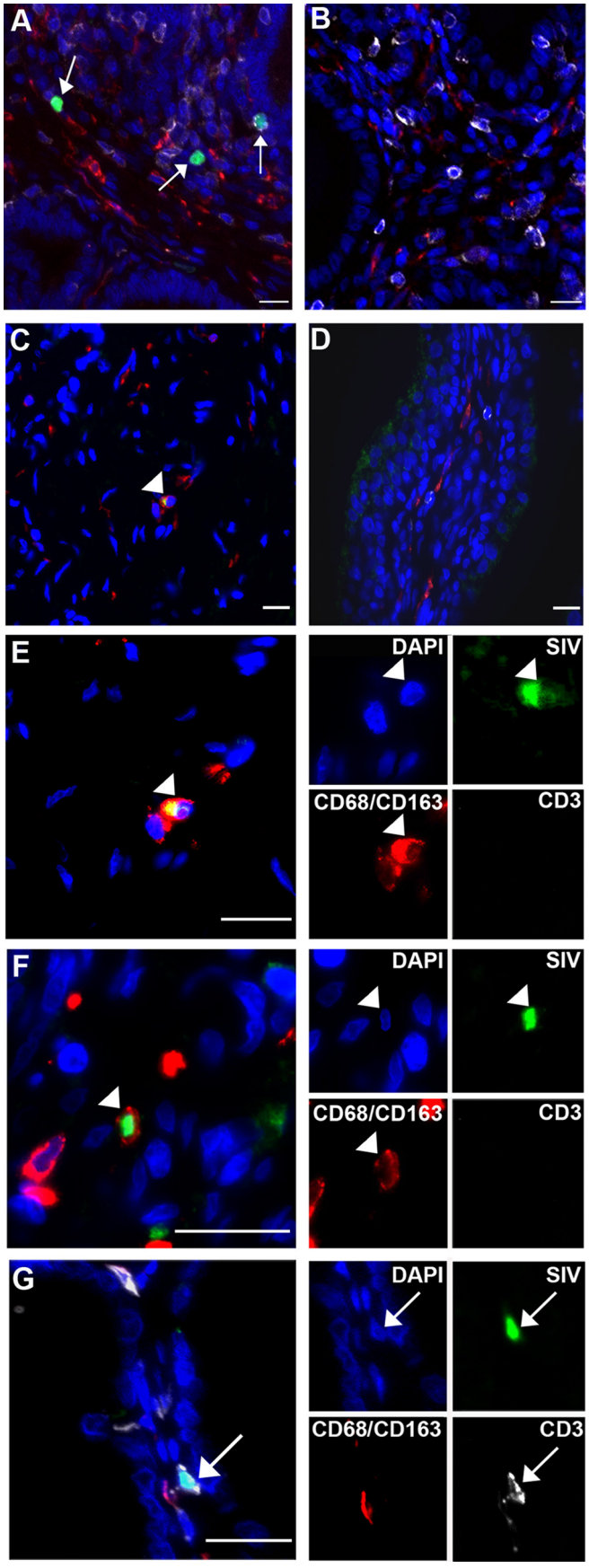


FIG 7 Quantification of SIV target cells and immune cell foci in the MGT tissues. (A) Quantitative analysis of the number of scattered macrophages (identified using a combination of antibodies against CD68 and CD163) and $CD4^+$ T lymphocytes in the urethra, epididymis, and prostate from untreated (white bars) and treated (red bars) animals ($n = 3$ in each group). (B) Semiquantitative analysis of the number of HLA-DR^+ immune cell foci in epididymis and prostate from untreated (white bars, $n = 8$) and HAART-treated (red bars, $n = 6$) macaques. Cell foci were divided into three categories based on the number of cells they encompassed: small foci (between 15 and 49 HLA-DR^+ cells), medium foci (between 50 and 200 cells), and large foci (over 200 cells). Numbers of cells in each focus category are indicated on the *x* axis. Histograms represent the means ± standard errors of the mean (SEM) of the number of cell foci in each category. Statistical analysis with a Mann-Whitney nonparametric test.

treatment was tested in macaques, as it was reported to induce a rapid decrease of semen viral load compared with that of standard HAART in men (14). Whereas raltegravir had no impact on seminal SIV load in the two animals displaying intermittent shedding, SIV was no longer detected at the end of intensified HAART in the semen of a continuous persistent shedder. Although it cannot be ruled out that the decrease in the semen SIV load of this animal was simply following its natural course, differences in seminal virus sources (i.e., systemic compartment versus MGT) among animals may explain the differential effect of intensification, and more generally of ARVs, on semen viral load. Thus, an intermittent shedding pattern (as observed in the two persistent shedders) has been associated in therapy-naive individuals with the presence of viral strains in semen distinct from those in blood, indicative of local production. In contrast, seminal strains similar to blood strains were observed in patients with continuous shedding of HIV in semen (81). Prolonged shedding in semen during potent HAART is frequently intermittent (6, 16–18, 78, 79), with seminal viral strains reported to be distinct from blood strains (6). We postulate that the resistance to treatment observed in the two intermittent shedders compared with the continuous shedder may reflect local compared to systemic origin of the seminal virus. Phylogenetic analysis of seminal and blood viral strains would be required to substantiate this assumption but was beyond the scope of this study.

Semen is composed of cells and secretions originating from the MGT, which itself comprises several exocrine organs (namely the testes, the epididymides, the seminal vesicles, the prostate, and the bulbourethral glands) and ducts (the efferent duct, the vas deferens, the ejaculatory ducts, and the urethra). We performed a thorough investigation into the infection of the MGT organs and ducts from treated and untreated animals and found a differential effect of HAART depending on the tissues. HAART led to undetectable median levels of vRNA in the prostate and vas deferens and significantly decreased vRNA levels in the epididymis and seminal vesicle. The testis infection level was extremely low after HAART. Using *in situ* hybridization, infected cells could not be detected in those tissues despite



extensive screening. In contrast, in the urethra, we did not observe any significant difference in vRNA level and infected cells were still readily detected after HAART.

One limitation to our study is the relatively short course of HAART (17 weeks) compared to the human treatment and the fact that viremia was not fully suppressed (i.e., below 12 copies/ml) at all time points in the animals, except for one macaque (23014). Thus, seeding of MGT organs from the periphery during the HAART treatment cannot be completely ruled out. Of note, however, is the fact that animal 23014 with consistently suppressed viremia had vRNA levels in MGT organs similar to those of other animals and infected macrophages were detected in the urethra.

Low drug penetration has been shown in tissues displaying residual productive infection in the face of HAART, such as lymphatic organs and brain (60, 82, 83). Due to the poor accessibility to the male genital organs, ARVs have never been assayed in male genital tissues. Similar ARV concentrations were found among all the MGT organs tested. Unfortunately, because of its small size and the fact that other experiments had to be performed, drug levels in the urethra could not be measured. Slightly lower drug levels were generally measured in the testis, as expected due to the presence of numerous drug efflux pumps and of the blood-testis barrier (84–86). The testis has been postulated as being able to constitute a viral sanctuary within the MGT (reviewed in reference 86). However, the very low level of infection of this organ following HAART, together with the localization of infected cells in the interstitium distant from the seminal lumen (reported by us and others [45, 47]), argues against the testis being an important contributor to virus in semen during HAART. It can be assumed that ARV concentrations in the MGT organs analyzed were sufficient to control viral replication, since SIV RNA levels and SIV RNA$^+$ cell detection were impacted by HAART. Whether this control eventually leads to full viral suppression remains to be determined through longer time course experiments. Animals persistently shedding SIV in semen, compared to nonshedders, did not display significantly different drug concentrations in the MGT tissues tested, indicating that the release of virus in semen during HAART is not associated with suboptimal drug concentrations in these organs (i.e., testis, epididymis, prostate, and seminal vesicle).

The urethra displayed a high number of SIV target cells (primarily macrophages) in both treated and untreated macaques and was generally the organ displaying the highest level of viral RNA within the MGT. Such a high number of macrophages was also reported for the urethra from healthy uninfected men (87–89). The majority of T lymphocytes in macaques' urethras were CD4

FIG 8 Phenotyping of SIV RNA$^+$ cells in the MGT tissues by combined immunofluorescence and *in situ* hybridization. Quadruple label fluorescence microscopy to identify macrophages (CD68/CD163, red), T lymphocytes (CD3, white), and SIV RNA expression (ISH, green). Cell nuclei are stained by DAPI (blue). (A) Positive control, tissue sections hybridized with antisense probe showing infected T lymphocytes (arrows) in the seminal vesicle from a highly (acutely) infected animal; (B) negative control, parallel tissue sections of seminal vesicle hybridized with SIV sense probe showing no signal for SIV RNA; (C, E, F) urethra from treated macaques displaying SIV RNA$^+$ macrophages (arrowheads); (D) negative control, hybridization of parallel sections of urethra with SIV sense probe showing no specific signal for SIV RNA; (G) prostate from untreated chronic macaque displaying infected T lymphocyte (arrow). Large panels represent merged images combining all channels. Side panels represent individual channels. Scale bar = 20 μm.

negative, as previously reported for human urethral tissue (87, 88). We evidenced the presence of SIV RNA$^+$ macrophages in the urethra from HAART-treated macaques with reduced or undetectable viremia. In agreement with our findings in macaques *in vivo*, the preferential infection of resident macrophages was recently described in human urethral tissue exposed *ex vivo* to HIV-1 (87). It was recently suggested that myeloid cells in the gut and lymphoid tissues from SIV-infected primates are resistant to infection and acquire SIV DNA primarily through the phagocytosis of infected T cells (90). However, it was reported that macrophages in lymph nodes and mucosal tissues get heavily infected in macaques depleted in CD4$^+$ T cells (91) and that the uptake of HIV-infected T lymphocytes by blood-derived macrophages leads to their infection (92). Macrophages are functionally and phenotypically extremely heterogeneous among anatomical sites. For instance, whereas intestinal macrophages have been shown to be resistant to HIV infection, vaginal macrophages are permissive (93). Infected myeloid cells in the brain are also widely believed to play an important role in neurocognitive disorders. Although we cannot be sure that SIV RNA detection in urethral macrophages reflects their productive infection, the fact that we did not evidence SIV RNA$^+$ T lymphocytes in the urethra argues against phagocytosis of infected CD4$^+$ T cells as the main mode of SIV RNA acquisition in those macrophages. Tissue-resident macrophages have the potential to constitute long-term viral reservoirs. In addition to being much more resistant to cytopathic effects of lentiviral replication than activated T cells, tissue macrophages can live from several weeks up to years (94) and have been shown capable of self-renewal (95). Therefore, long-lived, and possibly self-renewing, urethral macrophages may be able to produce virus for extended time periods. To determine whether the urethra constitutes an as-yet-unrecognized anatomical reservoir for HIV, an investigation into urethra infection in infected men with prolonged suppressed viremia under HAART would be required. In addition, measurement of ARV concentrations in this organ would be easier performed in men than macaques due to the bigger size of this organ.

The relatively high level of infection of the urethra evidenced in our study compared to infection of other MGT organs suggests that the urethra may play a substantial role in the seeding of semen with infected leukocytes and virions. Indeed, several studies in therapy-naive men support such a role: urethral swabs were found to contain significant amounts of HIV RNA (96), and HIV RNA and DNA were detected in the preejaculatory fluid coming from the urethra (97, 98). Phylogenetic analysis would be needed to demonstrate that the urethra is indeed a source of virus in semen during HAART. However, the relatively low semen viral load in treated animals, together with the very limited semen volume extractable from macaques (maximum of 500 μl/ejaculate), represents a major obstacle against obtaining sufficient seminal viral amplicons for reliable phylogenetic analysis (42, 99). As an alternative, phylogenetic comparison of viral strains in the semen, MGT organs, and blood from therapy-naive animals with compartmentalized seminal strains and high SVL would help establish the role of the urethra and other MGT organs in semen contamination.

We previously demonstrated in both men and therapy-naive macaques the infection of T lymphocytes and macrophages in the epididymis, prostate, seminal vesicles, and testis using *in situ* hybridization combined with immunostaining (43, 45, 47, 54, 100). Infected T lymphocytes predominated in the prostate (100), whereas infected macrophages were encountered primarily in the epididymis, seminal vesicle, and testis, together with some infected T lymphocytes (43, 45, 47). Such infected cells were no longer detected in these organs after 4 months of HAART in macaques. Productively infected T lymphocytes are short-lived, and their eradication during HAART in MGT tissues further indicates that antiretroviral drug concentrations in the MGT are efficient. A substantial proportion of infected macrophages in the MGT of therapy-naive animals may be short-lived infiltrating macrophages cleared during HAART. The much lower proportion of resident macrophages in MGT organs other than the urethra most likely explains the failure to detect infected macrophages after HAART in these tissues. We hypothesize that the residual level of SIV RNA detected in the epididymis, seminal vesicle, and testis after HAART is due to infected resident macrophages, too scarcely distributed to be detected on tissue slides. Alternatively, seeding of genital tract organs by ongoing viral replication in the periphery may be involved in the low levels of residual vRNA observed in those organs, as the duration of HAART (17 weeks) was relatively short and the macaques' viremia levels were not consistently below 12 copies/ml, except for one animal. Activated immune cell infiltrates encompassing SIV target cells were encountered in the epididymis, prostate, and seminal vesicles of untreated animals, in agreement with previous findings of our team and others in infected men and macaques (45, 101, 102). We previously showed that infection level was a crucial determinant of leukocyte recruitment in these tissues, as leukocyte infiltrates were absent in uninfected animals and in animals with low viremia and low MGT tissue infection (45). The number of inflammatory infiltrates was significantly reduced in the prostate and to a lesser extent in the epididymis of treated animals, in line with their decreased level of SIV RNA. Immune cell foci were never observed in the testis, which may be due to its naturally immunosuppressive environment. As for urethra, although cell foci were not observed, we cannot rule out an increase in immune cells caused by the infection.

We showed that the course of HAART performed in this study had no major impact on the detection of vDNA in MGT tissues in which vRNA levels were drastically decreased, reflecting the preferential removal of productively infected cells. In line with our findings, the persistence of vDNA in the absence of vRNA detection was found in the testis, prostate, and seminal vesicles from juvenile rhesus macaques infected with reverse transcriptase simian-human immunodeficiency virus (SHIV) treated by HAART for 26 weeks (52). A similar differential effect of HAART on vDNA and vRNA levels has been reported in the brain, a well-established HIV/SIV reservoir (67). The lack of HAART impact on vDNA suggests the presence of long-lived cells containing very low vRNA copies, such as latently infected memory T cells (which may be reactivated over time to produce virus and prevent full eradication in the MGT), macrophages producing low level of SIV RNA, or cells harboring defective vDNA. The detection of SIV DNA in MGT tissues was paralleled with the detection of SIV DNA in seminal cells from treated animals. Determining whether this detection results from tissue-resident cells or migration into the MGT of peripheral cells encompassing vDNA requires phylogenetic analyses of vDNA in blood and MGT tissues. We recently showed that both CD4$^+$ T lymphocytes and macrophages present in the semen of therapy-naive macaques contain SIV DNA and

can produce virus (29). In men under suppressive HAART, the presence of replication-competent virus has been shown in seminal leukocytes for up to 3 years after treatment initiation (7, 10, 23–26, 103, 104). On the basis of the present results and of our previous demonstration of the infection of female macaques following vaginal exposure to infected cells (28), a future study into the ability of persistently infected seminal cells to transmit infection in a sexual partner in macaques is of strong interest.

In conclusion, we showed that SIV shedding occurs in the semen of macaques despite a course of HAART leading to drastically decreased viremia. This shedding is not linked to lower drug concentrations in semen, testis, epididymis, seminal vesicle, or prostate. Drug concentrations in the urethra remain to be tested. The effect of HAART was variable among the MGT organs. Our results suggest that seminal shedding during HAART could result from the production of virus by long-lived cells within the MGT, such as the infected macrophages detected within the urethra of treated animals. Long-term HAART leading to fully suppressed viremia and phylogenetic analyses are required to fully substantiate this assumption. Our results pave the way for future studies into (i) the male genital organs and especially the urethra as a source of HIV in semen, through phylogenetic comparison of strains in semen and genital organs, (ii) the kinetics of virus decay in the male genital tract through the longer follow-up of animals receiving HAART, (iii) the infectiousness of the remaining free virus and infected cells in semen, and (iv) the factors triggering seminal shedding from the persistently infected genital organs during HAART.

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