Published in final edited form as:

Am J Reprod Immunol. 2015 December ; 74(6): 508–522. doi:10.1111/aji.12422.

Characterization of the Genital Microenvironment of Female Rhesus Macaques Prior to and After SIV Infection

Whitney A. Nichols, B.S.,

Department of Microbiology, Immunology, & Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA

Leslie Birke, D.V.M.,

Division of Animal Care, Louisiana State University Health Sciences Center, New Orleans, LA

Jason Dufour, D.V.M.,

Division of Veterinary Medicine, Tulane National Primate Research Center; Covington, LA

Nisha Loganantharaj, M.D.,

Department of Microbiology, Immunology, & Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA

Gregory J. Bagby, Ph.D.,

Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, LA

Steve Nelson, M.D.,

Department of Medicine, Louisiana State University Health Sciences Center, New Orleans

Patricia E. Molina, Ph.D., and

Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, LA

Angela M. Amedee, Ph.D.

Department of Microbiology, Immunology, & Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA

Whitney A. Nichols: wnich1@lsuhsc.edu; Leslie Birke: lbirke@lsuhsc.edu; Jason Dufour: jdufour@tulane.edu; Nisha Loganantharaj: nlogan@lsuhsc.edu; Gregory J. Bagby: gbagby@lsuhsc.edu; Steve Nelson: snelso1@lsuhsc.edu; Patricia E. Molina: PMolin@lsuhsc.edu; Angela M. Amedee: aamede@lsuhsc.edu

Abstract

Problem—HIV infection among women is frequently modeled in female rhesus macaques.

Longitudinal studies on genital compartment and hormonal factors that can influence susceptibility to SIV infection are lacking in this animal model.

Methods of Study—Genital specimens and menstruation of indoor-housed female rhesus macaques were analyzed prior to and after SIV-infection.

Results—Median menstrual cycle length averaged 27 days, although highly variable cycle lengths and frequent periods of amenorrhea were observed during summer months. The vaginal

Corresponding author: Angela M. Amedee, Department of Microbiology, Immunology, & Parasitology, LSUHSC, 1901 Perdido St., Box P6-1, New Orleans, LA 70112, Phone: (504) 568-5608, Fax: (504) 568-2918, aamede@lsuhsc.edu.

Conflicts of Interest: No conflicts of interest declared.

microbiota, characterized by adapted Nugent scoring, showed predominance of small gram-variable rods and gram-positive cocci. Highly variable vaginal cytokine levels were observed pre- and post-SIV infection. Vaginal viral loads correlated with plasma viral loads, but were not associated with progesterone levels.

Conclusion—These results provide an integrated characterization of important factors in the vaginal microenvironment that are relevant to the experimental design of HIV prevention and transmission studies in female rhesus macaques.

Keywords

Heterosexual Transmission; HIV; Nonhuman Primate Model; Vaginal

Introduction

Women primarily acquire HIV-1 infection through heterosexual contact, and they now account for 50% of people living with HIV/AIDS worldwide.^{1–3} A better understanding of the unique dynamics affecting HIV acquisition and disease progression in this growing population is critical for prevention efforts and disease management. Non-human primates (NHP) infected with simian immunodeficiency virus (SIV) have served as an invaluable animal model for studies of HIV transmission, with the rhesus macaque (*Macaca mulatta*) being the most widely used animal.⁴ Due to similarities in reproductive tract anatomy and physiology, as well as the ready access to genital mucosa sampling, the female macaque has provided insight to the early events following cervicovaginal transmission.^{5–7} This animal model is frequently used for investigations of transmission, prevention, and therapeutic efficacy of antimicrobial and anti-viral agents.^{8–11}

The female genital compartment is a dynamic environment comprised of innate and adaptive immune factors and several of these mediators have been shown to affect HIV transmission.^{12–14} In women, an increased risk of HIV acquisition is associated with the presence of cervicovaginal inflammation, which is commonly seen with sexually transmitted infections (STIs).¹⁵ These conditions are associated with increased levels of proinflammatory cytokines (IL-6, TNF α , IL-1 β) in the genital mucosa, which recruit CD4+ target cells to the underlying submucosa, disrupt the genital epithelial barrier, and activate HIV replication.^{16–19} An increased risk of HIV infection has also been associated with bacterial vaginosis (BV), as the vaginal microbial flora contributes to the genital mucosal defense against sexually transmitted pathogens.^{20, 21} In particular, the presence of *Lactobacillus* species has been shown to be protective against HIV transmission.^{22, 23}

The genital mucosal immune response is regulated by female sex hormones to balance immune protection and reproduction.^{12, 24} This includes modulation of the mechanical protection afforded by the vaginal epithelium, antibody production, and levels of soluble innate mediators.^{14, 25} High progesterone levels, as observed during the luteal phase of the menstrual cycle and with the use of hormonal contraceptives, have also been suggested to influence viral acquisition and genital HIV shedding.^{26–31} Although the effects of sex hormones on HIV transmission remain controversial, the menstrual cycle phase clearly impacts the inflammatory status of the genital environment and is an important variable to

consider in the context of HIV infection and in the design of preclinical studies on transmission, susceptibility, and prevention.^{24, 25} In studies with the macaque model, many of these variables in the genital compartment that affect HIV susceptibility can be readily assessed; however, limited studies have examined the stability and inter-animal variability of this environment in female macaques.^{32, 33}

Because the genital environment or milieu is influenced by hormonal levels, menstrual cycle variation in macaques is an important variable that can impact studies of HIV/SIV infection and pathogenesis. Female rhesus macaques are seasonal breeders and early studies reported a median menstrual cycle length of approximately 28 days, with more regular menstruation occurring in the fall/winter (October-February).^{34–38} However, many of these studies were performed in outdoor-housed rhesus macaques that were exposed to environmental and social cues.³⁹ More recent observations with indoor housed animals suggest that animals undergo year-round cycling, but limited data characterizing this pattern is available.^{39, 40} Elevated genital viral load is an independent risk factor for HIV-1 transmission; however, the relationship between the menstrual cycle and viral expression remain unclear in women.^{28, 29, 41, 42} While macaque studies have provided valuable insight into the impact of hormone treatment on viral acquisition, the influence of biological hormonal cycles on genital SIV shedding and their implications on transmissibility remains unknown.^{43–46}

The vaginal flora of the rhesus macaque has recently been described using both bacterial culture isolation and ribosomal 16s sequencing.^{47, 48} Several diverse bacterial species commonly seen in women have been identified in female rhesus macaques, with considerable variability between animals noted.⁴⁹ As the composition of the vaginal microbiome can influence the innate immunity of the genital compartment, inter-animal variations and longitudinal changes in the macaque vaginal flora are important variables in the context of SIV transmission. Limited published reports have detailed the variation in the genital microenvironment of female rhesus macaques; therefore a more thorough understanding of the inherent variability would aid effective experimental design with this valuable resource.

In this study, we sought to provide an integrated evaluation of parameters in the genital mucosal environment of female rhesus macaques that are critical to HIV transmission and pathogenesis. Utilizing two different cohorts of female rhesus macaques enrolled in our recent SIV pathogenesis studies, we obtained clinical observational data on the menstrual cycle and assessed the inflammatory state of the genital tract through measures of cytokine/chemokine levels in vaginal fluids and enumeration of cellular infiltrates.^{50, 51} Additionally, we characterized the microbial flora using Gram stains and an adaptation of the Nugent score. Following SIV-infection, viral levels were quantified in vaginal fluids and correlated with plasma progesterone concentrations and with plasma SIV loads. We present a detailed characterization of several factors relevant to SIV transmission, as well as shedding of virus in genital fluids, which will inform future studies with the rhesus macaque to model HIV infection in women.

Materials & Methods

Animals

Twenty-four 3.5–10 year old female rhesus macaques (*Macaca mulatta*) of Indian origin were utilized (Table I). Eight animals were obtained from the California National Primate Center and housed at the LSU Health Sciences Center Animal Care Facility (LSUHSC, New Orleans, LA). Sixteen animals were obtained from Tulane National Primate Research Center (TNPRC, Covington, LA), where they were also housed for the duration of the study. These animals were part of two independent studies evaluating the effects of delta-9-tetrahydrocannabinol (Δ^9 -THC) [cohort I at LSUHSC] or chronic binge alcohol administration [cohort II at TNPRC] on SIV pathogenesis (Table 1).^{50, 51} The time of sample collection and applicable treatments received relative to study design are indicated for each of the measures. Animals were intra-rectally (n=14) or intravenously inoculated (n=10) with SIV_{mac251} (100 TCID₅₀) following a period of either chronic cannabinoid (treatment A) or alcohol (treatment B) administration or the appropriate vehicle control, as previously described.^{50, 51}

Prior approval was obtained for all animal experiments by both the LSUHSC and TNPRC Institutional Animal Care and Use Committees. All experimental approaches adhered to the NIH guidelines for experimental animal use. The animals were housed indoors in individual cages in a room with 4–8 other females, and maintained on a 12:12 hour light-dark cycle. The macaques' diet consisted of primate chow and fruit. Animals were monitored daily by trained technicians and underwent regular physical exams that included serial blood collection and genital sampling.

Menstrual Cycle

Included in the twice-daily clinical observations was gross assessment of menstruation by inspecting the perineum and cage pan for menstrual blood. Based on recorded observational vaginal bleeding, menstrual cycle length and frequency as well as menses duration were determined.^{52, 53} The beginning of a menstrual cycle was defined by the onset of bleeding after an absence of menstrual blood for more than 10 days. The menstrual period included the days when vaginal bleeding was detected, inclusive of break-through bleeding within 10 days of previous cycle. Using reverse cycle counting, we designated the time 14 days prior to the onset of menses as the projected time of ovulation, after which the levels of progesterone would begin to increase. Based on that time frame, specimens were classified as belonging to either the secretory (< 14 days) or proliferative phase (>14 days). Menstrual cycle data was obtained over a two to three year period, with observational periods for individual animals ranging from 8 to 24 months. Analysis was performed on data obtained from only twenty of the animals, as those receiving treatment A (n=4) were excluded due to a potential effect of treatment on cycling, as previously reported.⁵⁰ The observation period included 4–12 months prior to SIV infection and 4–18 months post-SIV. In order to account for the variability in cycling duration, and prevent skewing of data, the median is reported.³⁴

Genital Sample Collection

The first samples collected were genital fluids on ophthalmic sponges, either Merocel (Beaver Visitec International, Waltham, MA) or Weck-Cel (DeRoyal Industries, Powell, TN), for cytokine quantification. Two sponges were pre-wet with 50µl of PBS then inserted into the vaginal lumen and allowed to absorb fluids for a total of five minutes.⁵⁴ Sponges were stored at -80°C until analyzed. Next, additional vaginal specimens were obtained with the assistance of a speculum, permitting visualization of the vaginal vault and cervical os. Three polyester-tipped swabs (Citmed Corporation, Citronelle, AL) were rolled along the lateral vaginal wall and posterior fornix, and placed in either serum free RPMI media for assessments of cellular infiltrates or RNALater (Ambion) for viral load measures. Vaginal cells and fluids were eluted off the swab by vigorously mixing the swabs. Samples were divided into supernatant and cellular fractions prior to storage at -80°C. The third swab was used to prepare a vaginal smear for gram staining and assessment of flora. Genital samples for biological measurements were not collected when overt vaginal bleeding (menses) was evident.

Vaginal Inflammatory Markers

Cytokine and chemokine concentrations were determined in fluid eluted from ophthalmic sponges as described.⁵⁴ Briefly, frozen sponges were allowed to thaw and placed in a spin assembly comprised of an upper microcentrifuge tube with a pin hole and a lower collection tube. Next, 100µl of ice cold elution buffer, comprised of 1× Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL) and 0.5% Igepal in PBS solution, was applied and allowed to diffuse through for 10 minutes. The specimen was then centrifuged at 20,000g at 4°C for 45 minutes. This process was repeated for the second sponge using the same lower chamber collection tube. Total volume eluted varied per sample and to control for this samples were normalized to total protein levels. Protein levels of the secretions were determined using the Pierce BCA protein assay (Thermo Fisher Scientific Inc., Rockford, IL). Cytokine levels are expressed as pg/mg protein, and the average total protein concentration of the eluted secretions was 9.7 mg protein/mL ± 1.2 (0.7–28.1 mg protein/mL).

Concentrations of inflammatory cytokines and chemokines in vaginal secretions (IFN-γ, TNF-α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-4, IL-6, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), and macrophage inhibitory protein-1α (MIP-1α)) were determined using a Milliplex MAP non-human primate cytokine kit (Cat# PRCYTOMAG-40k, EMD Millipore, Billerica, MA) according to the manufacturer's protocol. An additional two-fold diluted standard was included to improve assessment at the lower end of detection. Reactions were performed in triplicate and run on the Bio-Plex® 200 system followed by analysis with Bio-Plex Manager (Bio-Rad, Hercules, CA). The lower limits of detection were: IFN-γ, 1.2 pg/mL; TNF-γ, 9.8 pg/mL; IL-1β, 1.2 pg/mL; IL-1Ra, 1.2 pg/mL; IL-4, 2.4 pg/mL; IL-6, 1.2 pg/mL; IL-8, 1.2pg/mL; IL-10, 6.1 pg/mL; MCP-1, 1.2pg/mL; and MIP-1α, 2.4 pg/mL, respectively. For statistical analysis, those values below the limit of detection were set at the midpoint between zero and the lower limit of detection.

Cellular infiltrates were assessed in vaginal fluids eluted from swabs collected from uninfected animals (up to 36 weeks prior to inoculation) and following SIV infection (8–10 weeks). Polymorphonuclear neutrophils (PMNs) present in vaginal secretions were identified using the Endtz staining method (myeloperoxidase assay) as previously described.^{51, 55} Total cell counts per sample were reported and specimens were excluded if >100 red blood cells were present.

Gram Stain

Macaque vaginal flora was assessed at similar time points detailed above. Slides smeared with vaginal secretions were stained using the Becton Dickinson gram stain kit, then evaluated and scored using the Nugent Scoring System.⁵⁶ Other bacterial morphotypes that were frequently identified in previous rhesus macaque studies were also quantified at 1000× objective in four different observational fields, and those values were averaged.^{19,20} Morphotypes included in the assessments were: gram-positive cocci (GPC), gram-positive diplococci (GPDC), small gram-variable rods (SGVR), curved gram-negative rods (CGNR) and large gram-positive rods (LGPR). The relative abundance of morphotypes within a sample was calculated by ranking the average levels of each morphotype as described previously.⁵¹

Viral Quantification

SIV RNA levels in plasma and vaginal secretions were determined by a quantitative real-time PCR assay (qPCR) using SIV gag primers and probe, as described.⁵⁷ Briefly, virions contained in 1mL of plasma or in 1 mL of vaginal fluid eluted from swabs were concentrated by high speed centrifugation. Viral RNA was purified with Trizol reagent (Life Technologies, Grand Island, NY), and reverse-transcribed to cDNA. PCR amplification was performed in duplicate using a TaqMan® assay (Life Technologies) and quantified using SIV gag RNA standards. As described previously, an exogenous internal control RNA template (IC_{BVM}) was added to each sample prior to RNA purification and quantified simultaneously with SIV in a multiplex assay.⁵⁸ This permitted monitoring of extraction efficiency, reverse transcription, and identified samples that contained PCR inhibitors.⁵⁸ The limit of quantification for this assay is 50 copies/mL. Samples with undetectable viral levels were assigned a value midway between zero and the lower limit (25 copies/mL) of detection. For statistical comparisons, viral loads were log₁₀ transformed.

Progesterone Concentration

Circulating progesterone levels were assessed in plasma samples obtained at the time of genital sampling using a solid-phase ¹²⁵I radioimmunoassay kit, Coat-A-Count® Progesterone (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). EDTA-treated plasma samples were processed according to the manufacturer's basic protocol and counted for one minute on a 1480 Wizard Gamma Counter (Perkin Elmer, Waltham, MA). Final concentrations were determined by extrapolation of counts per minute to a calibration curve. The limit of detection in this assay is 0.02 ng/mL.

Statistical Analysis

Values are presented as means $\pm$ SEM for each experimental group. The number of animals per group is indicated in each figure legend. Statistical analysis was performed in Prism 5 (GraphPad Software, INC., La Jolla, CA) that included Mann-Whitney U test and Spearman's rank correlation coefficients. The level of significance was set at $p = 0.05$.

Results

Menstrual Cycle Characteristics

To assess the menstrual cycle, observational bleeding data from twenty indoor housed female rhesus macaques were recorded longitudinally for 8 months to 2 years and included time points pre- and post-SIV infection. Age of the animals ranged from 3.5–10 years, and body weight ranged from 4.4–8.52 kg (Table 1). A total of 269 menstrual cycles were documented with an overall median cycle length of 27 days. Collectively, we observed an average of 12.1 ± 0.9 menstrual periods per year. All animals displayed a high degree of variability in cycle length over the course of observation (Figure 1A). Among individual animals, the median cycle length ranged from 18 to 36 days, with periods of amenorrhea of greater than 60 days observed in 75% of animals (15 of 20). Some females had multiple occurrences of amenorrhea and in six animals amenorrhea was observed for more than 100 days. Approximately 44% (118/269) of the cycles ranged from 25 to 34 days in length, while 7.4% of the cycles were greater than 60 days (Figure 1B). Median cycle length or periods of amenorrhea were not associated with age or weight of the animals.

The characteristics of the menstrual cycle were further analyzed to assess variation in the context of the rhesus macaque breeding season (designated Oct. to Feb.). Menstruation was observed throughout the year, with a slightly higher average number of cycle starts per month observed over the breeding season months (24.6 cycle onsets/month) versus the rest of the year (20.9 cycle onsets/month) (Figure 1C). In five of the animals with amenorrhea for more than 100 days, an absence of cycling was noted in the summer months (ranging from April to September), with menstrual bleeding returning in August ($n=1$), September ($n=1$), and October ($n=3$); the sixth animal experienced amenorrhea over the breeding season months, from August through February. Shorter periods of amenorrhea for 2 to 3 months ($n=13$) were documented in 11 different animals. While the majority of these short periods of amenorrhea were also observed in the Spring/Summer months (69%), they were documented throughout the year, with 31% occurring in breeding season (4/13). The effect of SIV infection on menstrual cycle characteristics was also assessed. No difference in individual animal's cycling pattern was observed between the pre- and post-inoculation period (data not shown).

We corroborated our reverse cycle calculations through limited serum progesterone measurements in available samples ($n=88$), as our study design allowed. Only 2% (2/88) of samples contained high progesterone levels when observational bleeding predicted they would be low, suggesting a menstruation period was missed in these animals.

Vaginal Inflammatory State

The levels of a selected panel of inflammatory cytokines were measured in vaginal secretions of naïve animals. A total of 18 specimens were evaluated from ten different macaques, with six of the animals sampled at multiple (2–3) time points. Cytokine levels were detectable in all of the genital fluid samples analyzed, and each of the assayed proteins was detected in more than 78% of the samples collected except for MIP-1 α (66%). Cytokine concentrations were highly variable between animals, as indicated by the wide range in baseline levels shown in Table II. We also evaluated ten samples collected post-SIV infection (8–16 weeks) from 10 animals (Figure 2B). The levels of IL-1Ra were significantly decreased ($p<0.01$) by approximately 4 fold compared to uninfected animals; no other differences were noted.

The inherent inflammatory state of the macaque vaginal mucosa was also characterized by enumeration of immune cells present in genital secretions. Samples were available from 14 uninfected macaques, with a subset of animals providing samples from 2 time points. PMNs identified by positive myeloperoxidase staining were observed in 36% (8/22) of the samples, with an average total of 2.75×10^4 neutrophils per sample among those with detectable neutrophils (Figure 2C). A second sample was collected from 12 of the animals to assess neutrophil infiltrate post-SIV infection (8–10 weeks). Neutrophil infiltrates were not statistically different relative to uninfected animals, although only two post-SIV samples contained detectable neutrophils ($p=0.26$).

Vaginal Microbial Flora

To evaluate the vaginal microbiota, we analyzed gram-stained smears of vaginal samples using an adaptation of the Nugent scoring system.⁵¹ Using this clinically relevant test, we assessed the bacterial morphotypes in 27 samples collected from 17 individual macaques at time points prior to any treatment or SIV infection. The majority of vaginal specimens analyzed were classified as either intermediate (40%) or bacterial vaginosis (BV) (37%) (Figure 3A). To further characterize the microflora, the distribution of bacterial morphotypes present in each sample was determined, and the relative abundance is collectively depicted in Figure 3B. The macaque microbiota was dominated by small gram variable rods (SGVR) and gram positive cocci (GPC) morphotypes. GPC morphotypes comprised 53% of the flora and were present in 15 of 17 macaques. Large gram positive rods (LGPR), which are suggestive of *Lactobacilli* spp., consisted of 13% of the overall flora and were observed in only 7 of 17 macaques. To further evaluate stability of the vaginal flora, replicate samples from 10 animals collected 2–4 weeks apart were compared. Overall, the distribution of morphotypes was highly similar in replicate samples, with a shift in the presence of two or more morphotypes only evident in one animal (Animal E) (Figure 3C). We also evaluated vaginal microbial flora post SIV-infection (8–10 weeks) to expand upon our previous study by including additional animals.⁵² SIV-infection did not significantly change the distribution of major morphotypes found collectively in the SIV-animals compared to that found in naïve animals (Figure 3B). While minor shifts in the overall composition of morphotypes were noted, variability in timing between the collections of these specimens and the inherent diversity among individual animals needs to be assessed with more frequent sampling.

Genital SIV Shedding

To evaluate levels of SIV shed in vaginal fluids, we compiled viral load data previously reported from the control animals of cohorts I and II to describe viral expression over the initial course of SIV infection (n=12).^{51, 59} A total of 91 samples, collected at time points from 1–10 weeks p.i. were evaluated. Comparable vaginal viral levels were detected over the course of infection from animals inoculated via intravenous (IV) or intra-rectal (IR) routes. Peak vaginal viral load (observed at 2, 3, or 4 weeks p.i.) averaged 3.5 log SIV copies/mL in IR-inoculated animals and 3.2 log SIV copies/mL in those IV-inoculated. Set-point vaginal viral loads (average of values from 7–10 weeks p.i.) were 1.9 log SIV copies/mL and 1.7 log SIV copies/mL, in IR and IV inoculated animals, respectively. Overall, vaginal viral loads were highly variable throughout the course of infection (Figure 4A). Vaginal SIV was undetectable in 26% of samples (24/91), with 9/12 animals having at least one sample with undetectable levels of SIV at time points post-peak viremia. Genital secretions are a heterogeneous mixture of multiple components, which may include inhibitors of qPCR. To rule out the possibility that this could account for the lack of viral detection in some vaginal samples, we used a previously described RNA internal control and observed PCR inhibition in 3 of the vaginal samples analyzed.⁵⁸ We were still able to amplify SIV RNA from 2 of the 3 collected vaginal secretions, but these values may be an underestimation due to the presence of PCR inhibitors in those specimens.

Similar to what has been observed in HIV-infected women, plasma viral levels showed a direct and positive correlation with vaginal viral levels ($r=0.514$, $p<0.0001$).⁶⁰ The plasma to vaginal viral load ratio was 2.2 at peak viremia and increased to a mean of 3.0 at set point, as a result of lower viral levels in vaginal fluids at this time point (Figure 4B). To evaluate if progesterone levels influenced the levels of virus shed into vaginal fluids, we compared serum progesterone levels at the time point that vaginal SIV levels were measured. No significant statistical correlation was observed between serum progesterone concentration and vaginal viral load ($r=0.047$, $p=ns$) (Figure 4C). Vaginal SIV levels were also examined in relation to the expected menstrual cycle phase as determined by reverse cycle-counting, and no association was observed. Additionally, we did not find an association between viral load in plasma or genital fluids at set point and relative abundance of specific bacterial morphotypes, Nugent score, PMN infiltrates, or cytokine concentrations.

Discussion

In this study, we sought to identify the diversity in several parameters relevant to the sexual transmission of HIV/SIV using genital samples and observational data from indoor housed adult female rhesus macaques. Our observations detail the variation in vaginal inflammation markers and microbial flora, as well as menstrual cycling. Additionally, they provide reference values that will be useful for HIV prevention/transmission studies with the rhesus macaque and detail methodology to efficiently monitor these variables longitudinally.

The female rhesus macaque cyclic pattern of sex steroid hormone levels (estrogen and progesterone), is analogous to that observed in women. Anovulatory periods have historically been observed during the spring and summer months in rhesus macaques, which have been attributed to both behavioral and environmental factors.^{34–36, 39, 53, 61} However,

study designs frequently require housing animals indoors in a controlled-environment with no exposure to natural light or temperature. Under these conditions, year-round menstruation has been observed, as was evident with the animals in this study.^{40, 52, 62} An average of 12 menstrual cycles per year, with a median length of 27 days, was documented in our animals. These observations are highly analogous to that of women and expand upon earlier rhesus macaque studies evaluating reproductive cycle characteristics.^{34, 39, 63} Irrespective of housing conditions, menstrual cycle length consistently ranged from 25 to 35 days.^{34, 39, 52, 53, 64} Although the animals described in this study were housed indoors in single cages and removed from environmental and social cues, our observations found that periods of amenorrhea were most common during the summer months, similar to what is reported from environmentally-exposed animal. While these observations provide mean reference values, they also underscore the inherent variability in the rhesus macaque menstrual cycle. Timing of experiments to occur during the winter months and breeding season provides the best opportunity for ensuring more consistency in cycle length.

The immunological properties of the female genital tract are hormonally regulated by estradiol and progesterone.^{25, 65, 66} Elevated progesterone levels are observed during the secretory phase of the menstrual cycle, which also coincides with the generation of a permissive mucosal environment for fertilization and implantation to occur. Several studies have associated periods of high progesterone with decreased antibody levels in cervicovaginal secretions, suppressed cytotoxic T-lymphocyte (CTL) and NK activity, altered immune cell recruitment, and potential thinning of the vaginal epithelium, as suggested by studies in the macaque.(Review in^{14, 67}) Consequently, this curtailed inflammatory response creates a potential period of vulnerability against pathogens.^{66, 68} This is supported by epidemiological data and animal studies that associate progesterone-treatment with increased susceptibility to sexually transmitted infections.^{44, 69, 70} Therefore, the menstrual cycle phase at the time of vaginal challenge or specimen collection should be considered for appropriate interpretation and subsequent conclusions of the outcomes being evaluated.^{14, 71}

The commonly utilized methods for establishing the cycle characteristics involve either gross assessment of perineal cycle-dependent changes (i.e. changes in sex skin coloration or overt vaginal bleeding) or measurement of hormone levels.⁷²⁻⁷⁵ Monitoring gonadal hormone levels provides a more accurate reflection of the timing of ovulation. However, necessary serial blood collection to ensure sufficient measures to construct a complete cycle is a significant limiting factor to conducting those studies. Furthermore, this process would need to be repeated over the course of multiple menstrual cycles to appropriately depict the inherent fluctuations in hormone levels and precisely determine ovulation. Few studies with the SIV-macaque permit the frequent blood sampling needed to establish cycle phase using this technique. In contrast, gross assessments are noninvasive and part of the daily monitoring. Using this information, the cycle phase (proliferative vs. secretory) can then be extrapolated under the assumption that ovulation occurs 14 days prior to the start of menses, as the luteal phase is more consistent in duration compared to the follicular phase.^{76, 77} We recognize that menstrual flow may not always be obvious and therefore is a limitation of observational vaginal bleeding.⁶⁴ We sought to confirm our cycle calculations with limited measures of serum progesterone. Only 2% of the samples contained high levels of

progesterone when anticipated to be low, suggesting cycles were rarely missed. However, a more thorough assessment of hormone levels is necessary to fully assess the accuracy of observational bleeding. More precise evaluations could be made by daily swabs of the vagina to identify periods of light menstruation. Despite the limitations, observational bleeding records provide a highly feasible and reliable method of retrospectively associating study time points with the secretory or proliferative phase of the menstrual cycle.

Due to the potential implications in SIV transmission studies, the preexisting cervicovaginal inflammatory state of the rhesus macaque is an important factor to consider and has recently begun to be defined with the analysis of specific immune components.³² Various collection methods are utilized for sampling of the genital mucosa, thus limiting comparisons among studies.^{54, 78, 79} We measured cytokine concentrations in genital secretions collected with sponges as opposed to cervicovaginal lavages for a better assessment of the vaginal mucosa, an immunologically distinct site from the cervix. Most specimens (>78%) contained detectable levels of each of the cytokines measured, suggesting that this method of collection provides sufficient material for cytokine quantification. Consistent with previous findings, a high degree of variation in mucosal cytokine levels was observed between animals suggesting repeated measures are needed to establish basal levels for an individual animal.^{32, 80}

The commensal vaginal microbial flora influences mucosal immune responses. *Lactobacillus* species are the most prevalent bacteria in the vaginal microbiota of healthy women, in contrast to women with BV whose flora is predominately colonized by anaerobic bacteria and *Gardnerella vaginalis*.^{81–83} Vaginal dysbiosis increases the expression of pro-inflammatory cytokines and has been associated with an increased risk of HIV transmission.^{20, 84–86} Therefore, the microbial flora is a relevant factor to be considered in vaginal transmission studies. Recently, the rhesus macaque vaginal microbiota has been characterized as BV-like and relatively stable over time based on 16s pyrosequencing.^{32, 48} We assessed the feasibility of the clinically relevant Nugent scoring system as a cost effective and less technically-challenging methodology to evaluate the macaque vaginal flora. Consistent with previous findings, a diversity of bacterial morphotypes was observed on gram-stained vaginal smears, and the macaque microbiota was primarily classified as either intermediate (41%) or BV (37%). Furthermore, longitudinal assessment indicated stability in the macaque flora, in which only 2 out of 10 animals differed in Nugent score interpretation between the two time points. Stability was also noted in the relative abundance of specific morphotypes detected. SIV-infection did not significantly alter the distribution of microbial flora, which is consistent with our previous report.⁵² These findings demonstrate the utility of gram stain in combination with adapted Nugent criteria, and provide a methodology for longitudinal monitoring of the macaque vaginal flora.

Cervicovaginal viral load is a predictor of HIV-1 sexual transmission risk independent of plasma viral levels as local viral replication can occur within the female genital tract.^{41, 87–89} As a relevant biomarker in HIV prevention and therapeutic studies, characterization of the viral dynamics in the macaque genital compartment over the course of infection is needed. Peak viral levels in genital fluids were approximately 2 logs lower than plasma levels, but at set-point, these levels were an average of 3 logs lower than plasma. Using a sensitive qPCR

assay with an internal control to monitor PCR inhibition, we frequently observed intermittent SIV vaginal shedding following peak viremia. Overall, vaginal viral loads were positively correlated with those in plasma. HIV genital shedding has been correlated with various factors within the cervicovaginal milieu of women; however, the effects of the menstrual cycle phase remain controversial.^{28–31, 90, 91} We evaluated the potential relationship between progesterone levels/ cycle phase and genital SIV shedding in our macaques, however we did not observe a correlation.

Women are disproportionately affected by HIV-1 infection as indicated by the increasing prevalence among people living with HIV/AIDS. A better understanding of the unique host: virus interactions in this growing population are necessary for targeted prevention efforts and disease management. The female rhesus macaque model has been an invaluable tool for deciphering the specific mechanisms of cervicovaginal HIV transmission as well as exploring potential therapeutic strategies. However, few studies have considered the inherent dynamics of macaque genital mucosa. Our comprehensive evaluation of factors within the cervicovaginal environment relevant to HIV/SIV infectivity provides a thorough characterization of parameters critical in the experimental design of transmission studies.

Acknowledgments

The authors would like to thank Drs. Nicole LeCapitaine and Elise Boos for their contributions to data collection. We are also grateful for excellent technical assistance from Jamie A. Hubbel, Nedra Lacour, Curtis Vande Stouwe, Jean W. Carnal, Rhonda R. Martinez, Jane A. Schexnayder, Amy B. Weinberg, and Peter B. Lewis. Supported by NIH P60 AA09803, T32 AA007577, P51-OD011104, R01 DA020419, and R01DA030053.

References

1. WaH Department of Gender. , editor. World Health Organization. Gender inequalities and HIV. 2014.
2. UN Joint Programme on HIV/AIDS. Global Report: UNAIDS Report on the Global AIDS Epidemic 2013. WHO Library Cataloguing-in-Publication Data.
3. CDC. Estimated HIV incidence among adults and adolescents in the United States, 2007–2010. HIV supplemental Report. 2012
4. Sui Y, Gordon S, Franchini G, Berzofsky JA. Nonhuman primate models for HIV/AIDS vaccine development. Current protocols in immunology / edited by John E Coligan [et al]. 2013; 102(Unit 12):14.
5. Buse E, Habermann G, Osterburg I, Korte R, Weinbauer GF. Reproductive/developmental toxicity and immunotoxicity assessment in the nonhuman primate model. Toxicology. 2003; 185:221–227. [PubMed: 12581697]
6. Miller CJ, Alexander NJ, Sutjipto S, Lackner AA, Gettie A, Hendrickx AG, Lowenstine LJ, Jennings M, Marx PA. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. Journal of virology. 1989; 63:4277–4284. [PubMed: 2778875]
7. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. Nature. 2010; 464:217–223. [PubMed: 20220840]
8. Abel K, Compton L, Rourke T, Montefiori D, Lu D, Rothausler K, Fritts L, Bost K, Miller CJ. Simian-human immunodeficiency virus SHIV89.6-induced protection against intravaginal challenge with pathogenic SIVmac239 is independent of the route of immunization and is associated with a combination of cytotoxic T-lymphocyte and alpha interferon responses. Journal of virology. 2003; 77:3099–3118. [PubMed: 12584336]

9. Veazey RS, Ketas TJ, Dufour J, Moroney-Rasmussen T, Green LC, Klasse PJ, Moore JP. Protection of rhesus macaques from vaginal infection by vaginally delivered maraviroc, an inhibitor of HIV-1 entry via the CCR5 co-receptor. *The Journal of infectious diseases*. 2010; 202:739–744. [PubMed: 20629537]
10. Otten RA, Adams DR, Kim CN, Jackson E, Pullium JK, Lee K, Grohskopf LA, Monsour M, Butera S, Folks TM. Multiple vaginal exposures to low doses of R5 simian-human immunodeficiency virus: strategy to study HIV preclinical interventions in nonhuman primates. *The Journal of infectious diseases*. 2005; 191:164–173. [PubMed: 15609225]
11. Kim CN, Adams DR, Bashirian S, Butera S, Folks TM, Otten RA. Repetitive exposures with simian/human immunodeficiency viruses: strategy to study HIV pre-clinical interventions in non-human primates. *Journal of medical primatology*. 2006; 35:210–216. [PubMed: 16872284]
12. Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS immunology and medical microbiology*. 2003; 38:13–22. [PubMed: 12900050]
13. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *Journal of reproductive immunology*. 2011; 88:185–194. [PubMed: 21353708]
14. Hel Z, Stringer E, Mestecky J. Sex steroid hormones, hormonal contraception, and the immunobiology of human immunodeficiency virus-1 infection. *Endocrine reviews*. 2010; 31:79–97. [PubMed: 19903932]
15. Galvin SR, Cohen MS. The role of sexually transmitted diseases in HIV transmission. *Nature reviews Microbiology*. 2004; 2:33–42. [PubMed: 15035007]
16. Trifonova RT, Bajpai M, Pasieczny JM, Chandra N, Doncel GF, Fichorova RN. Biomarkers of leukocyte traffic and activation in the vaginal mucosa. *Biomarkers*. 2007; 12:608–622. [PubMed: 17852080]
17. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. *Biochimica et biophysica acta*. 2009; 1788:864–871. [PubMed: 18952050]
18. Alfano M, Poli G. Role of cytokines and chemokines in the regulation of innate immunity and HIV infection. *Molecular immunology*. 2005; 42:161–182. [PubMed: 15488606]
19. Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86:2336–2340. [PubMed: 2494664]
20. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *Aids*. 2008; 22:1493–1501. [PubMed: 18614873]
21. Mirmonsef P, Gilbert D, Zariffard MR, Hamaker BR, Kaur A, Landay AL, Spear GT. The effects of commensal bacteria on innate immune responses in the female genital tract. *American journal of reproductive immunology*. 2011; 65:190–195. [PubMed: 21143335]
22. Martin HL, Richardson BA, Nyange PM, Lavreys L, Hillier SL, Chohan B, Mandaliya K, Ndinya-Achola JO, Bwayo J, Kreiss J. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *The Journal of infectious diseases*. 1999; 180:1863–1868. [PubMed: 10558942]
23. Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, Spear GT. Female genital-tract HIV load correlates inversely with *Lactobacillus* species but positively with bacterial vaginosis and *Mycoplasma hominis*. *The Journal of infectious diseases*. 2005; 191:25–32. [PubMed: 15592999]
24. Wira C, Fahey J, Wallace P, Yeaman G. Effect of the menstrual cycle on immunological parameters in the human female reproductive tract. *Journal of acquired immune deficiency syndromes*. 2005; 38(Suppl 1):S34–S36. [PubMed: 15867615]
25. Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *American journal of reproductive immunology*. 2010; 63:544–565. [PubMed: 20367623]

26. Phillips SJ, Curtis KM, Polis CB. Effect of hormonal contraceptive methods on HIV disease progression: a systematic review. *Aids*. 2013; 27:787–794. [PubMed: 23135169]
27. Heffron R, Donnell D, Rees H, Celum C, Mugo N, Were E, de Bruyn G, Nakku-Joloba E, Ngunjiri K, Kiari J, Coombs RW, Baeten JM. Partners in Prevention HSVHIVTST: Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. *The Lancet infectious diseases*. 2012; 12:19–26. [PubMed: 21975269]
28. Curlin ME, Leelawiwat W, Dunne EF, Chonwattana W, Mock PA, Mueanpai F, Thep-Amnuay S, Whitehead SJ, McNicholl JM. Cyclic changes in HIV shedding from the female genital tract during the menstrual cycle. *The Journal of infectious diseases*. 2013; 207:1616–1620. [PubMed: 23417658]
29. Villanueva JM, Ellerbrock TV, Lennox JL, Bush TJ, Wright TC, Pratt-Palmore M, Evans-Strickfaden T, Conley LJ, Schnell C, Hart CE. The menstrual cycle does not affect human immunodeficiency virus type 1 levels in vaginal secretions. *The Journal of infectious diseases*. 2002; 185:170–177. [PubMed: 11807690]
30. Benki S, Mostad SB, Richardson BA, Mandaliya K, Kreiss JK, Overbaugh J. Cyclic shedding of HIV-1 RNA in cervical secretions during the menstrual cycle. *The Journal of infectious diseases*. 2004; 189:2192–2201. [PubMed: 15181566]
31. Reichelderfer PS, Coombs RW, Wright DJ, Cohn J, Burns DN, Cu-Uvin S, Baron PA, Coheng MH, Landay AL, Beckner SK, Lewis SR, Kovacs AA. Effect of menstrual cycle on HIV-1 levels in the peripheral blood and genital tract. WHS 001 Study Team. *Aids*. 2000; 14:2101–2107. [PubMed: 11061650]
32. Spear G, Rothauesler K, Fritts L, Gillevet PM, Miller CJ. In captive rhesus macaques, cervicovaginal inflammation is common but not associated with the stable polymicrobial microbiome. *PloS one*. 2012; 7:e52992. [PubMed: 23285244]
33. Keele BF, Estes JD. Barriers to mucosal transmission of immunodeficiency viruses. *Blood*. 2011; 118:839–846. [PubMed: 21555745]
34. Rowell TE. Behaviour and Female Reproductive Cycles of Rhesus Macaques. *Journal of reproduction and fertility*. 1963; 6:193–203. [PubMed: 14073726]
35. Knobil E. On the control of gonadotropin secretion in the rhesus monkey. Recent progress in hormone research. 1974; 30:1–46. [PubMed: 4210416]
36. Walker ML, Gordon TP, Wilson ME. Menstrual cycle characteristics of seasonally breeding rhesus monkeys. *Biology of reproduction*. 1983; 29:841–848. [PubMed: 6416317]
37. Blakley GB, Beamer TW, Dukelow WR. Characteristics of the menstrual cycle in nonhuman primates. IV. Timed mating in *Macaca nemestrina*. *Laboratory animals*. 1981; 15:351–353. [PubMed: 7341846]
38. Treloar AE, Boynton RE, Behn BG, Brown BW. Variation of the human menstrual cycle through reproductive life. *International journal of fertility*. 1967; 12:77–126. [PubMed: 5419031]
39. Riesen JW, Meyer RK, Wolf RC. The effect of season on occurrence of ovulation in the rhesus monkey. *Biology of reproduction*. 1971; 5:111–114. [PubMed: 5000818]
40. McNicholl JM, Henning TC, Vishwanathan SA, Kersh EN. Non-Human Primate Models of Hormonal Contraception and HIV. *American journal of reproductive immunology*. 2014; 71:513–522. [PubMed: 24716832]
41. Baeten JM, Kahle E, Lingappa JR, Coombs RW, Delany-Moretlwe S, Nakku-Joloba E, Mugo NR, Wald A, Corey L, Donnell D, Campbell MS, Mullins JI, Celum C. Partners in Prevention HSVHIVTST: Genital HIV-1 RNA predicts risk of heterosexual HIV-1 transmission. *Sci Transl Med*. 2011; 3:77ra29.
42. Chakraborty H, Sen PK, Helms RW, Vernazza PL, Fiscus SA, Eron JJ, Patterson BK, Coombs RW, Krieger JN, Cohen MS. Viral burden in genital secretions determines male-to-female sexual transmission of HIV-1: a probabilistic empiric model. *Aids*. 2001; 15:621–627. [PubMed: 11317000]
43. Hild-Petito S, Veazey RS, Lerner JM, Reel JR, Blye RP. Effects of two progestin-only contraceptives, Depo-Provera and Norplant-II, on the vaginal epithelium of rhesus monkeys. *AIDS research and human retroviruses*. 1998; 14(Suppl 1):S125–S130. [PubMed: 9581896]

44. Marx PA, Spira AI, Gettie A, Dailey PJ, Veazey RS, Lackner AA, Mahoney CJ, Miller CJ, Claypool LE, Ho DD, Alexander NJ. Progesterone implants enhance SIV vaginal transmission and early virus load. *Nature medicine*. 1996; 2:1084–1089.
45. Smith SM, Baskin GB, Marx PA. Estrogen protects against vaginal transmission of simian immunodeficiency virus. *The Journal of infectious diseases*. 2000; 182:708–715. [PubMed: 10950763]
46. Sodora DL, Gettie A, Miller CJ, Marx PA. Vaginal transmission of SIV: assessing infectivity and hormonal influences in macaques inoculated with cell-free and cell-associated viral stocks. *AIDS research and human retroviruses*. 1998; 14(Suppl 1):S119–S123. [PubMed: 9581895]
47. Doyle L, Young CL, Jang SS, Hillier SL. Normal vaginal aerobic and anaerobic bacterial flora of the rhesus macaque (*Macaca mulatta*). *Journal of medical primatology*. 1991; 20:409–413. [PubMed: 1803013]
48. Spear GT, Gilbert D, Sikaroodi M, Doyle L, Green L, Gillevet PM, Landay AL, Veazey RS. Identification of Rhesus Macaque Genital Microbiota by 16S Pyrosequencing Shows Similarities to Human Bacterial Vaginosis: Implications for Use as an Animal Model for HIV Vaginal Infection. *AIDS research and human retroviruses*. 2010; 26:193–200. [PubMed: 20156101]
49. Mirmonsef P, Gilbert D, Veazey RS, Wang J, Kendrick SR, Spear GT. A comparison of lower genital tract glycogen and lactic acid levels in women and macaques: implications for HIV and SIV susceptibility. 2012
50. Amedee AM, Nichols WA, LeCapitaine NJ, Stouwe CV, Birke LL, Lacour N, Winsauer PJ, Molina PE. Chronic Delta(9)-tetrahydrocannabinol administration may not attenuate simian immunodeficiency virus disease progression in female rhesus macaques. *AIDS research and human retroviruses*. 2014; 30:1216–1225. [PubMed: 25113915]
51. Loganantharaj N, Nichols WA, Bagby GJ, Volaufova J, Dufour J, Martin DH, Nelson S, Amedee AM. The Effects of Chronic Binge Alcohol on the Genital Microenvironment of SIV-Infected Female Rhesus Macaques. *AIDS research and human retroviruses*. 2014
52. Downs JL, Urbanski HF. Neuroendocrine changes in the aging reproductive axis of female rhesus macaques (*Macaca mulatta*). *Biology of reproduction*. 2006; 75:539–546. [PubMed: 16837643]
53. Du Y, Fan TY, Tan Y, Xiong Z, Wang Z. Seasonal changes in the reproductive physiology of female rhesus macaques (*Macaca mulatta*). *Journal of the American Association for Laboratory Animal Science : JAALAS*. 2010; 49:289–293. [PubMed: 20587158]
54. Kozlowski PA, Lynch RM, Patterson RR, Cu-Uvin S, Flanigan TP, Neutra MR. Modified wick method using Weck-Cel sponges for collection of human rectal secretions and analysis of mucosal HIV antibody. *Journal of acquired immune deficiency syndromes*. 2000; 24:297–309. [PubMed: 11015145]
55. Endtz AW. A rapid staining method for differentiating granulocytes from "germinal cells" in Papanicolaou-stained semen. *Acta cytologica*. 1974; 18:2–7. [PubMed: 4129934]
56. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology*. 1991; 29:297–301. [PubMed: 1706728]
57. Molina PE, Amedee AM, Lecapitaine NJ, Zabaleta J, Mohan M, Winsauer PJ, Vande Stouwe C, McGoey RR, Auten MW, Lamotte L, Chandra LC, Birke LL. Modulation of Gut-Specific Mechanisms by Chronic Delta-Tetrahydrocannabinol Administration in Male Rhesus Macaques Infected with Simian Immunodeficiency Virus: A Systems Biology Analysis. *AIDS research and human retroviruses*. 2014
58. Henning TR, Lacour N, Amedee AM. Efficient methodologies for sensitive HIV-1 RNA quantitation from plasma and vaginal secretions. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*. 2009; 46:309–313. [PubMed: 19783472]
59. Amedee AM, Nichols WA, Robichaux S, Bagby GJ, Nelson S. Chronic alcohol abuse and HIV disease progression: studies with the non-human primate model. *Current HIV research*. 2014; 12:243–253. [PubMed: 25053367]
60. Hart CE, Lennox JL, Pratt-Palmore M, Wright TC, Schinazi RF, Evans-Strickfaden T, Bush TJ, Schnell C, Conley LJ, Clancy KA, Ellerbrock TV. Correlation of human immunodeficiency virus

- type 1 RNA levels in blood and the female genital tract. *The Journal of infectious diseases*. 1999; 179:871–882. [PubMed: 10068582]
61. Ross GT, Cargille CM, Lipsett MB, Rayford PL, Marshall JR, Strott CA, Rodbard D. Pituitary and gonadal hormones in women during spontaneous and induced ovulatory cycles. *Recent progress in hormone research*. 1970; 26:1–62. [PubMed: 4919092]
 62. Eckstein P, Kelly W. A survey of the breeding performance of rhesus monkeys in the laboratory. *Symp Zool Soc Lond*. 1966:91.
 63. Chiazze L Jr, Brayer FT, Macisco JJ Jr, Parker MP, Duffy BJ. The length and variability of the human menstrual cycle. *JAMA : the journal of the American Medical Association*. 1968; 203:377–380. [PubMed: 5694118]
 64. Valerio DA, Pallotta AJ, Courtney KD. Experiences in large-scale breeding of simians for medical experimentation. *Annals of the New York Academy of Sciences*. 1969; 162:282–296. [PubMed: 4979552]
 65. Rodriguez-Garcia M, Patel MV, Wira CR. Innate and adaptive anti-HIV immune responses in the female reproductive tract. *Journal of reproductive immunology*. 2013; 97:74–84. [PubMed: 23432874]
 66. Wira CR, Fahey JV, Rodriguez-Garcia M, Shen Z, Patel MV. Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. *American journal of reproductive immunology*. 2014; 72:236–258. [PubMed: 24734774]
 67. Wira CR, Rodriguez-Garcia M, Patel MV. The role of sex hormones in immune protection of the female reproductive tract. *Nature reviews Immunology*. 2015; 15:217–230.
 68. Wira CR, Fahey JV. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *Aids*. 2008; 22:1909–1917. [PubMed: 18784454]
 69. Baeten JM, Nyange PM, Richardson BA, Lavreys L, Chohan B, Martin HL Jr, Mandaliya K, Ndinya-Achola JO, Bwayo JJ, Kreiss JK. Hormonal contraception and risk of sexually transmitted disease acquisition: results from a prospective study. *American journal of obstetrics and gynecology*. 2001; 185:380–385. [PubMed: 11518896]
 70. Kaushic C, Ashkar AA, Reid LA, Rosenthal KL. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. *Journal of virology*. 2003; 77:4558–4565. [PubMed: 12663762]
 71. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunological reviews*. 2005; 206:306–335. [PubMed: 16048557]
 72. Hadzic SV, Wang X, Dufour J, Doyle L, Marx PA, Lackner AA, Paulsen DB, Veazey RS. Comparison of the vaginal environment of *Macaca mulatta* and *Macaca nemestrina* throughout the menstrual cycle. *American journal of reproductive immunology*. 2014; 71:322–329. [PubMed: 24521395]
 73. Poonia B, Walter L, Dufour J, Harrison R, Marx PA, Veazey RS. Cyclic changes in the vaginal epithelium of normal rhesus macaques. *The Journal of endocrinology*. 2006; 190:829–835. [PubMed: 17003283]
 74. Vishwanathan SA, Guenther PC, Lin CY, Dobard C, Sharma S, Adams DR, Otten RA, Heneine W, Hendry RM, McNicholl JM, Kersh EN. High susceptibility to repeated, low-dose, vaginal SHIV exposure late in the luteal phase of the menstrual cycle of pigtail macaques. *Journal of acquired immune deficiency syndromes*. 2011; 57:261–264. [PubMed: 21546848]
 75. Kersh EN, Henning T, Vishwanathan SA, Morris M, Butler K, Adams DR, Guenther P, Srinivasan P, Smith J, Radzio J, Garcia-Lerma JG, Dobard C, Heneine W, McNicholl J. SHIV susceptibility changes during the menstrual cycle of pigtail macaques. *Journal of medical primatology*. 2014
 76. Fehring RJ, Schneider M, Raviele K. Variability in the phases of the menstrual cycle. *J Obstet Gynecol Neonatal Nurs*. 2006; 35:376–384.

77. Lenton EA, Landgren BM, Sexton L. Normal variation in the length of the luteal phase of the menstrual cycle: identification of the short luteal phase. *British journal of obstetrics and gynaecology*. 1984; 91:685–689. [PubMed: 6743610]
78. Jespers V, Harandi AM, Hinkula J, Medaglini D, Le Grand R, Stahl-Hennig C, Bogers W, El Habib R, Wegmann F, Fraser C, Cranage M, Shattock RJ, Spetz AL. Assessment of mucosal immunity to HIV-1. *Expert Rev Vaccines*. 2010; 9:381–394. [PubMed: 20370549]
79. Castle PE, Rodriguez AC, Bowman FP, Herrero R, Schiffman M, Bratti MC, Morera LA, Schust D, Crowley-Nowick P, Hildesheim A. Comparison of ophthalmic sponges for measurements of immune markers from cervical secretions. *Clin Diagn Lab Immunol*. 2004; 11:399–405. [PubMed: 15013994]
80. Promadej-Lanier N, Smith JM, Srinivasan P, McCoy CF, Butera S, Woolfson AD, Malcolm RK, Otten RA. Development and evaluation of a vaginal ring device for sustained delivery of HIV microbicides to non-human primates. *Journal of medical primatology*. 2009; 38:263–271. [PubMed: 19476564]
81. Hillier SL. Diagnostic microbiology of bacterial vaginosis. *American journal of obstetrics and gynecology*. 1993; 169:455–459. [PubMed: 8357044]
82. Hillier SL, Krohn MA, Nugent RP, Gibbs RS. Characteristics of three vaginal flora patterns assessed by gram stain among pregnant women. *Vaginal Infections and Prematurity Study Group*. *American journal of obstetrics and gynecology*. 1992; 166:938–944. [PubMed: 1372474]
83. Sha BE, Chen HY, Wang QJ, Zariffard MR, Cohen MH, Spear GT. Utility of Amsel criteria, Nugent score, and quantitative PCR for *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *Journal of clinical microbiology*. 2005; 43:4607–4612. [PubMed: 16145114]
84. Mirmonsef P, Krass L, Landay A, Spear GT. The role of bacterial vaginosis and trichomonas in HIV transmission across the female genital tract. *Current HIV research*. 2012; 10:202–210. [PubMed: 22384839]
85. Thurman AR, Doncel GF. Innate immunity and inflammatory response to *Trichomonas vaginalis* and bacterial vaginosis: relationship to HIV acquisition. *American journal of reproductive immunology*. 2011; 65:89–98. [PubMed: 20678168]
86. Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, Wabwire-Mangen F, Serwadda D, Li C, Kiwanuka N, Hillier SL, Rabe L, Gaydos CA, Quinn TC, Konde-Lule J. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet*. 1997; 350:546–550. [PubMed: 9284776]
87. Ellerbrock TV, Lennox JL, Clancy KA, Schinazi RF, Wright TC, Pratt-Palmore M, Evans-Strickfaden T, Schnell C, Pai R, Conley LJ, Parrish-Kohler EE, Bush TJ, Tatti K, Hart CE. Cellular replication of human immunodeficiency virus type 1 occurs in vaginal secretions. *The Journal of infectious diseases*. 2001; 184:28–36. [PubMed: 11398106]
88. Cu-Uvin S, DeLong AK, Venkatesh KK, Hogan JW, Ingersoll J, Kurpewski J, De Pasquale MP, D'Aquila R, Caliendo AM. Genital tract HIV-1 RNA shedding among women with below detectable plasma viral load. *Aids*. 2010; 24:2489–2497. [PubMed: 20736815]
89. Neely MN, Benning L, Xu J, Strickler HD, Greenblatt RM, Minkoff H, Young M, Bremer J, Levine AM, Kovacs A. Cervical shedding of HIV-1 RNA among women with low levels of viremia while receiving highly active antiretroviral therapy. *Journal of acquired immune deficiency syndromes*. 2007; 44:38–42. [PubMed: 17106279]
90. Homans J, Christensen S, Stiller T, Wang CH, Mack W, Anastos K, Minkoff H, Young M, Greenblatt R, Cohen M, Strickler H, Karim R, Spencer LY, Operskalski E, Frederick T, Kovacs A. Permissive and protective factors associated with presence, level, and longitudinal pattern of cervicovaginal HIV shedding. *Journal of acquired immune deficiency syndromes*. 2012; 60:99–110. [PubMed: 22517416]
91. Blish CA, McClelland RS, Richardson BA, Jaoko W, Mandaliya K, Baeten JM, Overbaugh J. Genital Inflammation Predicts HIV-1 Shedding Independent of Plasma Viral Load and Systemic Inflammation. *Journal of acquired immune deficiency syndromes*. 2012; 61:436–440. [PubMed: 22878424]

Figure 1. Menstrual Cycle Characteristics of Twenty Indoor Housed Rhesus Macaques

(a) Range of observed menstrual cycle lengths for individual animals; cohort median length depicted by the dotted line. Range of cycle length is depicted with box and whisker plots (min, max); solid line indicates the median value. (b) Histogram of the cycle length distribution of 269 observed cycles. (c) Median cycle length (days) observed throughout the calendar year and respective range in cycle duration based on month of menses onset. Number (n) of cycles observed is indicated below.

Figure 2. Quantification of Inflammatory Markers in the Female Rhesus Macaque Vaginal Mucosa

Protein concentrations (pg/mg protein) of cytokines and chemokines in vaginal secretions evaluated with a cytometric bead array. (a) Samples obtained from 10 uninfected macaques, with 6 animals repeatedly sampled, providing a total of 18 specimens. (b) Samples obtained 8–16 weeks post-SIV from 10 animals. (c) Polymorphonuclear cells (PMNs) in genital secretions (identified with Endtz stain) collected prior to (n=22) and post-SIV infection (n=12) (p=0.26). Values represent total cell numbers observed in vaginal swab specimen. Unique symbols correspond to specific animals as identified in the insert.

Figure 3. Nugent Score and Relative Abundance of Vaginal Bacterial Morphotypes in Genital Samples

Macaque vaginal microbiota was characterized using Gram stained smears (n=27) from 17 uninfected macaques prior to treatment (a) Nugent score distribution. (b) Relative abundance of detected bacterial morphotypes in naïve and SIV-infected animals. (c) Relative abundance of detected morphotypes per individual animal. Duplicate samples from 10 animals were obtained 2–4 weeks apart to assess stability. Cycle phase at time of sample collection is indicated below. Proliferative (P), Secretory (S) phase of menstrual cycle; gram-positive cocci (GPC), gram-positive diplococci (GPDC), small gram-variable rods (SGVR), curved gram-negative rods (CGNR), large gram-positive rods (LGPR).

Figure 4. Genital SIV Shedding Over the Initial Course of Infection

Vaginal fluids of control animals (cohorts I and II) were serially sampled over 10 weeks post-SIV infection, for a total of 91 specimens. (a) Vaginal SIV levels and (b) Plasma:vaginal viral load ratio throughout the acute phase of infection. (c) Correlation of serum progesterone level and vaginal viral load; data analyzed with Spearman's rank correlation coefficients ($r=0.047$, $p=0.7656$).

Table I

Cohort I and II Demographics

Monkey ID	Experimental Group	Age (years)	Weight (kg)	Route of Inoculation	Gravidity (#)	Median Cycle Length (days)	Pre-SIV Nugent Score	Set Point Vaginal Viral Load (log SIV copies/vaginal sample) ^a
Cohort I								
A	Treatment A	3.8	4.52	IV	0		N/A	
B	Treatment A	3.8	4.64	IV	0		4	
C	Treatment A	3.8	5.02	IV	0		4	
D	Treatment A	3.7	5.10	IV	0		6	
E	Control	3.9	4.94	IV	0	26.5	3	1.76
F	Control	3.8	5.08	IV	0	29	5	1.56
G	Control	3.7	6.18	IV	0	27.5	9	1.55
H	Control	3.7	4.74	IV	0	30	3	1.55
Cohort II								
I	Treatment B	8.8	5.60	IR	4	27	N/A	
J	Treatment B	8	8.20	IR	3	27	N/A	
K	Treatment B	3.7	4.40	IR	0	29	5	
L	Treatment B	3.5	5.15	IR	0	23	5	
M	Treatment B	5.8	5.20	IR	0	19	N/A	
N	Treatment B	4.7	8.52	IR	0	26	7	6
O	Treatment B	3.9	5.70	IR	0	31.5	6	6
P	Treatment B	3.8	5.55	IR	0	29	5	5
Q	Control	3.9	5.50	IR	0	36	3	2.80
R	Control	9.8	7.80	IR	2	39	10	9
S	Control	3.8	5.65	IR	0	24	9	1.40*
T	Control	4.0	4.70	IR	0	29	7	1.84*
U	Control	7.9	7.15	IR	2	26.5	N/A	2.43
V	Control	8.9	5.60	IR	2	31	N/A	1.40
W	Control	7.9	6.70	IV	2	26	N/A	1.75
X	Control	3.8	5.00	IV	0	18	10	2.35

Notes: Blank cells represent samples not included due to potential treatment effect.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

N/A for samples not available; IV, intravenous; IR, intrarectal
Average VL between 7 and 10 weeks pi
* progesterone data unavailable

Table II

Baseline cytokine levels (pg/mg protein) in vaginal secretions collected from eight rhesus macaque.

Cytokine	Median	Mean	Minimum	Maximum	SD	SEM	Lower 95% CI	Upper 95% CI
IFN γ	1.61	2.20	0.83	5.79	1.78	0.63	0.72	3.69
IL-1 β	3.03	5.47	0.35	24.18	7.93	2.80	-1.16	12.10
IL-1Ra	645.1	715.1	107.4	1542	535.7	189.4	267.3	1163
IL-6	0.47	1.01	0.16	3.8	1.26	0.44	-0.04	2.06
MCP-1	43.31	107.9	19.48	478.9	154.6	54.66	-21.36	237.2
TNF- α	33.1	40.31	4	98.05	32.76	11.58	12.92	67.70
IL-4	3.93	5.18	1.89	11.57	3.66	1.29	2.12	8.24
IL-8	1234	1329	237.6	2733	819.8	289.8	643.4	2014
MIP-1 α	2.17	4.74	0.31	18.78	6.26	2.21	-0.50	9.97
IL-10	3225.1	736.2	49.58	2326	830.6	293.7	41.86	1431

SD, standard deviation

SEM, standard error of the mean