

Differential susceptibility of HIV strains to innate immune factors in human cervical-vaginal secretions

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Abstract: The female reproductive tract (FRT) is protected by innate and adaptive immune mechanisms, which work in concert to defend against human immunodeficiency virus (HIV) and other sexually transmitted infections (STIs). Under the control of sex hormones throughout a woman's life, the immune system in the FRT has evolved to meet the challenges of protection against STIs, coupled with the need to sustain the development of new life. The studies presented in this review focus on the threat of HIV infection and the levels of protection present in the FRT during the menstrual cycle. Studies from our laboratory and others, examined the presence and variability of immune components against viral infection in the FRT. Our findings indicate that there are some factors in the FRT secretions that inhibit and enhance infectivity of individual strains of HIV. Given the complexities of hormonal regulation, identification of the elements involved in susceptibility to and protection against HIV in women must involve a careful analysis of transmitted viruses and a clear understanding of immune protection in the FRT.

Keywords: HIV susceptibility, CVL

HIV and the human female reproductive tract

With 25 million deaths and an additional 33.4 million people (of which approximately 50% are female) currently infected worldwide, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) has become one of the world's worst pandemics (UNAIDS 2009 Global Report).¹ More than 80% of the new HIV infections are acquired through heterosexual transmission,¹ and women are more likely than men to be infected in heterosexual intercourse.² Despite these findings, the rate of HIV transmission in women per coital act remains strikingly low at 1:122–1:1,000.^{3–5} We and others have reached the conclusion that in addition to viral load,⁶ exposure time following seroconversion,⁷ and preexistence of other sexually transmitted infections (STIs),^{8–10} the immune protection in the human female reproductive tract (FRT) influences the transmission of HIV.¹¹

The lower FRT (cervix and vagina) is generally considered a primary site of HIV-1 infection, but recent observations suggest that the upper FRT (uterus and fallopian tube) might also be a portal for the entry of HIV-1 following sexual intercourse. Depending on the site analyzed, factors that play a role in inhibiting or enhancing infectivity include coinfections, microabrasions, secreted immune factors, genetics, and menstrual status, all of which affect the local immune system. In addition, disease state and local viral load of the donor are very important determinants.¹² Genetic, behavioral, and economic risk factors also play a major role in determining susceptibility to HIV.

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HIV laboratory strains and transmitted viruses

To completely understand how the HIV infects, researchers have taken advantage of the available viral isolates, some of which were adapted to grow in cell culture. These HIV strains, as well as clinical isolates, are often characterized by their preferential attachment to the target cells that express CXCR4 chemokine coreceptors (X4), CCR5 chemokine coreceptors (R5), or both (X4/R5). Only recently has HIV research undergone a paradigm shift. By investigating HIV patients early after infection, Hahn, Shaw, and colleagues used single genome amplification to identify transmitted/founder viruses that were responsible for heterosexual infection.¹³ It is generally accepted that the donor seminal fluid contains multiple HIV variants, among which only the macrophage-tropic/R5 viruses cause infection. This was thought to be due to T-cell-tropic/X4 virus recognition and inhibition by mucosal epithelial cells (ECs), which is evaded by R5 viruses, which pass through the ECs via transcytosis without getting detected.¹⁴ Hahn and Shaw's studies led to the concept of a bottleneck for the sexual transmission of HIV, through which a single variant of R5 transmitted/founder virus traverses the mucosal barrier to infect women.¹⁵ Current studies are directed toward understanding the nature of these transmitted or founder viruses as to what makes them successful in invading the host.

The mucosal immune system of the FRT: layers of protection against HIV infection

The mucosal immune system in the FRT contains an array of protective mechanisms that extends through both the upper and the lower tracts. The immune protection is provided by both the innate and the adaptive immune systems. These systems consist of resident ECs, supportive stromal cells, and immune cells, which migrate into the uterus, cervix, and vagina. Among those cells pivotal in conferring immune protection, ECs are found to be pluripotential with abilities to confer protection against HIV. ECs, in addition to providing barrier protection, transport immunoglobulins (IgA and IgG) into FRT secretions and produce antimicrobials that are both bactericidal and viricidal.^{16,17} Through the production of cytokines and chemokines, these cells signal the recruitment and activation of other cells of the innate and adaptive immune systems that are resident throughout the FRT. Our goal in the following sections is to highlight some of the abilities of these epithelial and immune cells in the FRT in protecting against HIV and to point out that all these cells

are responsive to sex hormones. In the second part of this review, special emphasis will be placed on HIV and our evolving understanding of the role of the FRT in protecting against viral infection via the FRT.

Barrier and pH protection

As the first cells to make contact with the pathogens, the columnar ECs of the endocervix, uterus, and fallopian tubes are polarized and form tight junctions that physically prevent pathogens from entering the body. In contrast, squamous ECs lining the lower tract (vagina and ectocervix) do not form tight junctions, but have multiple layers of cornified cells that retard pathogen penetration. In addition tight junctions, pathogens encountering the lower tract are exposed to an acidic pH that is maintained by commensal bacteria in healthy women. The envelope glycoprotein (gp120) of HIV is particularly sensitive to an acidic environment.^{18,19} Interestingly, gp120 of certain strains of HIV are more sensitive to acidic pH than other strains, which might allow some strains to survive and infect better than others.^{18,20} The loss of vaginal acidity and/or commensals is known to make women more susceptible to pathogens.²¹ Mucus overlaying the EC in the FRT also can trap HIV and prevent infection of underlying target cells or transcytosis through an epithelial layer.²²

Protection provided by secreted immune factors

Another level of protection is provided by the innate immune system that secretes cytokines, chemokines, and antimicrobials both constitutively and in response to pathogenic challenge. To respond to pathogens, immune cells in the upper and lower FRT express functional pathogen-recognition receptors including toll-like receptors, NOD-like receptors, and RIG-like helicases (RIG-1 and MDA5)^{23–27} (Ghosh et al unpublished results). The response to pathogens is mediated through the secretion of antimicrobials, cytokines, and chemokines. Several antimicrobial molecules have been reported in FRT secretions, which include α and β defensins, secretory leukocyte protease inhibitor (SLPI), elafin, and LL37, all of which inhibit HIV.^{27–32} Some factors such as MIP3 α function as a dual mediator that has both antibacterial and antiviral activities, as well as chemokine activity, which attracts T cells and immature dendritic cells (DCs) to the site of pathogenic invasion.^{33,34} Secreted immune factors can therefore act as recruiters or activators of other cells, some of which are targets for HIV infection.

Intracellular antiviral activity

In addition to the protection provided by the secreted immune factors, there exists a well-developed system of intracellular antiviral protection within the FRT. Within ECs and macrophages, for example, intracellular protection is interferon mediated. Upon viral infection, the interferons upregulate genes such as MxA, 2,5-oligoadenylate synthetase (OAS), and RNA-activated protein kinase (PKR). As discussed in previous studies, these genes belong to a large family of interferon-stimulated genes that inhibit multiple viruses at various stages of their life cycle.^{35–39} Some studies indicate that HIV-1 uses immune escape mechanisms to evade these intracellular antivirals, suggesting that these pathways are important for preventing successful infection by HIV.⁴⁰

Innate immune cells

Innate immune cells involved in FRT immune responses include the DC, macrophages, natural killer (NK) cells, and neutrophils. Found throughout the FRT,⁴¹ these cells are phenotypically and functionally distinct from their counterparts in the peripheral circulation and/or tissues.^{16,42,43} For example, cell–cell interactions in tissue microenvironments determine if the DCs adopt immunogenic or tolerogenic features. We recently found that the secretions of uterine ECs lower the DC expression of CD80 and CD86, reduce immunogenic cytokine secretion, enhance IDO expression, and decrease DC capability to induce allogeneic T-cell proliferation, all characteristic of tolerogenic DC.⁴⁴ Tolerogenic DCs may be critical for optimal reproductive function of the uterus and for the sustained presence of DCs in the uterus. Uterine macrophages are distinct from most monocyte-derived tissue macrophages. Circulating monocytes migrate into tissues and differentiate into macrophages that can participate in responses to pathogens by secreting proinflammatory effectors, such as interleukin-1 β (IL-1 β). However, monocyte-derived macrophages, as well as intestinal and alveolar macrophages, are deficient in their ability to release mature, active IL-1 β in response to lipopolysaccharide stimulation. Inefficient processing of precursor IL-1 β by these cells is due to the lack of caspase-1 (IL-1 β -converting enzyme) activity. In contrast to other macrophages, uterine macrophages have caspase-1 activity, which enable the secretion of mature IL-1 β that facilitates implantation and mediates protection from pathogens.⁴⁵ Thus, primary uterine macrophages are unique among other tissue macrophages. NK cells are known to “kill” tumor cells and secrete cytokines that link the innate and adaptive immune systems. Uterine NK cells have a unique phenotype compared with blood NK

cell subsets.⁴⁶ For example, NK cells in the fallopian tubes, endometrium, cervix, and ectocervix are CD56+bright and express CD9, while blood NK cells are CD56+dim and do not express CD9.⁴⁷ Alterations in the physiological levels of NK cells in the FRT have been linked to a number of clinical conditions leading to reproductive failure.⁴⁸ We have demonstrated that NK cells are present in various FRT tissues and that their phenotypes and regulation largely depend on the FRT tissue where they reside.⁴⁷ Furthermore, the number of NK cells varies with the menstrual cycle in the endometrium, but not in the cervix or ectocervix. Recently, using Affymetrix microarrays with probes representing approximately 47,400 transcripts, we showed that human decidual NK cells from gravid uteri and NK cells from cycling endometrium are distinct NK cell subsets.⁴⁹ These data suggest that a unique local environment in FRT tissues may account for the recruitment of different NK cell subsets. We also compared neutrophils derived from fallopian tubes with neutrophils isolated from peripheral blood. Fallopian tube neutrophils express significantly higher levels of CD64, human class II histocompatibility antigen DR, gamma-interferon, and vascular endothelial growth factor than those from peripheral blood.⁴³ Fewer fallopian tube neutrophils expressed IL-8 receptors compared with blood neutrophils, while more express the receptor for the bacterial-derived chemoattractant formyl-Met-Leu-Phe. The number of fallopian tube neutrophils containing the granule proteins matrix metalloproteinase-9, lactoferrin, and myeloperoxidase decreased relative to those in blood. Thus, macrophages, NK cells, and neutrophils exhibit a phenotype distinct from their blood counterparts, suggesting functional activation of innate immune defense that is unique to the FRT. Each of these cells in the FRT contributes to protection against pathogens, including HIV. As professional antigen-presenting cells in the FRT, macrophages and DCs capture HIV prior to their transfer to the T cells.⁵⁰ Macrophages secrete a number of chemokines, such as MIP-1 α and MIP-1 β that block HIV from binding to HIV coreceptors. NK cells along with neutrophils are known to secrete antimicrobials that inhibit HIV and other pathogens that threaten reproductive tract homeostasis.

Adaptive immune cells

The adaptive immune system has evolved to elicit specific defense mechanisms. An effective immune response to pathogens requires that the antigen-presenting cells process the antigen from a pathogen and present it to the T cells, thereby inducing T-cell activation. Following antigen

presentation, lymphocyte effector functions, including cytokine production, cytotoxicity, and antibody synthesis, are activated. Protection is mediated through antibodies produced by B cells (humoral immunity) or the destruction of specific pathogens directly or indirectly by T cells (cell-mediated immunity). The cell surface proteins are identified on T cells and are used routinely to identify all T cells (CD3) or T-cell subgroups that help either in the production of antibodies (CD4) or in the killing of infected cells (CD8). CD4⁺ T cells are believed to be the first cells to be infected in the FRT upon sexual transmission.⁵¹ With regard to HIV exposure, pathogen-specific antibodies (IgG and IgA) and cytotoxic T lymphocytes have been identified to play a major role in the protection of the FRT mucosa.^{52,53}

Hormonal regulation of innate and adaptive immune responses

It is not widely recognized that all the aspects of immune protection in the FRT discussed earlier are under hormonal influence.^{11,16} Control of local mucosal immune function by sex hormones, estradiol and progesterone, is precisely regulated to ensure successful fertilization and pregnancy, at the same time conferring protection against STIs, which threaten to compromise women's reproductive health and survival. Based on our previous studies and those of others, we found a pattern of local immune suppression of some but not all aspects of the immune system. These findings led us to propose the existence of a window of vulnerability extending to both the upper and the lower FRT, which, although essential for successful reproduction, makes women more vulnerable to infections during the middle of the menstrual cycle (days 14–21), when the reproductive tract is prepared to accept an immunologically distinct conceptus or fetus.^{11,54} Under these circumstances, HIV and other STIs may take advantage of this temporary modified "tolerogenic" environment to infect cells and cause illness.¹¹

Anti-HIV immune factors in cervical vaginal lavages and differential susceptibility of HIV strains

A valuable and noninvasive tool, used in our laboratory, for studying immune responses in the FRT is the collection of cervical vaginal lavages (CVLs) that can then be used for a number of different assays. In our studies, the cervical vaginal area was rinsed with 10 mL of pH-neutral saline prior to the collection of CVL samples. Later, the samples were centrifuged for the recovery of fluid and cellular components. The supernatants were collected from both HIV(–) women

(healthy) and HIV(+) women (healthy, not on antiretrovirals) and were used to determine the extent to which they neutralize HIV-1 infection of a TZM-bl indicator cell line. We used 4 reference viruses (X4: IIB, NL4.3, R5: BaL, and YU-2c) and the R5 transmitted/founder virus CH077.c. As shown in the upper portion of Table 1, CVL from some healthy HIV(+) patients (A, B, C) had broad-spectrum inhibitory activity against all HIV strains tested, including the transmitted virus. Unexpectedly, we also found a tremendous variability in the neutralizing capacity of a given CVL sample toward different strains of HIV. For example, some healthy HIV(+) CVL samples were found to be highly inhibitory against one HIV-1 strain, whereas the same CVL failed to inhibit another strain (Table 1: D–F). Moreover, we found a few CVL (Table 1: G–I) from the same cohort that enhanced infection of several HIV strains, including the transmitted virus (Table 1).

When we compared the mean percent inhibition between the HIV(+) and HIV(–) groups, the results differed depending on the viral strain tested. IIB (X4), NL4.3 (X4), and BaL (R5) were similarly inhibited by CVL from HIV(+) and HIV(–) individuals. In contrast, activity against YU-2.c (R5) and CH077 (R5, transmitted/founder) was significantly higher in HIV(–) CVL than in HIV(+) CVL (data not shown). Given the number of immune factors present in the CVL,⁵⁴ our data suggest that different factors have differential activity against the strain of HIV tested. Specifically, in our study, we measured protein concentrations of HBD2, MIP3 α , SLPI, and elafin in the HIV(+) CVL and found

Table 1 CVL that enhanced infection of several HIV strains

Patient no.	X4 (% inhibition)		R5 (% inhibition)		
	IIB	NL4.3	BaL	YU-2.c	CH077.c
CVL with broad inhibitory activity					
A	100	100	98	82	78
B	100	99	90	91	96
C	95	100	86	92	96
Mean	98	100	91	88	90
SEM	2	0	4	3	6
CVL with variable inhibitory activity					
D	63	46	14	0	36
E	79	0	15	65	41
F	0	69	–32	82	63
Mean	47	38	–1	49	47
SEM	24	20	16	25	8
CVL with enhancing activity					
G	–60	–46	–58	–86	–43
H	11	–20	14	–45	–6
I	–61	29	–65	25	–31
Mean	–37	–12	–36	–35	–27
SEM	24	22	25	32	11

that MIP3 α levels correlated with anti-HIV activity against all 3 R5 viruses, but not the X4 viruses. However, HBD2 concentrations were correlated with IIIB and BaL, but not YU-2c, NL4.3, and CH077 (data not shown; Ghosh et al unpublished results).

Some of the known anti-HIV factors present in CVL are SLPI and defensins.^{55–57} Previous studies have shown that these factors can operate through multiple mechanisms. SLPI can block HIV fusion and entry by interacting with cell surface phospholipid-binding proteins annexin II and phospholipid scramblase 1 and 4.^{58,59} HBD2 and HBD3 have been shown to inhibit HIV infection through direct interaction, modulation of CXCR4 coreceptor,⁶⁰ and post – reverse transcription mechanisms.⁶¹ In contrast, alpha defensins inhibit HIV infection by interfering with the binding of envelope protein gp120 to the CD4 receptor.⁶² HBD2 and HBD3 have been shown to inhibit X4 HIV more efficiently compared with R5 strains of HIV.⁶⁰ SLPI is known to inhibit both X4 and R5 strains of HIV with reduced inhibitory capacity toward strains with broad coreceptor usage.⁶³ Data from our laboratory, however, indicate that HIV inhibition by factors in the CVL is likely to be more complex than viral tropism. The 12–20 innate immune mediators present in the FRT secretions vary with the stage of the menstrual cycle.^{11,54} For example, King et al³⁰ have shown that in the human endometrium, HBD1, HBD3, and SLPI are maximally expressed during the secretory phase; HBD4 is expressed highest during the proliferative phase; whereas HBD2 and elafin expressions peak during menstruation. This might explain the kind of variability in inhibition that we observed in our samples. The studies in progress in our laboratory are designed to identify the factor(s) in CVL from healthy women responsible for anti-HIV activity at different stages of the menstrual cycle.

Another explanation for the differential susceptibility observed among HIV strains in our study might be due to the differences in biological activity of anti-HIV molecules found in CVL. Many of these molecules are expressed as precursor proteins that must be cleaved for activation and/or release in secretions. Both defensins and elafin fall into this category. HD5 cleavage by trypsin is essential for both chemotactic and antimicrobial functions.⁶⁴ Elafin is expressed as trappin-2, attached to the cell surface. Upon activation by tryptase, it is released as elafin into the secretions.⁶⁵ In this case, both the cell-surface-attached and the secreted forms are antimicrobial. Multiple families of proteases are abundant in the secretions of the reproductive tract and are specific in their actions to activate and deactivate immune factors. The cathepsin

family of proteases is such an example. The cathepsins regulate the family of matrix metalloproteases, which are themselves responsible for activating and deactivating innate immune factors including the anti-HIV molecules CXCL12 and HNP1.^{66–69} Cathepsins are also responsible for directly regulating anti-HIV innate immune factors.^{70–72} For example, cathepsin D, a cysteine protease present in vaginal secretions,⁷³ has been shown to enhance HIV replication.^{74,75} Although the mechanisms are unclear, it is known that cathepsin D inhibits MIP3 α ,⁷⁰ a known anti-HIV factor in CVL.³⁴ Kallikreins (KLKs) are another family of serine proteases present in the genital mucosa that can activate/deactivate multiple immune factors in the FRT.^{76,77} For example, KLK5 has been shown to regulate the antimicrobial activity of LL37, a potent anti-HIV molecule.^{78,79} CD26/dipeptidyl peptidase IV is a serine protease responsible for the cleavage and inactivation of chemokines, such as regulated on activation, normal T cell expressed and secreted (RANTES) and stromal cell-derived factor-1 (SDF1), which are involved in blocking HIV entry.^{80,81} As discussed previously, many of these chemokines, including MIP3 α , MIP1 α , MIP1 β , RANTES, and SDF1, are antimicrobials with anti-HIV activity.^{34,82,83} Another level of complexity, beyond their ability to activate and inactivate FRT antimicrobials is that these protease families are regulated throughout the menstrual cycle by protease inhibitors present in the secretions. Several protease inhibitors, such as SLPI and elafin, are also known anti-HIV molecules.^{84–86} Overall, these findings indicate the several levels of complexity present in the FRT that have to be addressed as we identified the molecules in CVL responsible for anti-HIV immune protection.

Although hormone regulation plays a key role in the immune response to HIV (and other STI) infection of the FRT, it is as yet unclear whether estradiol and progesterone directly affect HIV replication/infection. Some studies have shown that estradiol can bind to the estrogen response element located within the HIV long terminal repeat and can enhance viral transcription directly.^{87,88} However, whether this takes place *in vivo* is unclear. Several studies have shown correlations between women taking oral contraceptives (OC) and an increased risk of seroconversion;^{89–91} however, others have failed to find any correlation.^{92,93} Higher progesterone levels, such as in pregnancy and OC usage, which change the commensal flora in the vagina, are believed to be risk factors for acquiring HIV.⁹⁴ In macaque studies, progesterone treatment increased viral acquisition, presumably through thinning of the vaginal epithelium that develops with OC usage.⁹⁵ This same study also found protective effects

of estrogen in terms of HIV acquisition. A recent study has shown that high progesterone, as found in pregnancy, can enhance the expression of CCR5 (HIV coreceptor for the sexually transmitted R5 HIV strains) on CD14⁺ and CD3⁺ HIV target cells in both peripheral blood mononuclear cells and vaginal biopsy samples.⁹⁶

Enhancement of HIV infection by immune factors in CVL

In our studies (Table 1), several CVL samples (20%) were found to contain factors that enhanced HIV-1 infection of TZM-bl cells. Enhancement of HIV infection of target cells in the presence of mucosal fluids has been described previously.^{97–100} Several studies have found a correlation of plasma viral load (PVL), as well as genital tract viral load (GTVL), with enhanced viral infection of target cells.^{101,102} Other studies, however, have shown that the presence of infectious virus in CVL is not related to the viral load in periphery or genital tract as measured typically by polymerase chain reaction (PCR).¹⁰³ In our studies (Ghosh et al unpublished results), we found that out of 57 HIV(+) women with both high and low PVL and GTVL measured by PCR, only 3 were capable of infecting TZM-bl cells. There were no correlations among viral load and the ability to infect TZM-bl cells in these 3 samples, although all had existing coinfections (2, BV; 1, Trichomonas). These data suggest that *in vivo* GTVL might not correlate with the shedding of infectious virus, which would be the primary determining factor in sexual transmission. This observation confirms the findings of others.^{104,105}

Bacterial and viral coinfections are important cofactors for HIV transmission and infection. Bacterial vaginosis is the most well-described cofactor that has been shown to enhance HIV replication.^{106,107} Infection by herpes simplex virus, Chlamydia, and other sexually transmitted pathogens has also been described to play a role in enhancing HIV infection and transmission.^{8,9} It is generally accepted that coinfecting pathogens result in a localized immune response and environment that favors HIV replication.^{10,106} Specific factors known to directly stimulate viral replication include proinflammatory cytokines, such as IL-6, tumor necrosis factor- α , and IL1 β .^{108–111} Other mucosal innate immune factors that have been reported to have an enhancing effect on viral replication include mucosal factor MRP8^{98,112} and scavenger receptor gp340.¹⁰⁰ Similarly, Levinson et al¹¹³ and Kaul et al⁹ demonstrated that the presence of anti-HIV molecules, such as RANTES and defensins, in genital secretions can enhance viral replication by attracting target cells that can be infected by HIV. Cathepsin D has been shown to enhance

HIV replication^{74,75} possibly through inhibition of MIP3 α , a known anti-HIV factor present in CVL.³⁴ It is also known that cathepsin D and its receptor are regulated by estradiol.¹¹⁴

Overall, the challenge in determining the exact mechanisms by which HIV infection is inhibited or enhanced in a given CVL sample lies in the sheer number of innate immune factors and their regulators present in CVL and the extent to which they are present throughout the menstrual cycle. The closest to come in examining these complexities was a study undertaken to deplete all cationic components of CVL (given that several of the innate anti-HIV factors are cationic in nature).¹¹⁵ Although depletion of cations resulted in the loss of anti-HIV activity, the complexities of hormonal effects were not taken into account. The fact that many of these factors affect HIV strains in a differential manner further emphasizes the need for a thorough examination of the antimicrobials present, their biological activities, and relative contribution to anti-HIV activity under various endocrine conditions.

Conclusion

Our goal in this review was to examine the spectrum of innate factors in the FRT that most likely contributes to the differential susceptibility to infection of target cells with HIV-1 strains. The FRT is a complex tissue with fine-tuned mechanisms of immune regulation that optimize conditions for fertilization and pregnancy, while providing protection against pathogens. Although the HIV pandemic continues to expand with women being affected disproportionately, we have yet to fully understand what makes a woman more or less susceptible to HIV infection. With the flurry of recent publications on transmitted/founder viruses, there is intensive interest in identifying the unique properties of these viruses that enable infection through the genital mucosa. Our findings presented in this review indicate that there are factors in the FRT secretions that differentially affect individual strains of HIV. Given the complexity of hormonal regulation of these factors and their regulators in CVL, identification of the elements involved in enhanced susceptibility of women to specific strains of HIV will involve both careful analysis of transmitted viruses and a clear understanding of immune protection in the FRT. Our findings suggest that the identification of biologically active anti-HIV immune molecules of the FRT during the different stages of the menstrual cycle will be instrumental in advancing our understanding of sexual transmission of HIV in women and will offer avenues not previously considered for preventing the sexual transmission of HIV.

Disclosure

The authors report no conflicts of interest in this work.

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