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TESI DI DOTTORATO DI RICERCA

Determinants of Human Immunodeficiency Virus-1 Transmission to the Female Genital Mucosa: Role of Co-infecting Pathogens and Cytokines in Semen

Settore MED/04 – Patologia generale

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SOMMARIO

Background. Nel seme Human immunodeficiency virus (HIV)-1 e co-patogeni sono presenti in una complessa rete di citochine che influenza la replicazione locale e l'infettivita' dei patogeni sessualmente trasmissibili, e, allo stesso tempo, è influenzata dalle infezioni del tratto genitale maschile (MGT). La comprensione di queste interazioni richiede un'analisi approfondita dei co-patogeni che infettano il MGT e delle citochine presenti nel liquido seminale, e delle relazioni intercorrenti tra questi e HIV-1.

Metodi. La carica virale di 6 herpesvirus e HIV-1 e' stata valutata nel plasma seminale e sanguigno di 83 soggetti HIV-1 infetti cronici non in terapia, e di 33 soggetti non infetti mediante real-time PCR. Un saggio multiplex basato su beads è stato utilizzato per misurare i livelli di 21 citochine. I rapporti tra citochine sono stati definiti mediante il calcolo del coefficiente di correlazione di Spearman per tutte le possibili coppie di citochine. Blocchi di tessuto cervico-vaginale e linfoide umano sono stati inoculati con HIV-1 e trattati con interleuchina (IL)-7. La replicazione di varianti HIV-1 R5 o X4 è stata monitorata mediante misurazione della produzione dell'antigene $p24_{gag}$. Cellule isolate da blocchi di tessuto al giorno 6 e 9 post-infezione sono state caratterizzate per l'espressione dei marcatori CD3, CD4, CD8, e HIV-1 p24gag, e l'apoptosi è stata valutata misurando l'espressione delle proteine APO2.7 e Bcl-2 mediante citofluorimetria a flusso.

Risultati. La maggioranza dei campioni di seme, ma non di sangue, dei soggetti HIV-1-infetti e' risultata positiva per EBV e CMV (56% e 70%). Sangue e seme sono compartimenti immunologici separati e con l'infezione da HIV-1 il seme e' il compartimento interessato dai maggiori cambiamenti nella composizione citochinica (16 vs 9 citochine alterate nel sangue). La riattivazione di CMV nel MGT è associata ad un aumento dei livelli delle citochine CCL5, CCL11 e CXCL9 nel seme. L'analisi dei rapporti tra citochine ha rivelato un numero maggiore di correlazioni e un aumento dell'intensita' di correlazione per la maggior parte delle citochine sia nel seme che nel sangue dei soggetti HIV-1-infetti, rispetto ai non infetti. IL-7 e' risultata essere una tra le citochine più concentrate nel seme e l'infezione da HIV-1 ne aumenta ulteriormente i livelli seminali. Abbiamo dunque utilizzato un sistema di infezione ex vivo di tessuti umani per studiare il ruolo di IL-7 nella trasmissione di HIV-1. IL-7 e' risultata promuovere la replicazione di varianti R5 e X4 di HIV-1 in modalita' dose e tempo-dipendente. In blocchi di tessuto trattati con 25 ng/mL di IL-7 e' stato osservato un numero maggiore di cellule T CD4$^+$ infette e una riduzione della deplezione cellulare, rispetto a blocchi di tessuto non trattati con IL-7. Il trattamento con IL-7 e' risultato inoltre in una riduzione della frazione di cellule T CD4$^+$ infette esprimenti il marcatore apoptotico APO2.7 e in un aumento dei livelli di espressione del fattore anti-apoptotico Bcl-2.

Conclusioni. HIV-1 è associato ad una alterazione generale dello spettro citochinico nel seme e alla riattivazione locale di CMV e EBV nel MGT, due fenomeni che sembrano essere correlati e potrebbero dunque influenzarsi reciprocamente. La ridotta flessibilita' dei rapporti tra citochine potrebbe ulteriormente contribuire alla riattivazione di tali infezioni latenti. Tra le citochine nel seme, IL-7 esercita un effetto protettivo sulle cellule T CD4$^+$ infette, favorendo lo stabilirisi di una infezione produttiva nel tratto genitale femminile. La comprensione di come il complesso di citochine, di concerto con i co-patogeni presenti nel seme, influenzano la trasmissione sessuale di HIV-1 sarà oggetto di ulteriori indagini.

ABSTRACT

Background. *In semen Human immunodeficiency virus (HIV)-1 and co-infecting pathogens are immersed in a complex network of cytokines that affects the replication and infectivity of sexually transmitted pathogens, and, at the same time, is affected by local infections in the male genital tract (MGT). Understanding the mechanisms of these interactions requires a comprehensive analysis of co-infecting pathogens and cytokines in semen, and of the relations with HIV-1.*

Methods. *Load of HIV-1 and 6 herpesviruses in semen and blood plasma of 83 HIV-1 chronically infected individuals naïve to therapy and 33 HIV-uninfected individuals was evaluated by real-time PCR. A multiplex beads-based assay was used to measure the levels of 21 cytokines. The cytokine network was defined calculating the Spearman`s rank correlation coefficient for all pairwise combinations of cytokines. Human cervico-vaginal and lymphoid tissue blocks were inoculated with HIV-1 and treated with interleukin (IL)-7. Replication of R5 or X4 HIV-1 variants was monitored over a period of 12 days measuring HIV-1 $p24_{gag}$ antigen by a beads-based assay. Cells isolated from tissue blocks at day 6 and 9 post-infection were characterized for the expression of the markers CD3, CD4, CD8, and HIV-1 $p24_{gag}$ antigen, and apoptosis was evaluated measuring the expression of the proteins APO2.7 and Bcl-2 by multicolor flow cytometry.*

Results. *Epstein-Barr virus (EBV) and cytomegalovirus (CMV) DNA was found in semen of the majority of HIV-1-infecetd individuals (56% and 70%), but not in their blood. Blood and semen are separated immunological compartments, and with HIV-1 infection the major changes in cytokine composition occur in semen (16 vs 9 cytokine levels altered in blood). CMV reactivation in the MGT was associated with increased levels of the cytokines CCL5, CCL11 and CXCL9. Analysis of the cytokines network revealed a higher number of correlations and increase in correlation strength for most of the cytokines in semen and blood of HIV-1-infected compared to HIV-uninfected individuals. Of note, interleukin (IL)-7 resulted to be one of the most concentrated cytokines in semen and HIV-1 infection further increased seminal IL-7. Thus we employed our system of human tissues ex vivo to study the effect of IL-7 on HIV-1 replication. IL-7 enhanced the replication of R5 and X4 HIV-1 variants in dose and time-dependent manner. In tissue blocks treated with 25 ng/mL of IL-7 we observed higher number of HIV-1 infected $CD4^+$ T cells and reduced $CD4^+$ T cell depletion, compared with tissue blocks infected and maintained in the absence of IL-7. The fraction of HIV-1 infected $CD4^+$ T cells expressing the apoptotic marker APO2.7 was reduced and the levels of the anti-apoptotic factor Bcl-2 in infected cells were increased in the presence of IL-7.*

Conclusions. *HIV-1 infection is associated with a general alteration of the cytokine spectrum in semen, and with the local reactivation of CMV and EBV in the MGT, two phenomena that appear to be related and may affect each other. The reduced flexibility of the cytokine network may favors the reactivation of such latent infections. Among seminal cytokines, IL-7 exerts a protective effect on HIV-1-infected $CD4^+$ T cells in the early stages of infection, preventing their death and thus promoting the establishment of a productive infection in the female genital tract. Understanding how other cytokines in concert with co-infecting pathogens found in semen affects HIV-1 sexual transmission will be object of further investigations.*

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Abbreviations

AIDS: acquired immunodeficiency syndrome
APC: antigen-presenting cell
ART: anti-retroviral theraphy
Bcl-2: B-cell lymphoma 2
CXCR4: CXC-chemokine receptor 4
CCR5: CC-chemokine receptor 5
CMV: cytomegalovirus
CTL: cytotoxic T lymphocyte
DCs: dendritic cells
DC-SIGN: dendritic cell specific intercellular adhesion molecule 3-grabbing non-
	integrin
EBV: Epstein-Barr virus
FGM: female genital mucosa
FGT: female genital tract
HHV: human herpesvirus
HIV: human immunodeficiency virus
HSV: herpes simplex virus
Ig: immunoglobulin
LCs: Langerhans cells
LTR: Long terminal repeat
MGT: male genital tract
NF-kB: Nuclear factor kappa B
NHP: nonhuman primate
PAMP: pathogen-associated molecular patterns
PRR: pathogen-recognition receptor
TLR: Toll-like receptor
SIV: simian immunodeficiency virus
STD: sexually transmitted disease
SLPI: secretory leukoprotease inhibitor

Cytokine nomenclature

CCL: chemokine (C-C motif) ligand
CXCL: chemokine (C-X-C motif) ligand
Eotaxin-1: CCL11
G-CSF: granulocyte colony-stimulating factor
GM-CSF: granulocyte-macrophage colony-stimulating factor
IFN-γ: interferon-γ
IL: interleukin, IL-8 (CXCL8)
IP-10: IFN-γ-inducible protein 10 (CXCL10)
MCP-1: monocyte chemotactic protein-1 (CCL2)
MIG: monokine induced by interferon-γ (CXCL9)
MIP: macrophage inflammatory protein-1α (CCL3), MIP-1β (CCL4),
    MIP-3α (CCL20)
RANTES: regulated on activation normally T-cell expressed and secreted (CCL5)
SDF-1: stromal cell-derived factor-1α (CXCL12a); SDF-1β (CXCL12b)
TNF-α: tumor necrosis factor-α
TGF-β: transforming growth factor-β

1. INTRODUCTION

1.1 The Human Immunodeficiency Virus

1.1.1 Structure and genes of HIV

The human immunodeficiency virus (HIV) is a Retrovirus belonging to the family of Lentivirus. HIV is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) that results in extremely variable clinical outcomes, such as a severe immunodeficiency accompanied by the establishment of opportunistic infections and tumors, organ decay and central nervous system degeneration [1]. Two different HIV viruses exist: HIV-1, the pandemic type, and HIV-2, more represented in West Africa [2]. The clinical manifestations of HIV-1 and HIV-2 infection are similar, although their genome and antigenicity are different. The HIV virion is round shape with a diameter of about 100 nm. In the mature virion the viral genome is contained in a protein shield, called core, which is surrounded by a lipid bilayer, known as envelope (Fig. 1.1.1).
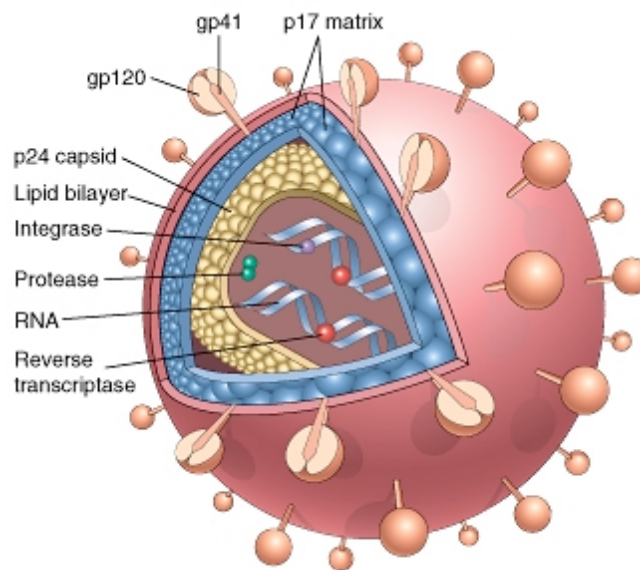
Figure 1.1.1 Schematic illustration of the human immunodeficiency virus virion. (From http://arapaho.nsuok.edu).

1.1.1.1 Genome

The HIV genome consists in two identical positive-sense single stranded RNA (ssRNA+) molecules, each 9.2 Kbasis in lenght. HIV-1 genome comprises three essential genes *gag*, *pol* and *env* and the regulatory genes *tat*, *rev*, *vif*, *nef*, *vpr* and *vpu* (Fig. 1.1.2). HIV-2 genome organization is identical, but *vpu* that was replaced by *vpx* [3]. The genetic information is highly organized in the HIV genome: different genes use common sequences that are differently read by the RNA polymerase. Moreover, the genes *tat* and *rev* comprise sequences dispersed into the genome

that require an alternative splicing event to form a coding transcript [4]. At each end of the ssRNA molecule there is a sequence called Long Terminal Repeat (LTR) that mediates the integration of HIV genome into the genome of a host cell and regulates viral replication. The LTRs contain promoters and enhancers of gene transcription specific for host transcription factors such as Nuclear Factor kappa B (NF-kB), activator protein 1 (AP1), nuclear factor of activated T cells (NFAT), as well as virus-encoded factors [4].
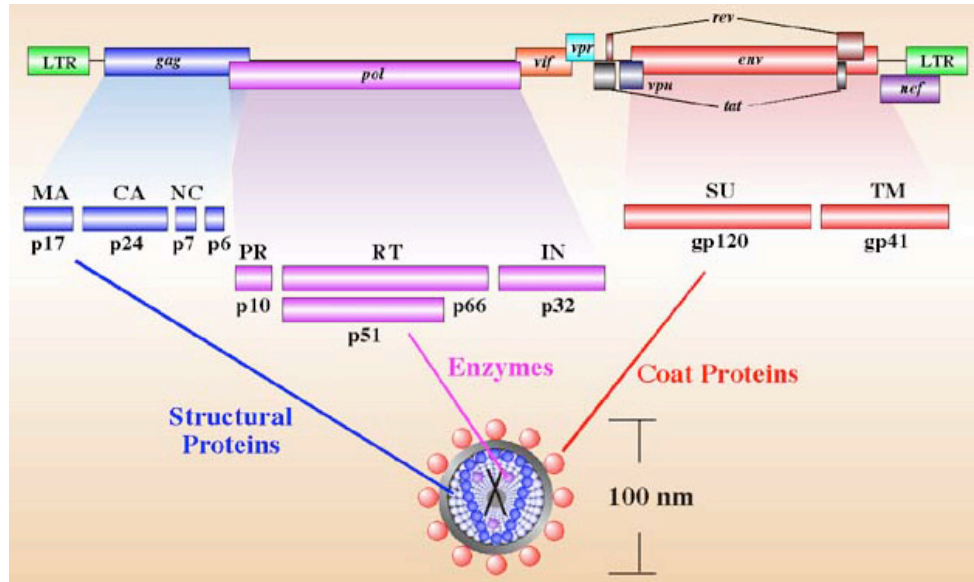
1.1.2 Organization of the HIV-1 genome. (From http://www.stanford.edu).

1.1.1.2 Core
The core of HIV is a nucleocapsid made of an elongated conical shell with icosahedral symmetry composed of proteins of 24 kDa in weight, called p24, which contains the HIV genome. The nucleocapsid is surrounded by a spherical shell, known as matrix, made of proteins of 17 kDa, called p17, anchored to the viral envelope through a myristic acid residue. The two ssRNA+ molecules in the capsid are associated with two proteins of 7 and 9 kDa, called p7 and p9 respectively, that function as packaging proteins. The proteins p7, p9, p17 and p24 are encoded by the gene *gag*, which transcript is translated in a poly-protein of 55 kDa, called p55 that is then cleaved by viral proteases in the above mentioned components during virion maturation [5]. In addition to the ribonucleoproteic complex, the core contains several virus-encoded enzymes: the reverse transcriptase (RT), a heterodimer constituted of two proteins of 66 and 51 kDa, the integrase, called p32, that facilitates the insertion of HIV genome into the host genome, and the protease, a homodimer composed of a protein of 10 kDa. These enzymes are encoded by the gene *pol*, which transcript is translated into a poly-protein of 180 kDa, called Gag-Pol, that is then cleaved by the viral potease itself [6]. The core also contains molecules that are important for the early stages of the biologic cycle of HIV and accessory proteins. Some of these proteins are virus-encoded such as Trans-

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activator of transcription (Tat or p14), Regulator of virion expression (Rev or p19), Negative regulatory factor (Nef or p27), Viral infectivity factor (Vif or p23), Viral protein U (vpu or p16) and Viral protein R (Vpr or p15) (or Viral protein X in case of HIV-2), and some derive from the host cell, such as cyclophilin A and a tRNA^{lys} that serves as primer for the RT to initiate HIV genome reverse transcription [7].

1.1.1.3 Envelope

The HIV envelope is a phospholypid bilayer acquired from the host cell during virion budding and release. The envelope contains host-derived proteins, including Human leukocyte antigens (HLAs) and adhesion molecules, and a series of spikes of 9-10 nm in length, called Env. Each spike is a trimeric complex of a glycoprotein of 120 kDa, called gp120, that from a cap non-covalently bound to a stem of a trimeric complex of transmembrane glycoprotein of 41 kDa, called gp41. The *env* gene encodes a protein called gp160 that, after glycosilation in the Golgi apparatus, is cleaved into gp120 and gp41. Respectively, these proteins enable HIV to attach to and fuse with the target cell membrane [8, 9].

1.1.2 Replication cycle of HIV-1

The replication cycle of HIV-1 begins with a high affinity binding between gp120 and the receptor CD4 expressed on the target cell surface. CD4 is a protein mainly expressed by lymphocytes with 'helper-inducer' activity, also known as CD4 T cells, that use CD4 as co-receptor for recognizing the antigen-HLA complex on the surface of an antigen presenting cell. CD4 is also expressed by monocytes and macrophages, dendritic cells, Langerhans cells, and some hematopoietic progenitors circulating in the blood stream and resident in the bone marrow. The interaction CD4-gp120 induces a conformational change in the structure of gp120 that leads to the exposure of the binding site for another receptor, called co-receptor, which presence on the host cell surface is required for viral entry. There are two main receptors which HIV-1 gp120 recognizes: CXC-chemokine receptor (CXCR) 4, that binds the chemokines stromal cell-derived factor (SDF)-1α (CXCL12a) and SDF-1β (CXCL12b); CC-chemokine receptor (CCR) 5, that binds the chemokines, also known as β-chemokines, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and regulated on activation normally T-cell expressed and secreted (RANTES, CCL5). The HIV-1 variants that bind to CXCR4 or CCR5 are called X4- or T-tropic, and R5- or macrophage (M)-tropic respectively [10, 11]. In addition to these two receptors, it was demonstrated that the receptors that HIV-1 can use to enter a target cell are more than seven. Mutations in the aminoacidic sequence of gp120 may modify the tropism of the virus: is some HIV-1-infected patients a progressive evolution from R5-tropic viruses, associated to transmission and acute infection, to X4-tropic viruses during late stages of the disease has been reported [12]. The interaction between gp120 and the co-receptor induces the exposure and activation of the lypophlic N-terminal region of gp41, called fusion peptide, that mediates the fusion of the viral envelope with the plasma membrane of a target cell [13]. Upon HIV-1 entry into the target cell, the core dissolves and a reverse-transcription complex (RTC) assembles in the cytoplasm. The RTC, comprising p17, viral enzymes, genomic RNA, and factors of cellular origin, allows the reverse transcription of the two identical molecules of ssRNA+ in double stranded DNA. HIV-1 RT lacks proof-reading activity and this results in a high rate of mutation that allows the virus to evade the host immune

defenses since the early stages of infection. The RTC then evolves in pre-integration complex (PIC) that crosses the nuclear membrane [14]. Inside the nucleus, the HIV-1 integrase promotes the insertion of the viral DNA in the host cell genome. At this stage the virus is called provirus and it can persist in a status of transcriptional latency for long periods of time [15]. The transcriptional status of the provirus largely depends on the metabolic status of the host cell [16, 17]. Antigenic stimulation through activation of the T-cell receptor signaling or stimulation of the host cell by pro-inflammatory cytokines can lead to activation and nuclear translocation of host transcription factors, such as NF-kB, that turn to activate or enhance HIV-1 provirus transcription [18]. This and other factors, including co-infecting pathogens, can break latency and initiate HIV-1 transcription, thus affecting progression to AIDS. The transcription of proviral genes is operated by the host enzyme RNA polymerase II, starting from the LTR at the 5' of the provirus. This sequence contains the promoter and the binding site for activators and regulators of transcription. Initially, the RNA polymerase is extremely inefficient in transcribing the proviral genes and most of the times transcription is terminated 100 basis from the promoter, when the nascent strand of RNA forms a stem-and-loop structure and the enzymes detaches from it [19]. When a complete transcript is produced, it translocates to the cytoplasm where the synthesis of the proteins Tat, Rev and Nef occurs.

Tat binds to the 5'LTR enhancing the efficiency of the RNA polymerase, preventing the premature termination of the transcription of HIV-1 genes [20].

Rev inhibits the host RNA splicing machinery thus controlling the nuclear export of intron-containing HIV-1 RNA. Depending on the amount of Rev in the nucleus, the production of HIV-1 components can switch from regulatory and accessory protein to structural proteins, and the unspliced HIV-1 genome can enter the cytoplasm to be incorporated into new virions [21].

Nef performs a striking number of activities that cooperate to delay the elimination of HIV-1-infected cells by the immune system to turn them into more effective producers of fully infectious virions. Of note, Nef down-regulates the expression of the CD4 and HLA molecules on the surface of the infected cells, by recruiting these receptors to the endocytic machinery or by rerouting them to lysosomes for degradation [22, 23]. These Nef functions protect virally infected T cells against cytotoxic T lymphocyte (CTL) lysis, reduce their migration in response to the chemokines CXCL12a and CXCL12b, prevent superinfection, and may facilitate the release of infectious virions.

Vpr is a virion-associated factor which reported activities include activation of proviral transcription, cell-cycle arrest in the G2 phase, induction of cell death, and enhancement of the fidelity of reverse transcription [24, 25]. Vpu is produced together with Env during the late stage of the viral life cycle. Vpu interacts with newly synthesized CD4 in the endoplasmatic reticulum and recruits an ubiquitin ligase complex to its cytoplasmic tail to mediate polyubiquitinylation and proteasomal degradation [26, 27]. CD4 degradation may facilitate virus release, avert superinfection, and enhance the incorporation of functional Env proteins into progeny virions by preventing the formation of gp120/CD4 complexes in virally infected cells. Vif is a factor essential for viral replication in primary T cells and *in vivo.* Its role is to disrupt the antiviral activity of the human enzyme APOBEC3G by targeting it for ubiquitination and cellular degradation. In the absence of Vif, APOBEC3G is incorporated into HIV-1 virions, inhibits viral DNA synthesis during

reverse transcription, and catalyzes deamination of cytidine to uridine during negative-strand DNA synthesis [28]. These changes lead to the degradation of the viral DNA and/or become fixed as guanosin-to-adenosin transitions in the proviral sequences (known as G-to-A hypermutations).

The final step of the viral replication cycle occurs on the plasma membrane of the host cell. The Env polyprotein (gp160) is cleaved in the Golgi by the HIV-1 protease and processed in the two envelope glycoproteins gp41 and gp120. Once they are transported to the plasma membrane of the host cell, gp41 anchors gp120 to the plasma membrane. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane, along with the HIV-1 genomic ssRNA+ as the forming virion begins to bud from the host cell. At this stage the HIV-1 accessory proteins and some host factors, such as cyclophilin A and tRNA[lys], are incorporated in the budding virus. Finally, the cleavage of the polyproteins Gag (p55) and Gag-Pol (p160) produces the enzymes necessary for the next round of infection, and the structural components p7, p17 and p24 that assemble to produce a mature virion [29].
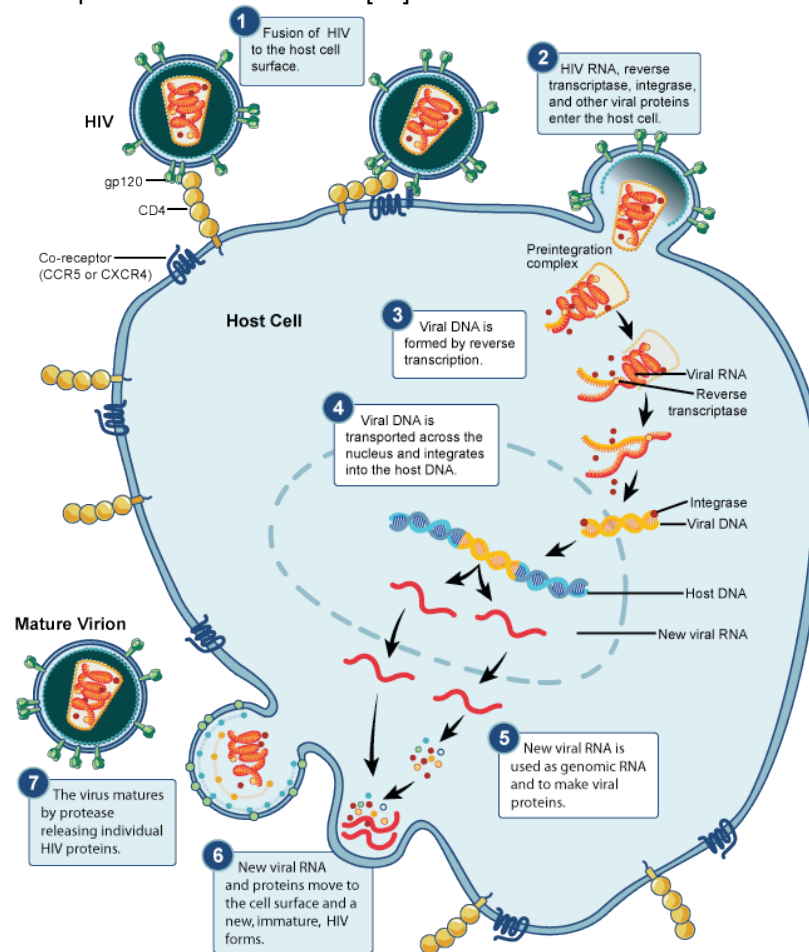
Fig 1.1.3 Replication cycle of HIV. (From http://www.niaid.nih.gov).

1.1.3 Pathogenesis of HIV-1 infection

The pathogenesis of HIV-1 infection begins as an acute infection that after a long period of latency, which can last 3 to 20 years, leads to a severe status of immunodeficiency, called acquired immunodeficiency syndrome (AIDS) [30]. The major determinant of the pathogenesis and disease caused by HIV-1 is the virus tropism for CD4$^+$ T cells, which results in a reduction in the number of these cells and consequent abolition of the helper function of adaptive immunity. HIV-1 induces several cytopathologic effects that may kill the infected T cell. In the the acute phase of infection, HIV-induced cell lysis and killing of infected cells by CTLs account for CD4$^+$ T cell depletion, although apoptosis may also be an important factor [31]. During the chronic phase, the consequences of generalized immune activation, coupled with the gradual loss of the ability of the immune system to generate new T cells, account for the slow decline in CD4$^+$ T cell number [32, 33].

The course of HIV disease parallels the reduction in CD4$^+$ T cell numbers and the amount of virus in the blood (Fig. 1.1.4).
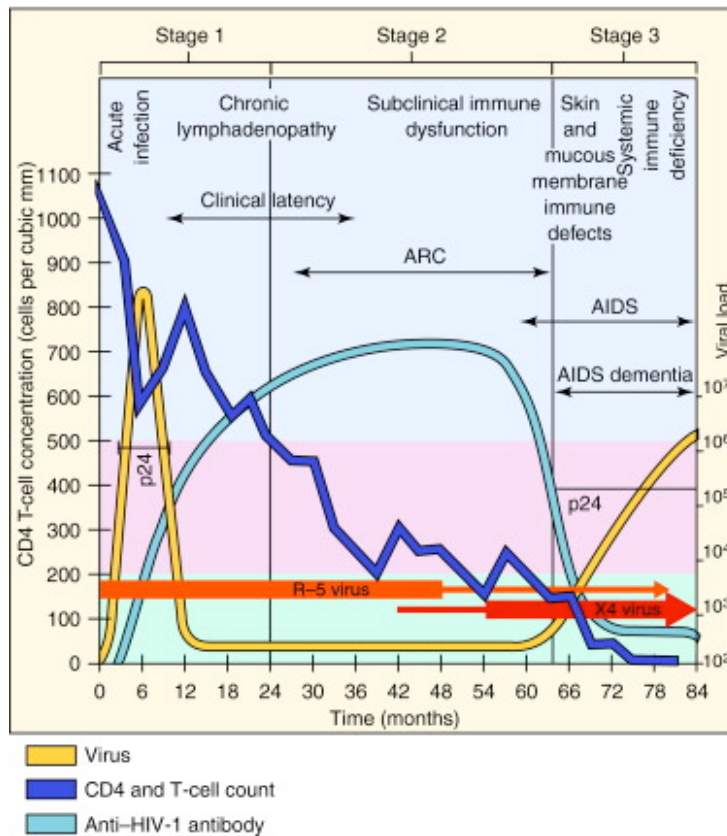
Figure 1.1.4 Time course and stages of HIV disease.
(From Murray et al., 2008 [34]).

During the acute phase, there is a large burst of virus production and infection spread systemically, in particular to secondary lymphoid organs. T cell proliferation and responses to the infected lymphoid and myeloid cells promotes a mononucleosis-like syndrome. The CD8^{+} T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4^{+} T cell counts recover. Virus levels in the blood decrease during a clinically latent period, but viral replication continues in the secondary lymphoid organs [35]. At the same time, virus can remain latent in macrophages, dendritic cells and resting T cells, establishing reservoirs in many organs. Continuous HIV-1 replication results in a state of generalized immune activation persisting throughout the chronic phase [32]. Immune activation, which is reflected by the increased activation state of immune cells and release of pro-inflammatory cytokines, results from the activity of several HIV-1 gene products and the immune response to ongoing HIV-1 and co-infecting pathogen replication. Immune activation has also been linked to the breakdown of the defenses of the gastrointestinal mucosal barrier caused by the depletion of mucosal CD4^{+} T cells during the acute phase of disease [36, 37].

Late in the disease, virus levels in the blood increase, CD4^{+} levels are significantly decreased, CD8^{+} levels also decrease, X4-tropic virus rises (in about 50% of the infected individuals), the structure of the lymph nodes is destroyed [38], and the patient becomes immunosuppressed (Fig 1.1.4). Stage IV or AIDS, as defined by the World Health Organization, is characterized by severe symptoms which includes toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma, and a CD4^{+} T cell count of less than 200/μL [39].

The central role of the CD4^{+} helper T cells in the initiation of an adaptive immune response is indicated by the extent of the loss of function caused by HIV-1 infection. When CD4^{+} T cells are unavailable or not functional, antigen-specific immune responses (especially cellular immune responses) are incapacitated, and humoral responses are uncontrolled. The loss of the CD4^{+} T cells responsible for activating macrophages allows the outgrowth of many of the opportunistic intracellular infections characteristic of AIDS (e.g., fungi and intracellular bacteria). Of importance, the decrease in number and the inability to activate CD8^{+} T cells increases the potential for recurrence of latent viruses, including JC polyomavirus progressive multifocal leukoencephalopathy (PML), herpes simplex virus, and cytomegalovirus infections, and even Epstein-Barr-associated lymphomas and human herpesvirus-8-associated Kaposi sarcoma [34].

1.2 AIDS epidemic and HIV transmission

After 30 years of epidemic the persons living with HIV are about 33.3 million worldwide [40]. The implement of preventive strategies and better access to therapeutic treatment, especially in low- and middle-income countries, led to decreased number of AIDS-related deaths and of new infections. Although the number of new infections has been constantly decreasing since the mid nineties, in 2009 there were still an estimated 2.6 million people newly infected with HIV (Fig. 1.2.1), among them 1.8 million in sub-Saharan Africa, which, with an estimated 22.5 million people living with HIV in 2009, remains the largest epidemic in the world (Fig. 1.2.2).

HIV-1 is the dominant HIV type worldwide, while HIV-2 is less infectious and aggressive virus, although it can cause AIDS as well as HIV-1, and remains restricted to West Africa [2]. For these reasons most of the studies, comprising ours, focused on HIV-1.
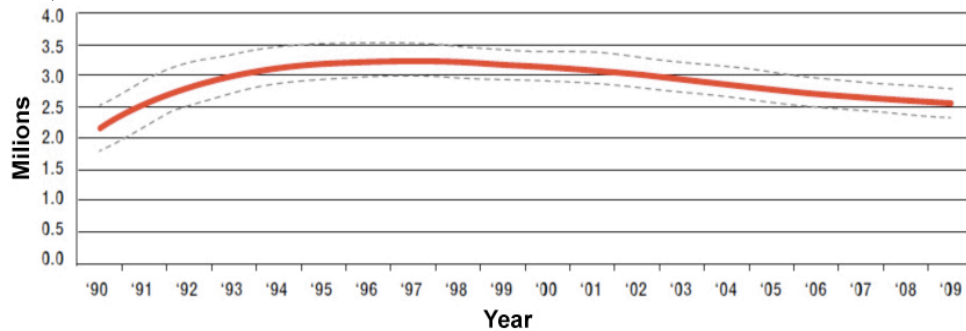
Figure 1.2.1 Number of peolple newly infected with HIV. (From UNAIDS 2010 [40]).
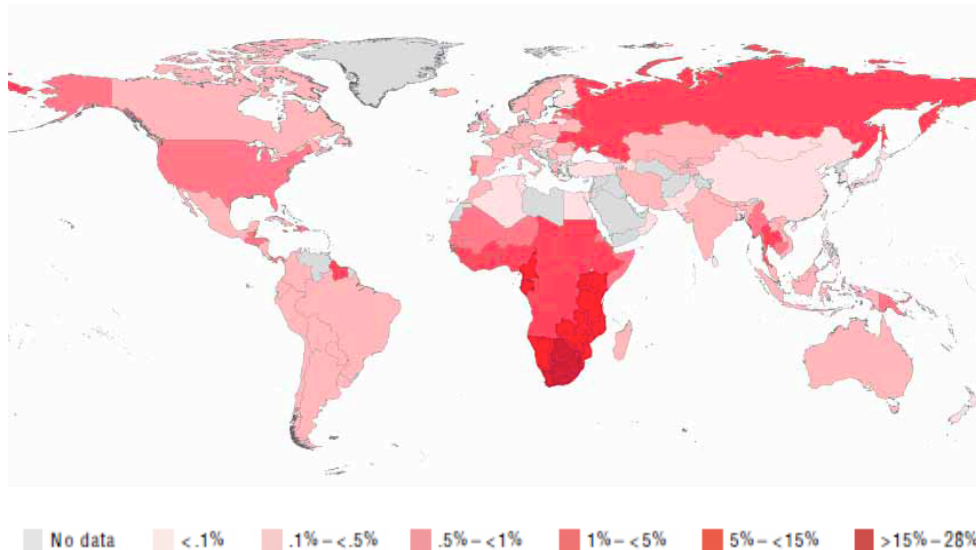
Figure 1.2.2 Global prevalence of HIV, 2009. (From UNAIDS 2010 [40]).

1.2.1 Routes of HIV-1 transmission

The major routes of HIV-1 transmission are mucosal exposure to infected body fluids such as blood, semen, vaginal fluids or breast milk, parenteral inoculation using infected needles or medical devices, and intrauterine mother-to-child transmission (Table 1.2.1). Despite the probability of HIV-1 sexual transmission is generally low, HIV-1 is primarily a sexually transmitted virus: about 80% of HIV-1-positive adults became infected through unprotected sexual intercourse [41]. In fact there are many factors that affect the efficiency of HIV-1 sexual transmission, first the anatomical site of infection. Unprotected receptive anal intercourse is associated with the higher risk of HIV transmission among all sexual practices, due to the thin monolayer of epithelial cells of the mucosa of the lower intestinal tract, which is highly susceptible of disruption during sexual intercourse and thus represents a weak barrier against HIV-1 breakthrough. Although this route of transmission accounts for the majority of new cases in high-income countries it is responsible for a small fraction of new cases globally. The penile and cervico-vaginal mucosa are mostly covered by stratified squamous epithelium that offers a better mechanical protection against HIV-1 entry. Indeed the efficiency of HIV-1 transmission via vaginal intercourse is 10 times lower than receptive anal intercourse (Table 1.2.1). Although its low efficiency, male-to-female HIV-1 transmission via unprotected vaginal intercourse accounts for the majority of all transmission events, more than the number of newly infected men due to exposure to cervico-vaginal fluids harboring HIV-1.

Slightly more than half of all people living with HIV are women and in sub-Saharan Africa young women are as much as eight times more likely than men to be HIV positive [40]. Understanding the mechanisms of semen-mediated HIV-1 transmission to the female genital mucosa is pivotal for the development of effective preventive strategies aimed to slow down the major driver of the pandemic spread of the virus.

HIV invasion site	Sub-location	Type of epithelium	Transmission medium	Transm. prob. per exposure event	Estimated HIV cases worldwide
Female genital tract	Vagina	Squamous, non-keratinized	Semen	1 in 200 - 1 in 2,000	12.6 million
	Ecto-cervix	Squamous, non-keratinized			
	Endo-cervix	Columnar, single layer			
	Other	Various			
Male genital tract	Inner foreskin	Squamous, poorly keratinized	Cervicovaginal and rectal secretions and desquamations	1 in 700 - 1 in 3,000	10.2 million[‡]
	Penile urethra	Columnar, stratified			
	Other	Various			
Intestinal tract	Rectum	Columnar, single layer	Semen	1 in 20 - 1 in 300	3.9 million[§]
	Upper GI tract	Various	Semen	1 in 2,500	1.5 million
			Maternal blood, genital secretions (intrapartum)	1 in 5 - 1 in 10	960,000
			Breast milk	1 in 5 - 1 in 10	960,000
Placenta	Chorionic villi	Two layer epithelium (cyto- and syncytio-trophoblast)	Maternal blood (intrauterine)	1 in 10 - 1 in 20	480,000
Blood stream			Blood products, sharps	95 in 100 - 1 in 150	2.6 million

Table 1.2.1 Contribution of HIV invasion sites to global HIV infections.
[‡]Includes men having sex with men (MSM), bisexual men and heterosexual men.
[§]Includes MSM, bisexual men and women infected via anal receptive intercourse.
(Adapted from Hladik & McElrath, 2008 [42]).

1.3 Semen

Semen is by classic definition the survival medium for spermatozoa, which main function is to protect and deliver sperm to the female genital tract (FGT). Semen also contains many molecules that actively signal in the FGT and modulate the local immune response to induce favorable conditions for fertilization and establishment of pregnancy. In addition to its reproductive tasks, semen serves as vector for pathogens originating from local infections in the male genital tract, which may modify the characteristics and immunologic properties of semen, thus affecting the transmissibility of pathogens themselves. Therefore, investigating the interplay between HIV-1, co-infecting pathogens and immunologic factors in semen is essential for a better understanding of HIV-1 sexual transmission and ultimately for the development of effective preventive strategies.

1.3.1 Origin and composition

Semen is a fluid composed of cells and secretions from the testes, epididymis, prostate, seminal vesicles and bulbourethral glands (Fig. 1.3.1).
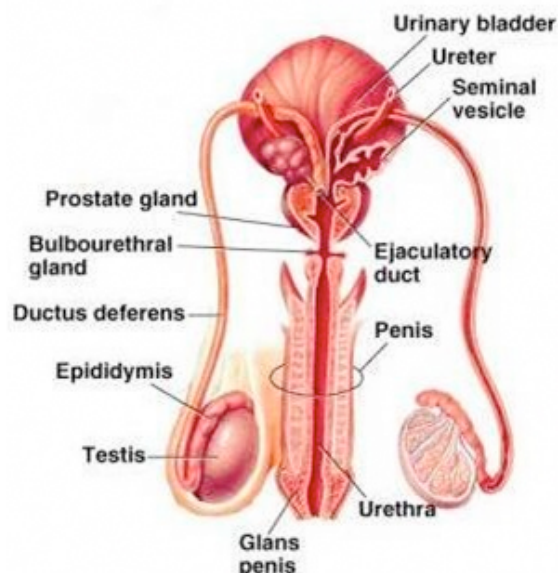
Figure 1.3.1 Schematic anatomy of the male urogenital tract.
(From http://nursingcrib.com).

The testis is the organ where androgens and spermatozoa are produced and it is in continuity with its extra-testicular pathways, comprising the epididymis, that transmit sperm to the urethra. Sperm make up only a small fraction, 1 to 5%, of whole semen. The prostate, seminal vesicles and bulbourethral glands are accessory glands, which secretions constitute the seminal plasma [43]. In fertile men, the sequence in which the accessory glands contribute their secretions to the ejaculate is fixed: the bulbourethral glands secrete a thin, mucoid, alkaline solution containing glycoproteins to neutralize the urinary tract and lubricate the tract during

the excitatory stage of sexual response. The bulbourethral glands contribute only a minimal amount to the ejaculate, less than 1%. Then, the prostate, epididymis and ducti deferentia contract together, discharging spermatozoa and prostatic secretions. The prostate secretion represents about 30% of the ejaculate volume, in the form of a slightly opaque fluid containing zinc, acid phosphatase, prostate-specific antigen, citric acid and proteolytic enzymes. Finally the seminal vesicles contract and expel the pellet of spermatozoa to the outside with their secretions, which are an alkaline, yellowish, viscid fluid that constitutes 60-70% of the ejaculate volume. Fructose and a variety of prostaglandis appear to be produced specifically by the seminal vesicles.

1.3.2 Physical and chemical characteristics

1.3.2.1 pH and buffering capacity
Semen has a very high buffering capacity and maintains its pH near neutral in the acidic vaginal environment, providing the sperm with the opportunity to enter the cervical mucus. On the basis of different reports, semen pH ranges between 7.4 and 8.4. The measured pH can depend on the length of time since ejaculation, and it tends to increase shortly after ejaculation as a result of loss of CO_2. Further aging of whole semen can result in a substantial decrease in pH resulting from fructolysis and the production of lactic acid. It has been calculated that HCO_3/CO_2 contributes 24.9%, protein contributes 28.5%, and low–molecular weight components such as citrate, inorganic phosphate, and pyruvate contribute half to the buffering capacity of semen [44].

1.3.2.2 Osmolarity
Semen is notable for its high osmolarity, which is substantially higher than that of blood plasma. The osmolarity of semen depends greatly on the concentration of sugars and other organics concentrations as well as ionic salt concentrations. Some researchers have noted that osmolarity increases measurably with semen aging [45].

1.3.2.2.1 Ions
Citrate is one of the most important anions present in human semen. Although citrate has high affinity for calcium, magnesium, and zinc, the citrate concentration is more than double the divalent metal concentration, consequently much of the seminal citrate is strongly anionically charged. The calcium concentration in semen is of great interest as a result of its relation to sperm motility, metabolism, the acrosome reaction, and fertilization itself. The other important ions found in human semen are magnesium, potassium, sodium and zinc. The concentrations of calcium, magnesium, and zinc are highly correlated. Magnesium and zinc are also found complexed with other molecules, which can sometimes be bound to the surface of the sperm cells [46]. Zinc is excreted from the prostate as a low-molecular weight complex with citrate. After ejaculation, 50% is redistributed and bound to medium- and high-molecular weight compounds from the seminal vesicles [45].

1.3.2.2.2 Fructose and glucose
Fructose is an important source of energy for the sperm and measurements of fructose concentration in whole semen can change over time as a result of fructolysis, the primary source of lactic acid in semen. Fructose is also likely involved in protein complexes, particularly in coagulated semen. Glucose may also

be an important source of energy to spermatozoa and is present in substantial concentrations. It should be noted that the variation in the measured values among studies is very large (range of 136–628 mg/100 mL for fructose, range of 4–300 mg/100 mL for glucose) [45].

1.3.2.2.3 Proteins

The bulk of the proteins found in semen derive from the seminal vesicles, although albumin is mainly of prostatic origin [46]. Albumin makes up about one third of the protein content of semen, the average albumin concentration is 1550 mg/100 mL and the average total protein concentration is 5040 mg/100 mL. The amino acid content of semen is much higher than that of plasma, and it increases rapidly (particularly glutamic acid) in the hours following ejaculation.

1.3.2.3 Viscosity

The rheological properties of semen change dramatically after ejaculation; the initial ejaculate quickly coagulates into a gelatinous material, and this material then liquefies. The coagulation factors derive from the seminal vesicles, while liquefying factors come from the prostate [47]. The relative contribution to sperm and seminal plasma to the viscosity of semen has not been clearly defined. Differences still exist as to what can be regarded as normal sperm concentration and many different cut-off values have been proposed, including 60, 20 and 10 millions of spermatozoa per mL [43] in a total average volume of 3.4 mL [45].

1.3.3 Immunological characteristics

1.3.3.1 Mechanisms of defense

Seminal plasma has strong bacteriostatic and bactericidal effects thanks to the presence of a variety of innate immune defense mediators including zinc, lysozyme, transferrin, and transglutaminase (Fig. 1.3.2) [48]. Studies of the gut and FGT immunologic milieus have begun to define the role of epithelial cell products such as chemokines, cytokines, mucins, and defensins in mucosal defense (see section 1.4.2.2), but there are few comparable published studies on immune defense mechanisms of male genital tract. The data produced to date indicate that epithelial cells in the male genital tract are capable of secreting antimicrobial peptides [49-51]. Furthermore, data are accumulating that innate defense mechanisms including defensins and cytokines are up-regulated in the male genital tract by infection [52]. β-chemokines (CCL3, CCL4, CCL5) are often detectable in semen from HIV-1-infected men [53]. Immunoglobulin (Ig) concentrations in normal human semen are much lower (~1-10%) than those in blood [54]. Both IgG and IgA isotypes are present in semen, but IgG predominates (total IgA = 9584 ng/mL vs. total IgG = 29741 ng/mL [55]). IgG and IgA are synthesized by plasma cells associated with the glands of Litre in the penile urethra [Pudney, 1995 #354], but much of the Ig in semen is apparently a transudate from the blood compartment [54]. The IgA found in mucosal secretions (sIgA) is different from serum IgA. It is composed of 10-S dimer and a J chain. The J chain is made by plasma cells and joins 7-sIgA monomers (the IgA normally found in serum) into 10-S dimers (and IgM monomers in pentameric sructures). A polymeric Ig receptor (p-IgR), primarily expressed in the genital tract by columnar epithelial cells in the penile urethra, binds polymeric IgA and IgM and transports it through the cell to the luminal mucosal surface [56]. Although sIgA are able to agglutinate bacteria, they do not have innate bactericidal activity. However, they can synergize with lysozyme and

complement to enhance antibacterial defense [57]. It was reported that IgA concentrations positively correlated with the seminal PMN count, suggesting that IgA concentrations are elevated in the male genital tract during episodes of genital tract inflammation and/or infection [55, 58].
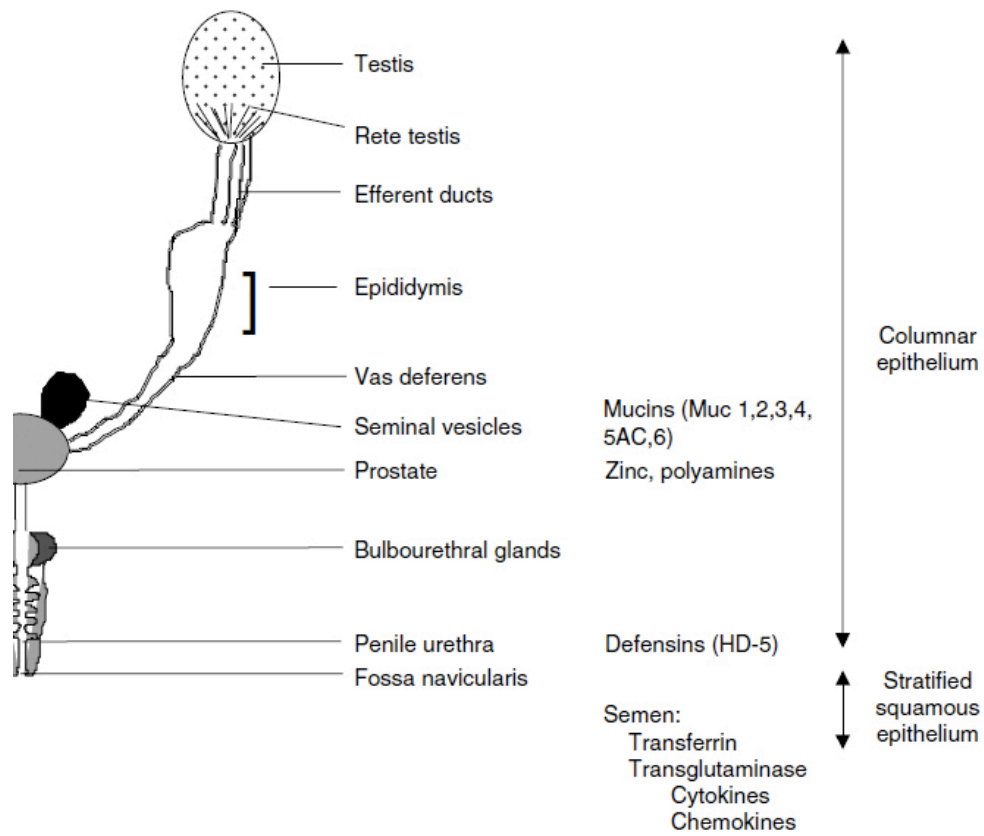
Figure 1.3.2 Mechanisms of innate defense in the male genital tract and in semen. (From Mucosal Immunology, 2005 [59]).

1.3.3.2 Cytokines

Semen is rich in several cytokines which biological significance is still poorly understood. Table 1.3.1 reports the results of one of the most comprehensive studies of semen from healthy fertile men, conducted by Politch at al. [55]. The men enrolled in the current study were at low risk for STIs and subfertility. Variables that could affect levels of cytokines and other immunologic factors in semen from different ethnic and demographic groups include genetic polymorphisms, differences in diet, hygiene, sexual practices and drug use. In addition, genital flora and sexually transmitted infections can influence seminal cytokine and antibody levels [60].

Cytokine	n	% positive	Median (pg/mL)	Range (pg/mL)
IL-1α	81	68	6.0	ND-214.0
IL-1β	83	54	2.0	ND-118.0
IL-2	77	1	ND	ND-24.0
IL-5	19	100	31.3	11.0-227.0
IL-6	79	99	6.0	ND-110.0
IL-7	19	100	2532.0	1109.0-3985.0
IL-10	80	9	ND	ND-32.0
IL-12	80	13	ND	ND-18.0
IL-13	19	53	3.1	ND-149.3
IL-17	19	74	11.6	ND-84.9
TNF-α	59	20	ND	ND-40.3
IFN-γ	82	37	ND	ND-130.0
IFN-α	60	55	3.3	ND-270.8
TGF-β1 (total)	77	100	85120.0	37520.0-192640.0
TGF-β1 (latent)	63	100	80118.0	36840.0-190230.0
TGF-β1 (active)	75	100	1096.0	168.0-9214.0
G-CSF	60	87	64.7	ND-5262.6
GM-CSF	60	7	ND	ND-1190.6
CXCL8	82	100	1305.0	384.0-14712.0
CCL3	82	15	ND	ND-136.0
CCL4	83	81	66.0	ND-1050.0
CCL5	83	98	126.0	ND-1480.0
CXCL12a	59	98	5741.9	ND-17955.4
CCL2	60	100	2980.9	292.4-81516.1

Table 1.3.1 Concentration of cytokines in semen of 'n' healthy fertile men. ND = non detectable. (Adapted from Politch et al., 2007 [55]).

1.3.3.2.1 Transforming growth factor-β

Transforming growth factor (TGF)-β is the highest cytokine in concentration present in human seminal plasma. Seminal plasma contains more active TGF-β than do other body fluids including blood plasma and breast milk [61]. In addition, seminal plasma contains a large amount of latent (inactive) TGF-β that can be

converted to the active form to achieve a sustained biologic effect. Indeed, TGF-β is thought to be synthesized in latent (inactive) form primarily in the prostate; following ejaculation, seminal TGF-β may be converted to its active form in the female tract by the acidic vaginal pH, or enzymes found in seminal plasma or vaginal secretions [62]. TGF-β is a key regulator of several aspects of the immune response (see section 1.3.4) and its role in HIV-1 transmission is controversial.

1.3.3.2.2 Interleukin-7

IL-7 is a member of the common gamma-chain cytokine family comprising IL-2, IL-4, IL-15, and IL-21 [63]. IL-7 plays a major role in modulating T cell development, and peripheral naïve and memory T cell homeostasis [64-66]. The main source of IL-7 in semen is believed to be the prostate, where IL-7 is important for maintaining T cells residing in prostate-associated lymphoid tissue [67]. Politch et al. were the first to report the presence of IL-7 in semen, at an average concentration of 2500 pg/mL, speculating that this cytokine plays a role in the maintenance of intraepithelial CD8^{+} T cells in the male genital tract [55]. A potential role of IL-7 during fertilization, and the effects of such an amount of IL-7 on the leukocytes resident in the female genital mucosa (FGM) remain to be investigated.

1.3.3.2.3 Chemokines

The three chemokines CXCL8, CXCL12a and CCL2 are present in semen at high concentration. CXCL12a is involved in the guidance, colonization, survival and proliferation of mammalian primordial germ cells, the progenitors of spermatozoa and oocytes [68]. Thus, it is also possible that CXCL12a may have a key role in the maintenance of male fertility. Although the function of CXCL8 and CCL2 in the male genital tract is not clear, these chemokines are thought to be involved in the inflammatory reaction that occurs in FGM after coitum (see section 1.3.4). Moderate levels of CCL4 and CCL5 were detected in semen. The β-chemokines CCL3, CCL4 and CCL5, and the chemokines CXCL12a and CXCL12b have been shown to inhibit HIV-1 entry into target cells by competing for the binding to the co-receptors CCR5 and CXCR4 respectively [69]. However, these chemokines may contribute to the recruitment of immune cells to the genital submucosa (i.e. CD4^{+} T cells by CXCL12a, both lymphocytes and macrophages by β-chemokines), which can be observed upon semen deposition onto the FGM. Thus, despite they can display anti-HIV-1 activity, their presence is semen may result in more target cells available for HIV-1 to infect during transmission.

1.3.3.2.4 Other cytokines

Prominent cytokines associated with T cell function, including IL-2, IL-10, IL-12 and IFN-γ, have been detected at elevated concentrations in semen of men with genital infections [70], while cellular immune activity is low in the genital tract of healthy men. Moderately high IL-5 concentration is present semen. This cytokine promotes the development of B cells and the production of IgA [71], and may play a role in humoral immune defense of the male genital tract.

1.3.4 Immuno-regulatory effects of semen on the FGT

In the vast majority of mammals, comprising humans, ejaculated spermatozoa are deposited in the vagina and have to reach the fallopian tubes to fertilize the egg at this location. Coagulation of semen occurs as a result of interaction of components of the prostate and a protein from the seminal vesicles (seminogelin), possibly to enhance the retention of semen in the vaginal canal. After 15-30 minutes, proteolytic enzymes contained in the prostatic secretions dissolve the seminal coaugulum (liquefaction), allowing spermatozoa to leave the seminal compartment and penetrate the cervix [43]. Although the great importance of semen as transporter and survival medium for spermatozoa, seminal fluid also contains signaling molecules that affect FGM immunity, thus promoting receptivity for embryo implantation. Cytokines, prostaglandins and other bioactive molecules present in semen initiate these events by interacting with the epithelial lining of the FGM triggering gene expression, leukocyte recruitment and activation of the innate and adaptive immunity in a sequence that resembles a classical inflammatory cascade [61].

The most immediate and evident effect of semen deposition onto the FGM is local induction of the secretion of pro-inflammatory cytokines, accompanied by a dramatic influx of inflammatory cells. These events were extensively described in mice and other mammals, while in humans the immune response to seminal fluid have received little attention. Leukocytosis was observed in samples recovered from the cervix of women artificially inseminated using a cervical brush, without providing any indication of the cellular changes within the genital mucosa [72, 73]. Seminal plasma, as opposed to sperm, mediates these events, since vasectomized mice, but not mice from which seminal vesicles have been surgically removed, elicit such response [74]. Recently, a study conducted by Sharkey et al. compared the composition of the leukocyte population, and the cytokine gene expression profile in cells recovered from needle biopsies from the ectocervix of ovulating women before and after vaginal intercourse, with or without coitus [75]. Macrophages and CD1a+ dendritic cells are among the major leukocyte populations recruited into the FGM after semen deposition. These cells are professional antigen-presenting cells and, in concert with cytokines, may play an important function in the induction of immune tolerance to male transplantation antigens contained within the ejaculate, as shown in mice [76]. Macrophages are also involved in tissue remodeling to facilitate implantation and placental development, because they produce matrix metalloproteinases (MMPs) under the influence of cytokines, extracellular matrix and prostaglandins [77]. The post-coitum inflammatory response mediates the emigration and efflux of neutrophils into the uterine lumen to ensure the clearance of microorganisms and seminal debris, promoting sperm survival by phagocytosing dead, abnormal and non-fertilizing sperm [78].

The chemokines present in semen, and those locally induced by semen in the FGM, trigger the recruitment of leukocytes into the FGM. Increased expression of the genes encoding the chemokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and CXCL8, and the pro-inflammatoty cytokines IL-1α and IL-6 has been reported in cervical tissue after unprotected coitus [75]. These and other genes, such as CCL2, macrophage inflammatory protein (MIP)-3α (CCL20) and IL-10, were previously demonstrated to be up-regulated by seminal plasma in cervico-

vaginal epithelial cell lines [79, 80] and tissue explants [81]. In the latter study up-regulation of CXCL8 and IL-10 in cervico-vaginal tissue by semen has been attributed to its abundant content in prostaglandin-E (PGE). Among the cytokines induced by semen deposition onto the FGM, GM-CSF targets the pre-implantation embryo to promote blastocyst formation, increasing the number of viable blastomeres by inhibiting apoptosis and facilitating glucose uptake [61].

To date, the active signaling components of human semen responsible for stimulating the FGM immune response are unidentified. Protein chromatographic techniques and neutralizing antibodies have identified TGF-β as the principal mediator of the induction of uterine inflammatory responses following mating in mice [82]. Recently a comprehensive gene expression study performed by Sharkey et al. [83], revealed that TGF-β accounts for the up-regulation of GM-CSF and IL-6 caused by exposure of cervico-vaginal epithelial cells to semen *in vitro*, while regulation of the production of cytokines such as CXCL8, CCL2, CCL20 and IL-1α depends on other seminal factors. TGF-β has also been identified as an important player in establishment of tolerance to paternal antigen by regulating the proliferation of T regulatory cells, which role is considered important for fertilization of the egg and early pregnancy [76].

1.3.5 Sources of seminal HIV-1

HIV-1 can be present in semen as cell-associated virus, carried by infected leukocytes or bound to spermatozoa plasma membrane, and cell-free virus. Genomic sequence analysis of cell-free HIV-1 variants found in semen of chronically infected individuals revealed that these viruses originate from multiple sources: direct import from the blood stream, oligoclonal amplification within the male genital tract, and compartmentalization [84, 85]. It was also shown that some of the cell-free HIV-1 variants were different from those isolated from infected leukocytes present in semen [86]. All these data support the idea that in the male genital tract there are sources of HIV-1 production other than the HIV-1 infected cells found in semen and infection of the genital organs actively contributes to viral shedding in semen. However, the infectiousness and the role of cell-free and cell-associated virus in HIV-1 transmission have been poorly investigated and remain matter of debate.

1.3.5.1 Cell-associated HIV-1

1.3.5.1.1 Leukocytes
Leukocytes enter semen from various sites along the reproductive tract, including the rete testis, epididymis, prostate, and urethra, where they play an immunosurveillance role. Concentrations of leukocytes in semen are highly variable. Leukocytospermia, an asymptomatic genital inflammatory condition characterized by more than 10^6 leukocytes/mL semen occurs in approximately 5-10% of healthy non-HIV-infected men and as many as 24% of HIV-infected men [87, 88]. Some studies indicate that semen from healthy non-HIV-infected men contains on the order of 10^5 leukocytes/mL, the majority of which are polymorphonuclear leukocytes [55] although substantial numbers of macrophages and CD4$^+$ T cells are also present. Macrophages usually outnumber CD4$^+$ T cells in semen, and this is especially the case in HIV-infected men in whom seminal CD4$^+$ lymphocytes are depleted (Tab 1.3.2) [89]. These data indicate that macrophages are the most abundant HIV-susceptible host cell in semen and a

likely principal mediator of cell-associated HIV transmission. HIV-infected cells have also been detected in pre-ejaculatory fluid, a urethral secretion secreted from the glands of Littre and Cowper glands during sexual stimulation, and these may also facilitate the sexual transmission of HIV [90].

	HIV-negative (n=17)	HIV-positive (n=98)
Total leukocytes	170000 [9000-20520000]	104000 [0-55380000]
Monocytes/ macrophages	52000 [300-998000]	22000 [0-24349000]
CD4$^+$ T cells	4000 [0-52000]	0 [0-6187000]
CD8$^+$ T cells	2000 [0-57000]	0 [1547000]

Table 1.3.2 Leukocytes concentration in semen. (From Anderson et al., 2010 [88]).

To date, the percentage of HIV-infected leukocytes in semen has not been determined. Only a few studies have used HIV DNA PCR assays to assess the prevalence or number of HIV-infected cells in semen. In these studies, the prevalence of HIV-1 proviral DNA in semen samples has ranged from 21 to 65% and the amount of HIV DNA has ranged from not detectable to 80000 copies/mL [88]. After initiation of HAART, levels of both HIV-1 RNA and DNA are reduced in semen, although HIV proviral DNA-bearing cells can persist in semen for several months and have been shown to be infectious in vitro [91].

1.3.5.1.2 Spermatozoa
The nature of HIV-1 interaction with spermatozoa is still matter of debate. Most studies failed to detect HIV-1 nucleic acids within purified motile spermatozoa, indicating that the few positive results reported represents either false positives or very rare events [88, 92]. It is generally accepted that HIV-1 does not productively infect motile spermatozoa. However, a recent study demonstrated the presence of HIV DNA in ejaculated spermatozoa with abnormal morphology [93]. This phenomenon may be explained by the fact that mammalian spermatozoa spontaneously take up foreign DNA or RNA in the absence of seminal plasma (e.g. epididymal spermatozoa). One hypothesis is that non-specific uptake of HIV RNA/DNA by epididymal spermatozoa would lead to abnormal spermatozoa prone to cell death. These defective sperm could potentially introduce HIV-1 to phagocytic macrophages or other cells in the female genital tract after intercourse. Alternatively, this detection may result from specific interactions between HIV and spermatozoa (see section 1.3.6.2.2).

1.3.5.2 HIV-1 detection within the organs of the MGT
Anatomical sites and sources of seminal HIV-1 are largely unknown. Most of our knowledge on this topic comes form indirect evidences, such as detection of viral RNA in secretions of genital organs and swabs, non-human primates models, and human explants infected *ex vivo*.
Recent data showed that the testis is infected early during the course of HIV-1 infection. Roulet et al. developed a system for infecting and maintaining in culture

human testis *ex vivo,* in which productive replication of the virus was observed [94]. The main virus-producing cells in this culture model are the resident testicular macrophages. An *in vivo* study of SIV-infected macaques confirmed the productive infection of the testis during the asymptomatic chronic stage and revealed that this infection occurs during the acute primary infection [95]. Because seminal shedding of HIV RNA and HIV DNA continues after vasectomy [96], distal genitourinary anatomic sites are likely important sources of seminal HIV. Early studies showed evidence of HIV and SIV in immune cells infiltrating the epididymis, prostate and seminal vesicles of men or macaques at the AIDS stage [92]. All these organs are infected very early and produce viral particles. The infection persists during the chronic stage and its intensity is positively correlated with the blood viral load. Infected cells are mainly T lymphocytes and to a lesser extent, macrophages. The infected immune cells are mainly localized within the stroma of the organs but are also found inserted within the epithelium, a finding most common within the epididymis. Their localization within the secretory epithelium could lead to the release of free viral particles and infected cells in the lumen, and therefore in the seminal plasma during ejaculation. As prostate and seminal vesicles secretions account for respectively 30% and 60% of the seminal fluid (see section 1.3.1), these two organs have been thought to represent the main source of virus in semen. It was confirmed that human prostate [97] and seminal vesicles [98] are susceptible to HIV-1 infection *ex vivo* and *in vivo*, although on the basis of a comprehensive study of prostatic secretions of men without urethritis who shed HIV in their seminal plasma, distal genitourinary sources other than the prostate appear to be the major source of seminal HIV-1 [99].

1.3.5.3 Infectiousness of seminal HIV-1

A number of laboratories have cultured HIV from both seminal cells and cell-free seminal plasma. Overall, the recovery rate of infectious HIV from seminal cells has been much higher (median 20%, range 4–55%) than that from seminal plasma (median 5.9%, range 3–11%) [88]. The relatively low HIV recovery rate from seminal plasma contrasts with quantitative PCR data indicating that HIV prevalence rates and viral copy numbers are higher in seminal plasma than in the semen cell fraction [88]. Although it has been proposed that this discrepancy would indicate that cell-free HIV in semen is replication incompetent or inactivated, the low culture rate could also reflect the toxicity of seminal plasma to peripheral blood mononuclear cell (PBMC) target cells used for culturing HIV [100]. Moreover, HIV-1 RNA levels in semen have been correlated to the risk of transmission (see section 1.5.2.2). Whether this reflects also changes in cell-associated HIV-1 is not known. To date no study was able to define the weight and the role of cell-free and cell-associated virus in HIV-1 sexual transmission and current models still need to be improved to address this important topic.

1.3.5.4 HIV-1 reservoir in the MGT

HIV-1 heterosexual transmission decreases remarkably following HAART. However, the existence of viral sanctuaries prevents the full eradication of the virus from the body. A viral sanctuary is either an anatomical or a cellular site (e.g. latently infected resting memory lymphocytes), impermeable to the action of one or several antiviral drugs and within which the virus replicates or persists despite treatment. Such sanctuaries are called reservoirs when they replenish the body in free virus or infected cells.

In semen, some viral inhibitors display sub-optimal concentrations, sometimes leading to the emergence of drug-resistant strains and to their sexual transmission [92]. The testis is a well-known pharmacological sanctuary in which HIV inhibitors have restricted access and several studies indicate that the MGT may constitute a viral reservoir responsible for HIV shedding in semen. Although in most patients HAART can reduce the semen viral load to an undetectable level [101], the persistence of HIV RNA and/or infected cells in semen has been reported in up to 10% of men under various antiretroviral treatment combinations, despite an undetectable blood viral load [91, 102]. The residual viral load detected in semen is generally low but appears to be extremely variable amongst individuals (range of 0.6 log to 5 log of HIV RNA copies/mL), because depends on many factors that globally affect the efficiency of sexual transmission (see section 1.5.2.2).

This persistence of HIV excretion in the semen of a subset of individuals under HAART has potentially important consequences for the transmission of the virus. Indeed, the possibility of transmitting HIV-1 despite an undetectable blood viral load following HAART remains in a subset of patients, as illustrated by the case report of an homosexual man under effective HAART who contaminated his partner [103].

1.3.6 Role of semen in HIV-1 transmission to the FGM

In vitro an *in vivo* studies identified a number of factors in human semen that can inhibit or enhance HIV-1 transmission. Most of these studies investigated the effect of whole semen or seminal plasma from HIV-uninfected individuals on the replication lab-adapted HIV-1 variants, thus ignoring some important changes in semen characteristic induced by HIV-1 infection of the MGT. Moreover, the choice of the system to investigate these effects is critical due to the nature of the reproductive act and of the female genital mucosa, and the global effect of semen on HIV-1 transmission may be different depending on many other factors that affect donor infectiousness and recipient susceptibility (see section 1.5.2).

1.3.6.1 Inhibitory effect

1.3.6.1.1 Cationic polypeptides

Cationic antimicrobial polypeptides produced by mucosal surfaces, such as secretory leukoprotease inhibitor (SLPI), defensins and lactoferrin, have been found to have anti-HIV-1 properties [104]. Martellini et al. demonstrated that SP contained 52 individual cationic polypeptides, which contributed to its aggregate anti-HIV-1 activity, and that SP maintained anti-HIV-1 activity, even when diluted 3200-fold [105]. However, this phenomenon was transient, as whole SP incubated for over 24 hr exhibited a reduction in anti-HIV-1 activity.

1.3.6.1.2 Seminal plasma interferes with the attachment of HIV-1 to DC-SIGN

DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) is expressed by stromal DCs in the FGM and appears to be a universal pathogen receptor, which was described to mediate the uptake of HIV-1 virions, protecting them from degradation, and efficiently promote infection of CD4$^+$ T cells (see section 1.5.1.2). Sabatte et al. found that human seminal plasma, even when used at very high dilutions (1:10^4 to 1:10^5), abrogates the recognition of HIV-1 by DC-SIGN [106]. Not only the binding of HIV-1 to monocyte-derived DCs (which express high levels of DC-SIGN) but also the transmission of HIV-1 from DCs to T CD4$^+$ cells was markedly inhibited by seminal plasma. The inhibitory effect appeared to

be specific for DC-SIGN since no inhibition was observed using DC-SIGN-negative target cells. Semen clusterin accounts, at least in part, for the inhibitory effect of semen on the capture of HIV-1 mediated by DC-SIGN [107].

1.3.6.2 Enhancing effect

1.3.6.2.1 Neutralization of acidic vaginal pH

The healthy vagina is colonized with lactobacilli producing lactic acid, which creates an acidic pH environment (pH 4.0-6.0) necessary to maintain the resident microbiome and combat pathogens. Cell-free and cell-associated HIV-1 and are rapidly inactivated *in vitro* at acidic pH levels [108]. O'Connor et al. demonstrated that laboratory strains of HIV-1 were uniformly stable at pH of 5.0–8.0, with mild reduction in infectivity (25%) at pH 4.5 [109]. After ejaculation, semen increases the pH of the vaginal fluid to neutral or higher levels within 30 seconds, maintaining an increased pH level for up to 2 hours [44, 110], and thus promoting HIV-1 survival and infection.

However, studies done with primary isolates instead of laboratory strains showed a high variability of HIV-1 to inactivation by acidic pH. In fact, for certain isolates, it was reported that the infectivity of HIV-1 was enhanced after exposure to pH 4.0–5.0 [111].

1.3.6.2.2 Spermatozoa bind and transmit HIV-1

Although CD4 is absent on spermatozoa membrane, alternative molecules have been described to bind HIV-1: GalAAG, a glycolipid related to galactosylceramide, CCR5, mannose receptors, and eparan sulfate [92]. Ceballos et al. reported that spermatozoa greatly enhance the infection of DCs by HIV-1 [112]. Spermatozoa efficiently attach and transmit the virus to DCs through heparan sulfate molecules expressed on the surface of spermatoza. Interestingly, spermatozoa-attached virions are also efficiently transmitted to macrophages and CD4$^+$ T cells. Of note, acidic pH similar to those found in the vagina after sexual intercourse markedly increase the binding of HIV-1 to the spermatozoa surface and the consequent transmission of HIV-1 to CD4$^+$ target cells.

1.3.6.2.3 Electrostatic interactions

Screening a complex peptide/protein library derived from human seminal fluid to determine possible inhibitors and enhancers of HIV-1 infection, Munch et al. found that naturally occurring fragments of the semen protein prostatic acidic phosphatase (PAP) form amyloid fibrils, termed semen-derived enhancer of virus infection (SEVI), which markedly increased HIV-1 infection [113]. SEVI has eight basic residues, which make it highly cationic (isoelectric point = 10.21), an important property for its attachment effects [114, 115]. The positively charged SEVI fibrils bind to both target cells and HIV-1 virions and augment infection by increasing physical contact between these entities, similar to the manner in which synthetic cationic polymers promote retrovirus attachment to target cells [115]. SEVI appears to act as a general enhancer of HIV-1 infection. It increases the infection by R5-, X4- and dual tropic HIV-1 clones in peripheral blood mononuclear cells (PBMCs), macrophages, DCs and human lymphoid tissue explants. Moreover, SEVI enhances HIV infection in a dose- and time-dependent manner, and its effects are seen across different envelopes [116]. Importantly, the enhancing effect of SEVI was most pronounced at low concentrations of virus, resembling conditions of sexual HIV-1 transmission. Infection enhancement, however, appears to be donor dependent. The effect of SEVI enhancement was

tested in hCD4/hCCR5-transgenic rats inoculated with either HIV-1 YU2 or SEVI-treated HIV-1 [113]. Tail vein inoculation with SEVI-treated HIV-1 increased the cDNA copy numbers in splenectomy extracts by five fold.

1.3.6.2.4 Proinflammatory effects

The immediate reaction of semen deposition into the mammalian reproductive tract is a dramatic influx of inflammatory cells, and a strong induction pro-inflammatory cytokines and chemokines (see section 1.3.4). Interestingly, it is becoming increasingly clear that different normal reproductive processes such as ovulation, menstruation, implantation and parturition display hallmark signs of inflammation [117, 118]. Whether or not the inflammatory responses induced by semen deposition in the mucosa affect the course of the early events involved in the fertilization or the implantation processes remains to be determined. Similarly, these inflammatory responses may contribute to the transmission of HIV-1 and other sexually transmitted infectious diseases through disruption of the epithelial barrier, and by inducing the local recruitment of CD4^{+} target cells.

Berlier et al. have demonstrated that seminal plasma also induces the expression of CCL20, a key chemotactic factor involved in recruitment and maturation of Langerhans cells and dendritic cells [79], which some investigators consider to be the first target cells for HIV genital mucosal infection [119] or somehow involved in promoting HIV-1 transmission in its early stage [120].

1.4 The female genital tract

The female genital or reproductive tract (FGT) has evolved into separate compartments consisting of ovaries, fallopian tubes, uterus, cervix and vagina (Fig. 1.4.1), which function is precisely regulated by the endocrine system. Each of these sites is designed to carry out different functions related to conception: production and transportation of the oocytes, fertilization, implantation, pregnancy and menstruation. In addition to its reproductive task, the FGT represents a barrier against pathogens and the female genital mucosa (FGM) displays unique features among mucosal sites, which allow the immune system to fight and control infections without interfering with the events that surround conception.
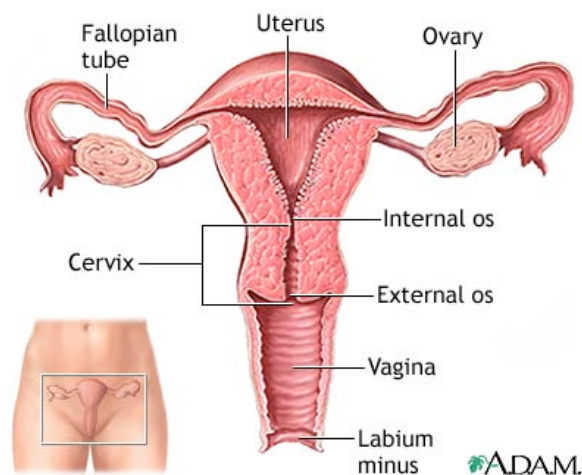
Figure 1.4.1 Anatomy of the female genital tract. (From http://health.allrefer.com).

1.4.1 Anatomy of the sites of HIV-1 infection in the FGT

Ex vivo studies using human tissues and pathogenic models of HIV-1 transmission in non-human primates (NHPs) showed that HIV-1 can infect the mucosa of the vagina and of the uterine cervix, either ectocervical and endocervical mucosa, although the relative contribution of these three sites to successful transmission remains unknown and debated. HIV-1 and its homologs in NHPs, the simian immunodeficiency viruses (SIVs), can establish infection solely penetrating the vaginal epithelium, as show in a woman born without a uterus [121] and in Rhesus Macaques challenged intra-vaginally after surgical hysterectomy [122]. However, studies in Rhesus Macaques demonstrated predominant early SIV RNA+ cell foci within the endocervix [120, 123]. Rather than a controversy, these data may reflect the ability and flexibility of HIV-1/SIV to circumvent different defensive barriers of the FGT.

1.4.1.1 The vagina

The vagina is a fibromuscular tube that extends from the uterus to the external genitals (vulva) and whose anterior (front) and posterior (rear) walls are normally in contact with one another [56]. The vaginal wall consist of three layers: the mucous membrane, composed of stratified squamous nonkeratinized epithelium and an underlying lamina propria of connective tissue; the muscular layer, composed of

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smooth muscle fibers disposed both longitudinally and circularly; the adventitia, a dense connective tissue that blends with the surrounding fascia. Although there is wide anatomical variation, the length of the unaroused vagina of a woman of child-bearing age is approximately 6 to 7.5 cm across the anterior wall, and 9 cm long across the posterior wall. During sexual arousal the vagina expands in both length and width. Its elasticity allows it to stretch during sexual intercourse and during birth to offspring.

The stratified squamous epithelium of the vagina is several layers thick. The basal layer is a single layer of cylindrical cells with oval nuclei. Above this area are several layers of polyhedral cells and above these are more layers of cells that are more flattened in appearance and accumulate glycogen in their cytoplasm. They also exhibit keratohyalin granules intracellularly, although this tendency to keratinization is not normally completed in the vaginal epithelium and the surface cells always retain their nuclei. The most superficial cells are desquamated into the vaginal lumen where their intracellular glycogen is converted into lactic acid, probably by the bacteria that normally colonize the vagina. The resulting acidity is important in protecting the FGT from infections.

Before puberty and after menopause, when estrogens levels are low, the epithelium is thin and the pH is higher than in the reproductive years. Indeed estrogens stimulate the production of glycogen, which results in low pH, and maintain the thickness of the entire epithelium, thus representing and important determinant of the FGM susceptibility to infection (see section 1.5.2).

1.4.1.2 The uterine cervix

The uterus has two major components: the expanded upper two-thirds of the organ, the body of the uterus, and the cylindrical lower one-third, the cervix [56]. These two parts are rather different from one another in their structure and function. The fundus is the rounded upper part of the body and the isthmus is the short, slightly constricted zone between the body and the cervix. Here we will focus our attention mainly on the cervix.

The cervix consists primarily of dense collagenous connective tissue. Only about 15% of its substance is smooth muscle. In the isthmus, the uterine lumen narrows down to form the internal os. Below this point the lumen widens slightly to form the cervical canal or endocervical canal that measures 7 to 8 mm at its widest. Finally, a constricted opening, the external os, at the lower end of the cervix provides communication between the lumen of the cervix and that of the vagina (Fig. 1.4.1). This protruding portion of the cervix projects into the vagina through the upper anterior vaginal wall and is referred to as *portio vaginalis* or ectocervix. On average, the ectocervix is 3 cm long and 2.5 cm wide.

The epithelium of the cervix is varied. The ectocervix is covered by stratified squamous nonkeratinized epithelium. Usually, in older women, this type of epithelium extends from a very short distance into the cervical canal, where it forms a rather abrupt junction with the simple columnar epithelium lining the rest of the endocervical canal (Fig. 1.4.2). The site of the squamocolumnar junction, called tranformation zone, varies as well. It may occur higher up in the cervical canal, or the columnar epithelium may actually extend out beyond the external os where it forms small patches known as physiologic eversion, or ectopy, on the vaginal surface of the cervix. Ectopy is usually present in adolescents, when the endocervix moves out of the uterus, and decreases in post-menopause women,

when the uterus shrinks moving the transformation zone upwards. Changes (metaplasia) of the transformation zone may occur also with the changes of the cervix associated with the menstrual cycle.
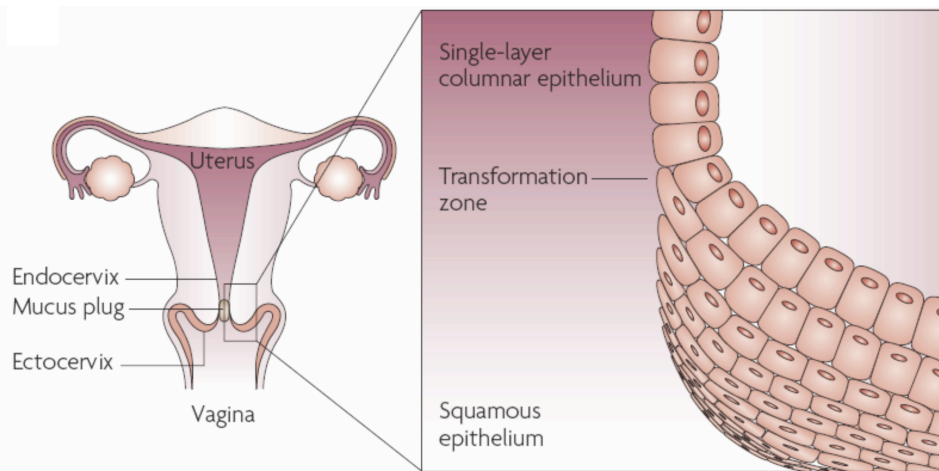
Figure 1.4.2 Transformation zone of the uterine cervix. This particular portion of the female genital mucosa is thought to be a preferential site for HIV-1 infection due to its physical and immunological properties. (From Hladik & McElrath, 2008 [42]).

Inside the cervix, the endocervical mucosa is arranged in a series of folds and ridges. The mucosa also contains large branched endocervical glands, that are not true glands but are merely grooves, called crypts, that serve to increase the surface area of the mucosa. During childbirth, contractions of the uterus will dilate the cervical canal up to 10 cm in diameter to allow the child to pass through. The epithelium of the both the mucosal surface and the crypts is of the simple columnar type in which almost all the cells are mucus secreting. A few ciliated cells are present. If the ducts of the glands become blocked, mucous secretion accumulate inside them to form small lumps just under the surface.

Unlike the mucosa of the body of the uterus the endocervical mucosa does not slough off at menstruation. It does, however, respond to cyclic changes in the levels of the ovarian hormones. It secretes up to 60 mg of mucus a day throughout much of the cycle, but near the time of ovulation (midcycle), when estrogen secretion reaches a peak, the secretion rate increases 10-fold and the aboundant clear mucus fills the cervical canal. It is less viscous then at other times during the cycle and it is easily penetrated by spermatozoa. The production of progesterone by the corpus luteum after ovulation (or during pregnancy) changes the quantity and properties of mucus produced. It becomes more viscous, less abundant and much less penetrable by spermatozoa, acting as a plug to seal off the uterine cavity.

1.4.2 Immune defenses of the FGM

The mucosa of the FGT is a component of the mucosal immune system, an integrated network of specialized cells and tissues that employs specialized immune defense mechanisms at mucosal surface (Table 1.4.1). There are 3 lines of defense that the FGM evolved to fight and control infections: the epithelial barrier, innate immunity, and adaptive immunity mechanisms.

	Cellular immunity			Epithelium	Genital secretions
	T	M	LC		
Endocervix	+++	+++	++	Simple columnar	Mucins, cytokines, lysozime, lactoferrin, defensins, SLPI, IgG, IgA, pH<0.5
Ectocervix	++	++	++	Stratified squamous	
Vagina	+	++	++		

Table 1.4.1 Some of the mediators of immunity in the female genital mucosa.
T = T cells; M = macrophages; LC = Langerhans cells; Ig = immunoglobulin;
SLPI = serine leukocyte protease inhibitor.

1.4.2.1 The epithelial barrier

1.4.2.1.1 Epithelium

The epithelial lining of the FGM, made up of epithelial cells and mucus, provides a robust physical and immunological barrier against pathogens. As discussed above, epithelial structures vary depending on their localization. The nonketarinized stratified squamous epithelia of the vagina and the ectocervix are multilayered structures of (up to 300 μm thick) consisting of a layer of basal germinative cells resting on a basal lamina (a 20-100 nm layer of extracellular material), which separates the epithelium from the underlying vascular connective tissue called the lamina propria. A lack of tight junctions in the squamous epithelial layers permits the movement of small molecules within epithelial spaces within the cells [124]. The continuous sloughing of the superficial layers of the stratified epithelium prevents many pathogens from colonizing and establishing infections.

The epithelium lining the endocervical canal is simple columnar, a single layer of polarized epithelial cells with apical tight junctions. A number of factors have been described to affect the integrity of this barrier. For example estradiol, the proinflammatory cytokine tumor necrosis factor (TNF)-α, and other factors secreted by the underlying stromal cells, which are also under hormonal control, compromise transepithelial resistance, leading to increased pathogen translocation across the epithelium [125, 126]. Physical and chemical trauma and ulcerating infections can cause epithelial disruption that provides pathogens access to unprotected cells in the germinal layer and lamina propria (see section 1.5.2.1). Epithelial disruptions and cervical ectopy are indeed major risk factors for HIV-1 transmission.

1.4.2.1.2 Mucus

Epithelial cells of the FGM secrete several biological factors that create an inhospitable environment for pathogens, including a layer of mucus that lubricates

and reinforces the epithelial barrier. Mucus obtains its structural characteristics from mucins, a family of hydrophilic glycoproteins that contain tandem repeats of serine- and threonine-rich domains, which are sites of attachment of O-glycan attachment. Encoded by MUC gene family, to date 18 mucin genes have been cloned and two classes of mucins have been identified: membrane-associated mucins and secreted mucins, which include large gel-forming and small soluble mucins [56]. Membrane-associated mucins are expressed at the apical surface of epithelial cells throughout the FGM and form a dense glycocalyx. Negatively charged carbohydrate residues on the mucin protein backbone confer disadhesive properties. Among secreted mucins, the gel-forming mucins are formed by multiple mucins monomers linked one another with lengths up to several microns and molecular weights ranging from 0.5 to 200 Mda.

The composition of the mucus in the FGT is affected by a number of factors comprising tissue-specific posttranslational processing, genetic polymorphisms, endocrine status, infection and inflammation. For example, the expression of the large gel-forming mucin MUC 5B by the endocervix and its glycosylation are upregulated by estrogen, accounting for the large volume of watery mucus in the endocervical canal at the time of ovulation that permits sperm movement [127].

Mucins can adhere directly to microorganism, such as *Candida albicans*, through their glycosylated domains and impede their attempts to colonize the epithelium []. Mucins fibers are densely packed in the glycocalix and exclude 30 nm-sized particles, representing an effective barrier against virus penetration. Mucins fibers are less dense in gel-forming secreted mucins and the space between midcycle cervical mucus fibers (20-200nm) is large enough for proteins and small viruses (HIV is 100 nm in diameter) to diffuse [128, 129]. Another important factor that affects microorganism penetration through cervicovaginal mucus is pH. The acidic environment of the vagina has been shown to slow the rate of HIV diffusion by abolishing the negative surface charge of the virus. I presence of semen, which has a pH closer between 7 and 8, HIV retains its negative surface charge resulting in higher rate of diffusion [130].

Intersperse within mucins fibers is an acqueus phase containing antimicrobials and immunoglobulins. Antibodies, especially polyvalent IgA and IgM, can agglutinate pathogens into clusters that are too large to diffuse through mucus. Secreted antibodies bind to mucins through low-affinity molecular bonds with the Fc and SC components of the molecules and through interactions with mucin-like-IgG Fc binding protein that is found in most mucosal secretions [131].

1.4.2.2 Innate immunity
The innate defenses of FGM consist of cells that constitutively express antimicrobial factors and/or rapidly mount antimicrobial responses triggered by recognition of highly conserved molecular motives present on microorganisms. The principal effector cells of innate immunity at the mucosal surface are dendritic cells (DCs), macrophages, neutrophils and natural killer (NK) cells.

1.4.2.2.1 Pathogen-recognition receptors
The cells of innate immunity are able to discriminate pathogen-associated molecular patterns (PAMPs), such as nucleic acids (i.e. double-stranded RNA, unmethylated CpG DNA sequences), aminoacidic patterns (N-formylmethionine), carbohydrates (tecoic acid), and lipids (lipopolysaccharide) that are found in microbes but not in mammalian cells, through cellular receptors known as

pathogen-recognition receptors (PRRs) [132]. These receptors are encoded in the germ line and have a more limited repertoire than receptors of adaptive immunity.

The proteins of the complement system represent a front line of defense in against extracellular pathogens by opsonizing microbes for clearance by phagocytes through their complement receptors. Another class of PRRs, the C-type lectins, allows phagocytes to ingest microbes via recognition of pathogens-associated carbohydrates. Members of this class of proteins found on DCs, such as langerin and Dendritic Cell-Specific Intercellular adhesion molecule 3-Grabbing Non-integrin (DC-SIGN), have been described to play a role in HIV-1 transmission (see sections 1.3.6.1.2 and 1.5.1.2.1). Intracellular PRRs include the Nucleotide-binding Oligomerization Domain (NOD)-like receptor gene family, active against intracellular bacteria, and a family of RNA helicases called Retinoic acid Inducible Gene I (RIG-I)-like receptors that detect viral infections leading to production of type I Interferons.

The most prominent and best characterized PRRs are the Toll-like receptors (TLRs), a family of trans-membrane proteins that are expressed by almost all the immune cells, and by some non-immune cells. To date more than 10 TLRs have been identified in humans and their localization in the cell is associated with the type of PAMP recognized: TLRs 1, 2, 4, 5 and 6 are expressed on the cell plasma membrane and recognize products of bacteria, fungi, protozoa and mycobacteria; TLRs 3, 7, 8 and 9 reside in endosomal and lysosomal membranes and recognize nucleic acids of viruses and bacteria [133]. The binding of TLRs leads to the activation of an acute inflammatory response mediated by the induction of genes coding for antimicrobial proteins, proinflammatory cytokines, type I interferons and chemokines, through a signal transduction pathway highly conserved. The consequential recruitment of effector cells, such as neutrophils and macrophages, favors the elimination of microorganisms and the induction of co-stimulatory molecules on antigen presenting cells, bridging innate and adaptive immunity [134]. Various TLRs have been characterized in female genital mucosa cells: TRLs are expressed by macrophages, DC, NK, B and T cells and can also be expressed by epithelial cells [135]. Despite the important anti-viral function of TLRs and other innate receptors, their role in HIV infection of the female genital tract has not been deeply investigated. Expression of TLRs 3, 7, 8 and 9, among other PRRs, has been detected in epithelial cells of the ectocervix and endocervix [136], thus they may play an important role in triggering the early immune response against viruses. It has been reported that plasmacytoid dendritic cells (pDCs) are among the first cells to sense HIV-1 through TLR9, inducing the production of type I interferons [137]. Although HIV-1 also activates the TLR 7 and 8 pathways [138], other PRRs specific for PAMPs from pathogens infecting the cervico-vaginal mucosa or present in semen may have an important role in affecting the local immune response during HIV-1 transmission [139].

1.4.2.2.2 Leukocytes

DCs and macrophages are professional antigen-presenting cells (APCs) and are important for the generation and regulation of the adaptive immune functions. Pathogen exposure and phagocytosis induces APCs maturation, through activation of their PRRs, and antigen presentation on type Human Leukocytes Antigens (HLAs) complexes to naïve T cells, which results in expansion of pathogen-specific adaptive immunity. Among DCs, Langerhans cells (LCs) are immature dendritic

cells localized within the layers of the stratified squamous epithelium of vagina and ectocervix [140]. They have high endocytic activity and low T cell activation potential and are characterized by the presence of Birbeck granules, which are cytoplasmic organelles which function is still unknown. LCs have dendrites that extend and retract through the intercellular spaces ad that can even reach up to the surface of the epithelium [141]. LCs express CD4, CCR5 and langerin, that have been described to mediate HIV-1 entry into these cells, although productive infection, if it occurs, appears inefficient (see section 1.5.1.2.1). Myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) are two other types of DCs that reside in the sub-epithelial stroma of the FGM [56]. mDCs, once activated by recognition of pathogens that penetrated through the mucosa, migrate to regional lymph nodes where they function as APCs and activat naïve T cells. pDCs, as reported above, have an important role in early detection and response against viral infection and are major producers of type I interferons. Stromal DCs exhibit different HIV-1 receptor expression patterns compared to LCs because they express DC-SIGN in addition to CCR5, thus potentially permitting alternative entry pathways for HIV (see section 1.5.1.2.1).

Macrophages localize in the lamina propria of the FGM and account for 10% of the leukocytes present in the FGT [135]. Long-lived tissue macrophages are derived form blood monocytes and recruited to the to the sub-epithelial stroma by endogenous chemoattractants in the non-inflamed mucosa. These resident macrophages have down-regulated expression of PRRs and low expression of pro-inflammatory cytokines, but retain their phagocytic and anti-microbial functions. A different population of macrophages is recruited to the site of infection in the FGM by inflammatory chemokines. These cells express abundant PRRs and secrete pro-inflammatory cytokines, amplifying the immune response [56].

The most represented granulocytes in the FGM are neutrophils. They are present throughout the FGT and their number progressively decreases from the upper to the lower genital tract [135]. Neutrophils express a wide array of TLRs and at the site of infection they respond to pathogens through phagocytosis, release of preformed cytoplasmic granules that contain antimicrobial peptides and of reactive species of oxygen and nitrogen, and secretion of cytokines and chemokines that recruit and activate additional immune cells [56].

NK cells constitute a major component of the innate immune system and play an important role in the recognition and killing of cancer cells and virally infected cells. Within the FGT, NK cells demonstrate different phenotypic characteristics. The number of NK cells varies in the endometrium across the menstrual cycle, reaching a peak of approximately 70% of the total leukocyte population in the secretory phase [135]. NK cells express most of the TLRs and NOD proteins, and kill target cells through the release of lytic proteins such as perforin and granzymes. Type I interferons play a critical role in NK cells activation, as well as IL-2, IFN-γ and IL-12 [56].

1.4.2.2.3 Anti-microbial peptides

Secretions of the FGT contain several peptides with broad anti-microbial activity that prevent and/or reduce infection by killing or controlling the growth of microorganisms by direct or indirect mechanisms [124]. They include defensins, enzymes (e.g. lysozime), anti-proteases and chemokines (e.g. β-chemokines). Defensins are small cationic antimicrobial peptides active against bacteria, fungi

and enveloped viruses. They are classified in two groups α- and β-defensins, depending on their structure, and are secreted by immune cells, such as neutrophils, and epithelial cells of the FGM [135]. Defensins exert their anti-viral activity by directly binding to the viral membrane of enveloped viruses or indirectly through the recruitment and activation of immune cells [142]. Serine Leukocyte Protease Inhibitor (SLPI) is a member of the trappin gene family and is produced primarily from epithelial cells, neutrophils and activated macrophages. It has a broad anti-microbial activity and it has been described to protect macrophages and CD4$^+$ T cells from HIV-1 infection [124].

1.4.2.3 Adaptive immunity

The cellular components of the acquired immune system of the FGT are two: T cells, and antibody secreting plasma cells. Adaptive immunity is induced specifically against one pathogen following presentation and stimulation of T cells by APCs. A number of cells in the FGT have been shown to present antigens on HLAs molecules. These include classical APCs including macrophages, dendritic cells and Langerhans cells, but also epithelial cells of the cervix and endometrium. Adaptive immunity includes Th1 (cell-mediated), Th2 (humoral), T regulatory and Th17 responses.

In the FGM, CD8$^+$ T cells (35-50%) predominate over CD4$^+$ T cells (25%) and are numerous within the epithelium of the ectocervix, vagina and transformation zone [122, 124]. Substantial infiltrates of CD8$^+$ and CD4$^+$ T cells are seen in the stroma of the transformation zone, but T cells are relatively sparse in the stroma of the ectocervix and vagina. The transformation zone of the cervix has been indentified as a major inductive and effector site of cellular immunity in the lower FGT due to the particularly high number of T lymphocytes and APCs resident therein [140]. Phenotypic analysis of the lymphocytic subsets isolated from human cervico-vaginal explants, revealed that FGM-resident CD4$^+$ T cells are predominantly effector memory and terminal effector memory, with almost complete absence of naïve and central memory cells [143]. These cells expressed high levels of CCR5 as well as CXCR4. The same study identified a higher proportion of terminal differentiated memory cells among CD8$^+$, compared to CD4$^+$ T cells, that express higher levels of the activation markers CD38, HLA-DR, and CD57.

CD4$^+$ helper T cells play a role in humoral immunity by driving B cell maturation to antibody secreting plasma cells. Plasma cells are concentrated in the subepithelial layers of the endocervix but substantial numbers are also found in the ectocervix and vagina [56]. Unlike other mucosal surfaces such as gastrointestinal and respiratory surfaces, where IgA secretion is the dominant isotype, both IgG and secretory-IgA (sIgA) are expressed in genital secretions and IgG predominate (see section 1.3.3.1).

1.4.2.4 Cytokines

Cervical and vaginal epithelial cells are primary source of cytokines comprising IL-1α, IL-1β, IL-6, IL-7 GM-CSF, M-CSF, TGF-β, CXCL8, CCL3, CCL4, CCL5 and CXCL12 [56, 144]. In most cases the secretion of cytokines from epithelial cells occurs preferentially towards the apical/luminal compartment resulting in a gradient that is important for attracting immune cells to the epithelial surface. For example, apically secreted CXCL8 induces neutrophil migration across the epithelium suggesting that, in the absence of a chemokine gradient, neutrophils would be less likely to cross the epithelial barrier, potentially reducing the level of protective α-

defensins in luminal secretions. Other cytokines and chemokines such as TGF-β are secreted into the basolateral/sub-epithelial compartment where they influence the development and function of resident immune cells [124].

Many of the above mentioned cytokines can be detected in cervico-vaginal secretions and provide important information on inflammatory and immune defenses mechanisms in the lower female genital tract [145]. The secretion of cytokines leads to rapid communication between the different cell types present in the female reproductive tract. Furthermore, these molecules elicit a potent innate immune response, which creates an environment hostile to pathogen survival. An important cytokine family involved in female reproductive tract immunity, particularly against viruses, are the type I interferons [146]. IFNs are rapidly induced in the presence of viral and bacterial pathogens, which up-regulate the production of hundreds of interferon-stimulated genes (ISGs) via autocrine and paracrine actions. Some chemokines are also active antimicrobial compounds, being able to directly interfere with microbial pathogenesis. As reported in section 1.3.3.3.3, CXCL12a and CXCL12b, and the β-chemokines CCL3, CCL4 and CCL5 may play opposite roles in HIV-1 transmission.

1.5 HIV-1 transmission to the FGM

### 1.5.1 Current model of HIV-1 transmission to the FGM

The vaginal mucosa and ectocervix are covered with a multilayered squamous epithelium that, if intact, provides a better mechanical protection against HIV-1 penetration than the single-layer columnar epithelium of the endocervix. Moreover, the transformation zone is likely an important site for HIV-1 transmission because of its peculiar immunologic features, and all the factors that affect its extension may represent important determinants of HIV-1 transmission. However, depending on the menstrual cycle and other factors, a barrier of mucus hinders the access of the virus to the endocervical epithelium, and the greater extension of the vaginal and ectocervical epithelia offers more potential sites for HIV-1 entry (see section 1.4.1). Although HIV-1 penetration and infection of the FGT was demonstrated in all 3 of these mucosal compartments using different experimental models, the preferential site, if any, of HIV-1 transmission *in vivo* remains unknown.

1.5.1.1 HIV-1 interaction with the epithelium

Both free and cell-associated viruses can establish HIV-1 infection *in vivo*. This observation has been confirmed with a number of experimental model, such as female macaques infected with SIV, mice infected with HIV, indirectly in humans through genetic matching of HIV viruses sequenced from acutely infected women and from seminal cells and plasma from their infected partners, and human cervical explants studies [41].

Initially, some of the deposited seminal cells or free virions are trapped in cervical mucus [123, 147]. However, it remains unclear whether this trapping promotes transmission by prolonging contact time of infected cells or free virions with the mucosa, or whether the trapping impedes transmission by immobilizing the cells and virions and increasing their ability to be attacked by innate antiviral substances. HIV virions, either inoculated cell free or released from infected donor cells, interact with epithelial cells and traverse the epithelium through several ways, including transcytosis, endocytosis and subsequent exocytosis, by establishing productive infection in epithelial cells, or merely by penetrating through the gaps between epithelial cells to encounter its primary target cells (Fig. 1.5.1).

By using epithelial cells derived from the lower female genital tract, it has been demonstrated the binding and entry of HIV-1, and the subsequent ability of HIV-1 to be transferred to susceptible CD4$^+$ T cells [148]. Another phenomenon that may contribute to viral transfer in primary genital epithelial cells is transcytosis [149]. During this cellular process, virions in polarized intact epithelial cells can cross from the apical to the basal region, where they are released and then can infect their primary targets. It has been reported that virions can also productively infect the cervical epithelial cells themselves [150], although this remains controversial. Conceivably, HIV-1 can also be transported through the cervico-vaginal epithelium to the draining lymphatics by donor infected leukocytes. Mechanical microabrasions of the mucosal surface induced by intercourse may also allow HIV to directly access the target cells at the basal epithelium and the underlying stroma. The vaginal epithelium, like squamous epithelia at other sites (eg, skin and oral mucosa), is not a structure of uniform thickness. The stromal papillae frequently project deep into the outer epithelium, creating considerable variations in distance between the luminal surface and the basal membrane (Fig. 1.5.1).
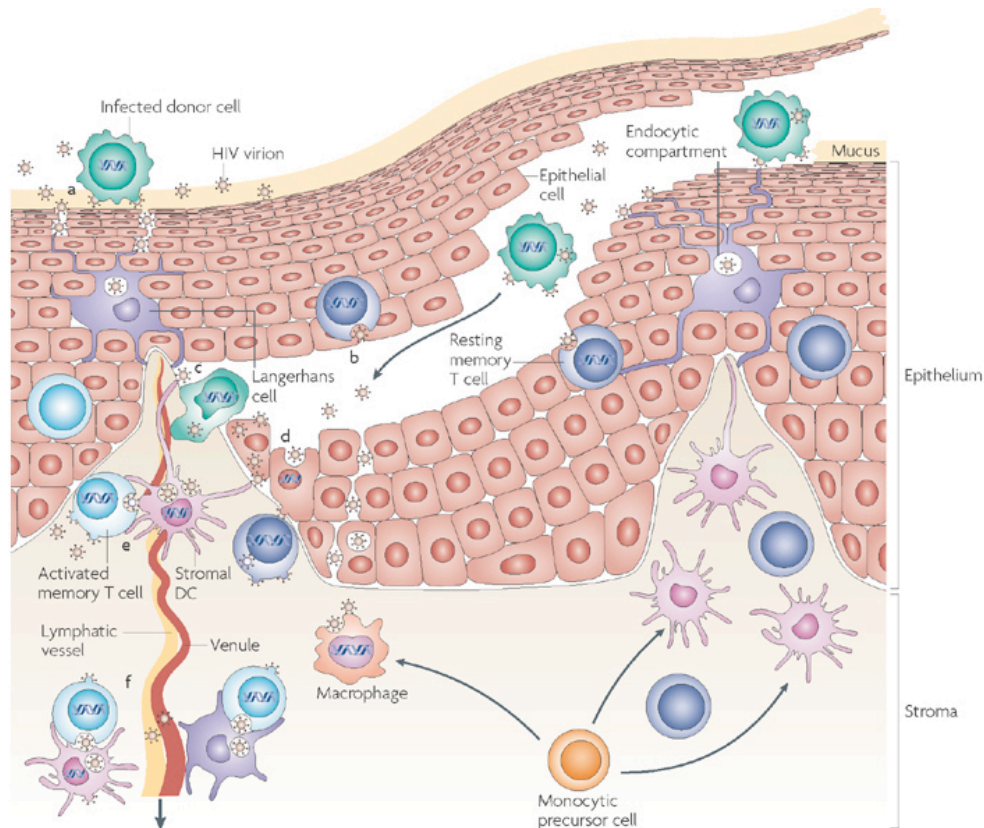
Figure 1.5.1 Pathways of HIV-1 invasion in the lower female genital tract mucosa. a) Viruses trapped in mucus and penetrating through gaps between epithelial cells. b) Infection of intraepithelial lymphocytes. c) Direct import of donor infected cells or virions to the mucosal stroma through micro-abrasion of the epithelium. d) Transcytosis. e) HIV-1 interacts with and infects the leukocytes resident in the mucosal stroma. f) Infected or virus harboring leukocytes migrate into the draining lymphatic and blood vessels. (From Hladik & McElrath, 2008 [42]).

Microabrasions in regions of minimally layered epithelium could more easily expose the basal layers of the epithelium or even the underlying stroma. In addition to the purely mechanical microabrasions, chemical and infectious alterations of the mucosa may open access routes for HIV to reach resident leukocytes (see section 1.5.2). Regardless of the mode of penetration, studies with SIV in macaques indicate that virus crosses the cervico-vaginal epithelium after exposure *in vivo* within 30 to 60 minutes [151]. This penetration may bring the virus in direct contact with two cell types that it may infect later: intraepithelial Langerhans cells (LCs) and CD4$^+$ T lymphocytes. Interestingly, LCs and, on the stromal side, T cells and macrophages cluster at the tips of the stromal projections into the epithelium, thus creating microareas with an increased likelihood of infection. The highly focal manner of initial SIV infection in the genital mucosa of macaques in vivo may

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reflect this notion [120, 123].

1.5.1.2 HIV-1 target cells in the mucosa

1.5.1.2.1 Dendritic cells
It was demonstrated that vaginal intraepithelial LCs are susceptible to HIV-1 entry and infection, either in macaques infected with SIV and in a model of human vaginal epithelial sheets, separated from the underlying stroma, infected and cultured *ex vivo* [119, 151]. The latter study demonstrated that vaginal LCs very efficiently internalize HIV-1 into cytoplasmic organelles and, when the cells subsequently exit the epithelium at the basal side, they can take intact virions with them. Vaginal LCs express CD4, CCR5 and the C-type lectin langerin, but not CXCR4 and another C-type lectin, DC-SIGN (see section 1.4.2.2.1) [152]. In contrast to vaginal LCs, HIV is efficiently captured by langerin in epidermal LCs [153]. Upon viral entry, langerin directs HIV-1 to Birbeck granules, where the virus is degraded. It has been propose that in vaginal LCs, HIV may bypass C-type lectins, including langerin, in favor of other routes of binding and endocytosis, allowing the virus to reach endocytic compartments that are less hostile to the virus than the Birbeck granules, although no productive HIV-1 infection of vaginal LCs has been reported [119].

In addition to LCs, DC-SIGN expressing DCs are present in the stroma of the FGM. HIV-1 has been reported to exploit the binding to DC-SIGN to directly infect DCs that may transmit the virus to CD4$^+$ T cells [154]. Of note, recent studies suggest that the role of DC-SIGN in promoting the spreading of HIV-1 is not only related to the ability of recognizing and attaching the virus to the DC surface. Binding to DC-SIGN also triggers a signaling pathway mediated by Raf-1 that is required for the generation of full-length viral transcripts and HIV-1 replication in DCs [155].

1.5.1.2.2 CD4$^+$ T cells
The lamina propria of the lower human FGT contains many CD4$^+$ T cells, which are often enriched in the superficial stroma, close to the basal membrane. CD4$^+$ T cells also infiltrate the vaginal and ectocervical squamous epithelium. The majority of these cells are effector memory T cells that express higher levels of CCR5 than those circulating in blood [143]. Investigators using human organ cultures of vaginal, ectocervical, and endocervical tissues, and SIV challenge experiments in macaques consistently found high numbers of CD4$^+$ T cells that were infected with HIV-1 early after virus exposure [120, 156, 157]. Interestingly, SIV productively infects not only activated (HLA-DR$^+$ Ki67$^+$) T cells but also resting (HLA-DR$^-$ Ki67$^-$) T cells [157]. Although it is impossible to selectively remove DCs from the epithelial sheets, several lines of evidence indicate that initial infection of intraepithelial CD4$^+$ T cells is not dependent on DC-mediated viral uptake and infection in trans [119].

1.5.1.2.3 Macrophages
According to some human explant studies, macrophages in the cervico-vaginal stroma were also identified as targets for HIV-1 infection [158-160]. However, immunostaining studies have never conclusively demonstrated that macrophages in the human uterine cervix or the vagina express CCR5 in situ. In contrast to experiments in the human explant model, vaginal SIV challenge experiments in macaques either rarely revealed SIV-infected macrophages in genital tissues or failed to detect them at all [151, 157]. Captured HIV can be archived within macrophages for several days and still be transmitted to T cells. Therefore, the role

of macrophages in early HIV-1 infection of the lower genital tract in women still needs to be defined.

1.5.1.3 Early events in HIV-1 transmission

Although there is some argument on the phenotype of the first cell populations that are productively infected in the female genital submucosa, HIV-1 may take advantage of multiple cellular targets to establish infection. Once the virus gains access to the host, the appearance of viral spread within the submucosae of the FGT is not typically demonstrable until 3-4 days after exposure of NHPs to a high dose of virus [123]. Infection then expands locally before detection of infection in the draining lymph nodes and systemically throughout the secondary lymphoid organs (Fig. 1.5.2).
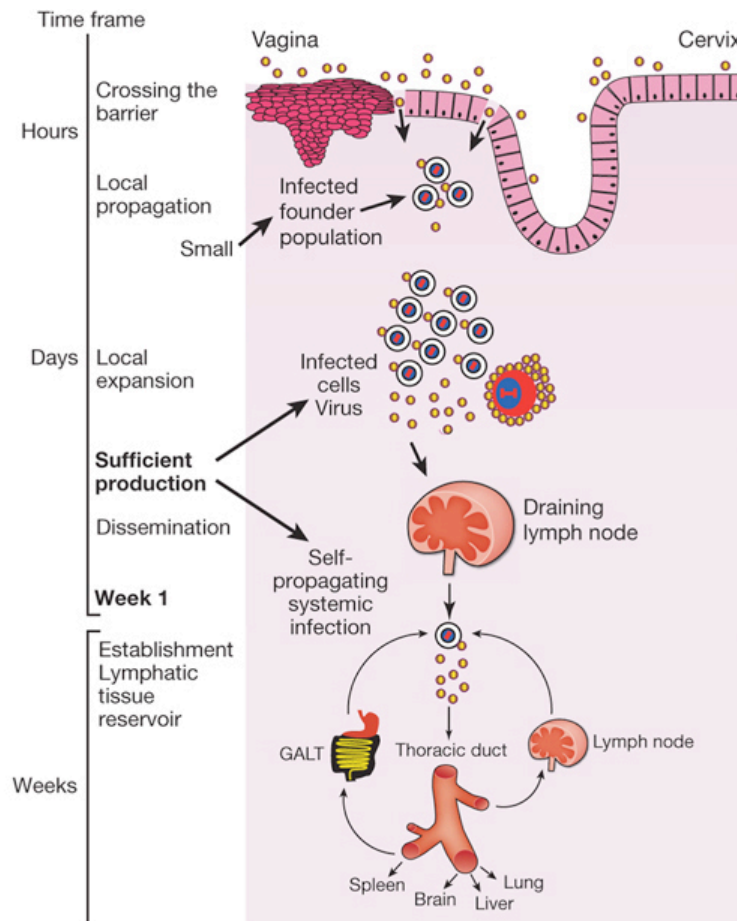
Figure 1.5.2 Major events in vaginal transmission of HIV-1. Within hours, virus may gain access through breaks in the mucosal epithelial barrier to susceptible target cells. The small focal infected founder population expands locally in 'resting' and in activated CD4$^+$ T cells. Local expansion is necessary to disseminate infection to the draining lymph node, and subsequently through the bloodstream to establish a self-propagating infection in secondary lymphoid organs. GALT = gut-associated lymphoid tissue. (From Haase, 2010 [161]).

Some groups have demonstrated the presence of both SIV or infected cells within the genital draining lymph nodes, albeit at very low levels, shortly after intravaginal SIV challenge, suggesting that rapid dissemination to lymphatic tissues can occur after mucosal exposure [123, 151]. However, there is little evidence of active viral replication in draining or systemic lymphatic tissues until 5-6 days after vaginal challenge. Apparently, although viral particles themselves might rapidly gain access to draining lymphatic tissues after intravaginal SIV inoculation through lymphatic drainage or carried by DCs, the threshold for a self-propagating infection is not crossed until virus and infected cells are produced locally in sufficient quantities to establish infection distal to the portal of entry, because the draining lymph nodes are not the site where productive infection is first detected [157]. Thus, dilution, trapping in cervical mucus, the physical mucosal epithelial barrier and other mechanisms transform exposure to a large quantity of virus in the inoculum to a small founder population of infected cells that must then expand locally for a few days before systemic infection is established [161] (Fig. 1.5.2). The 'eclipse' phase observed in humans between presumptive exposure and development of either detectable viremia or clinically apparent symptoms of an acute viral syndrome, and data showing increased HIV-1 incidence with sexually transmitted co-infections (see section 1.5.2), support the notion of a requirement for a "threshold" level of mucosal susceptible CD4$^+$ target cells to establish local infection. This CD4$^+$ target cells can result from active recruitment to mucosal tissues through early host inflammatory processes or as a result of preexisting inflammation. In this picture, DCs and macrophages in the FGM may play a key role in maintaining HIV in an infectious state until the arrival of activated CD4$^+$ T cells within the submucosa (see section 1.5.1.2).

1.5.1.4 The transmitted virus
One of the greatest obstacles in preventing HIV transmission is the enormous sequence diversity both within an individual and globally. However, during transmission this considerable diversity is reduced because the infecting virus undergoes a genetic bottleneck that results from the biology of mucosal transmission [162]. Recently developed methods for viral sequence analysis, often designated as single-genome amplification (SGA), have both documented and permitted a greater understanding of the genetic restriction during sexual transmission of HIV. It was discovered in cohorts self-identified as heterosexual that the number of transmitted variants can be traced back to a single founder variant in roughly 80% of infections [163]. To date, transmitted Envs have all been shown to be functional and capable of mediating entry via CD4, using CCR5 as the coreceptor in most cases. Furthermore, when full-length transmitted/founder viruses were cloned, each showed significant replication in CD4$^+$ T cells but did not replicate in monocyte-derived macrophages [164], findings that are consistent with CD4$^+$ T cells being identified as the earliest productively infected cell type *in vivo* in NHP studies and *ex vivo* in human tissues (see section 1.5.1.2.2).

In a recent study HIV-1 transmission to human cervical explants was evaluated comparing 6 chronic isolates and 2 transmitted/founder viruses and no difference in efficiency of transmission was observed between the two groups of viruses [165]. Interestingly, despite CD4$^+$ T cells resident in the FGM display high levels of expression of CCR5 as well as CXCR4, human cervico-vaginal explants infected *ex vivo* with HIV-1 preferentially support R5 variant productive replication [143].

These data suggest that the bottleneck observed *in vivo* may be more complex than a simple selection and transmission of one or few HIV variants, and rely on determinants other than viral genetic features and restriction factors in the FGT.

1.5.2 Determinants of the efficiency of HIV-1 transmission to the FGM

1.5.2.1 Host susceptibility

The efficiency of HIV-1 transmission across the mucosa is a function of both recipient susceptibility and donor infectiousness. Recipient susceptibility to HIV-1 is influenced by the mucosal integrity, availability of susceptible cells, and environmental and behavioral factors. As discussed in section 1.5.1, mucosal trauma during heterosexual contact may disrupt the epithelial barrier and provide HIV-1 direct access to the microcirculation of the mucosa and to the target cells resident in the lamina propria. Behavioral factors, such as increased frequency of sexual contacts, are associated with increased HIV-1 transmission [59].

#### *1.5.2.1.1 Pre-existing infections and genital microflora*

The integrity of the genital epithelium may be compromised by pre-existing sexually transmitted infections, that can also facilitate the recruitment and activation of immune cells in the genital submucosa, increasing the number of potential HIV-1 target and fueling the expasion of the local pool of infected cells [166]. Mucosal infections associated with increased susceptibility include chancroid, syphilis, herpes simplex virus (HSV) infection, and undiagnosed genital ulcers. Local infections may also enhance recipient susceptibility to HIV-1 infection by disrupting the natural equilibrium existing between FGM and commensal microflora [56]. For example, the acidic microenvironment of the vagina is maintained by lactic acid producing commensal bacteria, such as *Lactobacillus* [167]. A higher pH in some ethnic groups correlates with a higher proportion of anaerobic bacteria present in the vagina. In addition to regulating vaginal pH, a recent study by Ahmed et al. reported that specific commensal microdomes may affect HIV transmission by selective activation of TLR signaling pathways [139]. This concept requires further research but is an important observation on how commensal bacteria may directly alter the vaginal environment thus mediating infection. Moreover, it was reported that the amount, molecular size and morphology of mucin glycoproteins protecting the genital epithelium can be altered in disease states. Lactobacillus organisms that colonize the normal human vagina can stimulate the expression of mucins (MUC 2 and 3), whereas pathogens associated with bacterial vaginosis are capable of degrading cervicovaginal mucus, facilitating HIV-1 penetration to reach the mucosa [56].

1.5.2.1.2 Menstrual cycle

The FGM displays unique characteristics compared to other mucosal sites because in addition to protecting against pathogens, it must adapt to a spectrum of physiologic events that include fertilization, implantation, pregnancy and parturition. For this purposes, the menstrual cycle ensures a strict control of tissue growth and remodeling as well as of the immune defenses of the genital mucosa [168]. By examining multiple immunological parameters Wira and Fahey concluded that within the FGT during a normal menstrual cycle, there is a period lasting 7–10 days when important components of innate, humoral, and cell-mediated immunity are suppressed by estradiol and/or progesterone, enhancing the potential for HIV-1 infection [169]. The onset of this "window of vulnerability" coincides with an

increase in estradiol at about the time of ovulation (Fig. 1.5.3).
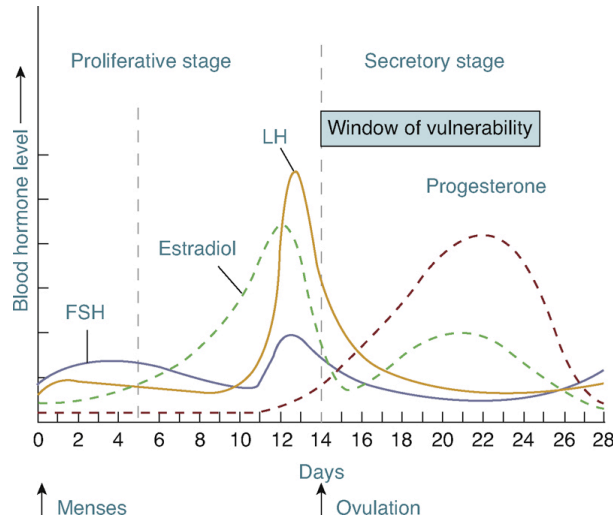
Figure 1.5.3 Relative changes in levels of estradiol and progesterone during the proliferative and secretory stages of the menstrual cycle. Indicated on days 14-23 is the window of vulnerability to HIV infection. FSH = follicle stimulating hormone; LH = luteinizing hormone. (From Wira & Fahey, 2008 [169]).

Analysis of the concentrations of cytokines, chemokines, and antimicrobials in cervico-vaginal lavage indicated that SLPI, defensins, and lactoferrin dropped significantly at midcycle (day 13) and remained depressed for 7–10 days, returning to proliferative stage levels just before menstruation [170]. It was also showed that the titers of anti-human papillomavirus 16 virus-like particle IgG in cervical secretions dropped approximately nine-fold at midcycle during ovulatory cycles [171]. It remains unclear whether sex hormones act directly on immune cells and their secretions in the lower FGT, or whether changes are due to alterations in mucus content or volume. Finally, it has been demonstrated that the immune system throughout the FGT is dampened at the time of fertilization to optimize conditions for implantation and pregnancy [124]. During the proliferative (estradiol-dominant) phase of the menstrual cycle leading up to ovulation, expansion of $CD25^+$ $Foxp3^+$ regulatory T cells occurs in the human uterus. This coincides with a decrease in cytolytic activity by uterine CD8+ cells after ovulation in the uterus. Thus, the modulation of innate and adaptive immune defenses in the FGT by sexual hormones may produce a particular status of immunity aimed to establish tolerance to the fetus, although this enhances host susceptibility to infection of HIV-1 and other sexually transmitted pathogens.

1.5.2.2 Donor infectiousness

Donor infectiousness reflects the probability that the donor will transmit HIV-1 infection to the recipient. The infectiousness of a donor is greatest during primary and late-stage HIV-1 infection, when blood levels of virus are maximal [59]. A high viral burden in blood is a key predictor of heterosexual transmission and appears to correlate directly with the level of virus in genital secretions and, consequently, the amount of virus inoculated onto a mucosal surface [172]. Increases in the genital viral load have great impact on the rates of sexual transmission. Using a probabilistic empirical model that accounts for semen viral load and the number of cervical CCR5 receptors, the probability of transmission was 3 per 10,000 episodes of intercourse when the semen viral load was 1,000 copies per ejaculate, but 1 in 100 when the semen contained 100,000 viral copies per ejaculate [173] (Fig. 1.5.4). The concept that viral shedding in genital fluids reflects the plasma viral load is furthered by findings that successful ART decreases the viral load in semen. However, some men with undetectable plasma viral loads shed HIV in their semen, and the compartmentalization of HIV-1 infection in the male genital tract results in the presence of viral variants in semen that are different from the ones detectable systemically (see section 1.3.5). These considerations highlight the importance of the intrinsic characteristics of semen and local factors restricted to the male genital tract of HIV-1-infected individuals as determinants of the efficiency of HIV-1 sexual transmission. Our lack in the understanding of such phenomenon largely depends on the fact that a model suitable for the study of seminal factors under controlled laboratory conditions has yet to be developed.
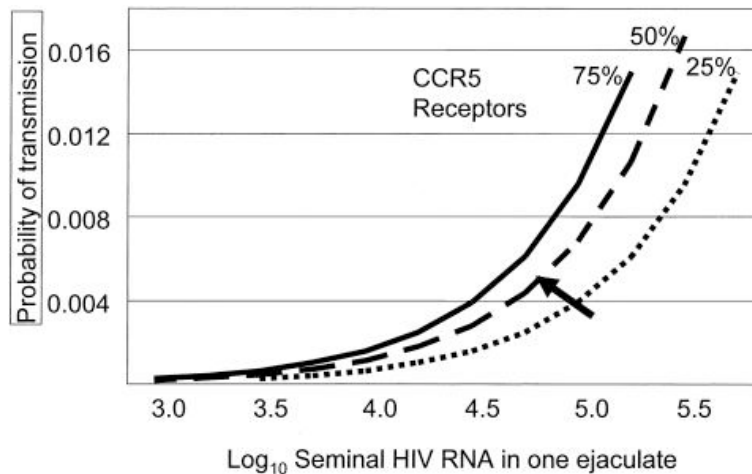

Figure 1.5.4 Estimated male-to-female per sexual contact HIV-1 transmission probability based on the load of HIV-1 in semen and CCR5 density on CD4$^+$ T cells resident in the female genital mucosa. (From Cohen, 2006 [174]).

1.5.2.2.1 Infections of the MGT

HIV-1 infection induces the disruption of the equilibrium between a human host and its virome, leading to two grave consequences: reactivation of ubiquitous symbiotic viruses, which start to replicate to higher levels, and infection by new viruses [175]. Male genital tract infections and local inflammation increase donor infectiousness, likely because of increased HIV-1 shedding and increased accumulation of virus-bearing cells [59, 166]. Sexually transmitted diseases (STDs) that cause ulcers generally increase shedding of HIV in the genital tract [176]. This can occur by direct shedding of HIV from the ulcerative lesion. As the most common causative agent of genital ulcer diseases worldwide, HSV has an important role in HIV infections. HIV-1 RNA was detected in ulcer swabs in 25 of 26 men with symptomatic HSV-2 infection, and the levels exceeded 10,000 copies/mL of swab sample in most cases [177]. Even asymptomatic HSV shedding was found to be associated with increased HIV-1 shedding. In general, STDs that cause inflammation increase the concentration of HIV-1 in the urethra and semen [166]. Treatment of STDs reduces the concentration of HIV-1 in genital secretions [178].

A modest number of experiments have been conducted *in vitro* to examine the direct effect of various sexually transmitted pathogens on HIV replication. Co-infection of macrophages with HIV-1 and certain opportunistic pathogens, such as *Mycobacterium avium* or cytomegalovirus (CMV), causes bidirectional up-regulation of both HIV-1 and the pathogen [59]. Infection of macrophages with opportunistic pathogens may also induce cytokines, such as TNF-α, that enhance HIV-1 replication, and CCR5 ligands, such as CCL3 and CCL4, as well as other chemokines that can recruit mucosal HIV-1 target cells at the site of transmission [59].

Ping and colleagues conducted a detailed study of viral diversity in variants that were harvested from the semen of HIV-1 infected men before and after antibacterial therapy for STDs [179]. The results showed that three-quarters of both STDs and control subjects had multiple HIV variants in their blood, with even more variability in semen. Subjects with STDs who received treatment had more changes in semen variants than blood variants at follow-up, showing that local genital conditions were affecting viral diversity in semen in a way that was not reflected in blood.

1.5.2.2.2 Human herpesviruses

Due to their ubiquity in the human population, human herpesviruses (HHVs) are among the most common viruses that undergo reactivation in HIV-1-infected patients [180]. Human herpesviruses can cause lytic, persistent, and latent/recurrent infections, and Epstein-Barr virus (EBV or HHV-4) and HHV-8 are associated with cancers. Once a patient has become infected by a herpesvirus, the infection remains for life. The initial infection may be followed by latency with subsequent reactivation. Although these viruses usually cause benign disease, they can also cause significant morbidity and mortality, especially in immunosuppressed people [34].

There are at least 25 viruses in the family Herpesviridae (currently divided into three sub-families). 8 herpesvirus types are known to infect man frequently (Table 1.5.1).

Virus	Primary Target Cell	Site of Latency	Means of Spread
Herpes simplex type 1 (HHV-1)	Mucoepithelial cells	Neuron	Close contact
Herpes simplex type 2 (HHV-2)	Mucoepithelial cells	Neuron	Close contact (sexually transmitted disease)
Varicella-zoster virus (HHV-3)	Mucoepithelial and T cells	Neuron	Respiratory and close contact
Epstein-Barr virus (HHV-4)	B cells and epithelial cells	B cell	Saliva (kissing disease)
Kaposi sarcoma–related virus (HHV-8)	Lymphocyte and other cells	B cell	Close contact (sexual), saliva ?
Cytomegalovirus (HHV-5)	Monocyte, lymphocyte, and epithelial cells	Monocyte, lymphocyte, and ?	Close contact, transfusions, tissue transplant, and congenital
Herpes lymphotropic virus (HHV-6)	Like CMV, salivary glands, neurons	T cells and ?	Saliva
Human herpesvirus 7 (HHV-7)	Like CMV	T cells and ?	Saliva

Table 1.5.1 Human herpesviruses classified by their target cells and location in the latent state. (From Murray et al., 2008 [34]).

HSV-1, HSV-2 and CMV are sexually transmitted pathogens and are extremely prevalent worldwide. Among HIV-1-infected people the seroprevalence of HSV-2 is ~70 to 90% [181], and seminal shedding of HSV is associated with higher HIV-1 RNA genital levels (see section 1.5.2.2.1). The effect of HSV infection on the viral burden of HIV in blood has been the subject of unresolved but important debate about the adjunctive potential of HSV antiviral therapy on the management of HIV. It was demonstrated that anti-herpetic therapy in individuals who are co-infected with HIV and HSV-2 reduced HIV blood and seminal plasma viral loads [182, 183]. However, a clinical trial conducted by Celum at al. showed that daily acyclovir therapy did not reduce the risk of transmission of HIV-1, despite a reduction in plasma HIV-1 RNA and a 73% reduction in the occurrence of genital ulcers due to HSV-2 [184].

The seroprevalence of CMV among HIV-infected men is even higher at 95 to 100%, and CMV is associated with HIV disease progression in both treated and untreated individuals [185]. A possible mechanism for this accelerated disease progression may be CMV enhancement of HIV replication, especially in the male genital tract, where CMV levels positively correlate with HIV levels [186]. Moreover, asymptomatic CMV coinfection is associated with higher T cell immune activation,

which is linked to blunted CD4^{+} T cell recovery during antiretroviral therapy and to premature mortality [187].

Compartmentalized replication of CMV in the genital tract, probably has consequences for CMV transmission, impacts localized immune responses and interacts with other sexually transmitted viruses, like HIV-1 [188]. Except for HSV-2 and CMV, limited information is available on herpersvirus infections in the male genital tract of HIV-1-infected individuals and their shedding in semen.

Human herpesviruses have been studied mainly in the context of disease progression. Many mechanisms have been reported through which herpesviruses contribute or interfere with HIV-1 replication. The strategies used by these viruses to evade the host immune response include: (1) downregulation of HLA (e.g., by CMV-encoded US2, 3, 6, and 11; HHV-6- and HHV-7-encoded UL-21); (2) induction of host cytokines (e.g., of CXCL8, CCL5, CCL2, and IL-1 by CMV or EBV); (3) interference with host cytokine signaling (e.g., TNF-α- and IFN-α-signaling modulated by EBV); (4) expression of virus-encoded chemokine homologs (virokines) and chemokine receptors (viroceptors) (e.g., EBV-encoded IL-10 homolog BCRF1, CMV-encoded IL-10 homolog US111, HHV-6-encoded CCL5 homolog U83, and HHV-7-encoded β-chemokine receptor homolog U12); (5) inhibition of apoptosis (e.g., by CMV-encoded v-Bcl-2 homolog or EBV-encoded v-ICA and v-MIA); and (6) altered activation of autologous complement (e.g., by modulation of CD46 expression by HHV-6 and HHV-7) [175]. All these effects may favor the immune evasion not only of these viruses, but also of HIV-1 itself.

On the other hand, some of the effects induced by HHV-6 and HHV-7 have been demonstrated to inhibit HIV-1 replication. Up-regulation of CCR5-binding chemokines CCL3, CCL4, and CCL5 was showed to mediate HIV-1 suppression by HHV-6 in co-infected human lymphoid tissue *ex vivo* [189], although a prolonged up-regulation of CCR5-binding chemokines *in vivo* favours the selection for CCL5-resistant viral variants [190]. CCL5 resistance, including the emergence of highly pathogenic CXCR4-utilizing HIV-1 variants, is associated with the late stages of HIV disease [191]. This association may explain how HHV-6, in spite of the ability to suppress HIV-1 infection, constitutes a co-factor in HIV disease progression [192]. A diminished CD4 expression has been reported for HHV-7 infection of human lymphoid tissues *ex vivo*. In these tissues, CD4 is down-regulated not only in HHV-7-infected cells, but also in bystander (uninfected) CD4^{+} T cells, making this effect massive. As a result, in HHV-7-coinfected human tissues, HIV-1 is suppressed [193].

1.6 Study models of HIV-1 transmission to the FGM

Understanding the mechanisms of HIV-1 transmission to the FGM requires knowledge of the functions and interactions of all immune cells and of the extracellular matrix in this tissue. Although conventional cultures of isolated cell lines or peripheral blood mononuclear cells have been useful in many areas of HIV research, these cultures are limited by the fact that they do not reproduce the spatial distribution of cells and their native communication within the tissue cytoarchitecture.

1.6.1 Non human primates

Experimental SIV infections of rhesus macaques have been used for decades to study the various aspects of the biology of primate lentivirus infection. Many aspects of human infection can be recapitulated in a NHP model. These models provide a unique opportunity to study early events in viral transmission and evolution, both systemically and within tissue compartments. The ability to sample tissues before infection and immediately after challenge make NHP models a valuable tool for helping to elucidate the contributions of different anatomical barriers and innate immune mechanisms to prevent infection.

Historically, the initial development of NHP models involved the identification of pathogenic viral isolates and the use of high-dose intravenous inoculation to reproducibly infect the animals, cause viremia typical of human infection, and eventually lead to AIDS-like pathology. Next, a high-dose intrarectal challenge model was adopted for basic transmission and vaccine efficacy studies designed to achieve 100% infection rate while providing a relevant mucosal route of infection of broader clinical relevance. Mucosal challenge models were further modified with a shift to repeated challenges with lower inocula, titered to more accurately simulate clinically relevant virus exposures [194].

Studies of vaginal infections in NHP models have been problematic because of the extensive inte-animal variability in establishing infection, even after uniform challenge conditions. Factors contributing to this variability may include changes in epithelial thickness and mucus that vary as a function of menstrual cycle stage, access point of the virus, preexisting inflammation, or target cell availability. Finally, although it has been reported that cell-associated virus can also initiate infection [195], it is difficult to rigorously rule out the potential contributions to transmission of cell-free virus derived from freshly produced virus even in thoroughly washed inoculums of infected cells. Although it is thus difficult to unequivocally demonstrate a role for infected cells in mucosal transmission, it is unequivocally clear that cell-free SIV is sufficient in these models to recapitulate the infection outcomes observed in HIV infection.

1.6.2 Human tissue explants

Typically, tissue fidelity and function are best achieved by attempts to maintain tissue biopsies or explants *ex vivo*, rather than attempts to reconstruct tissue from individual cell types. For the study of multiple aspects of HIV pathogenesis our laboratory has favored the use of raft culture, in which blocks of human tissue are cultured on collagen sponges at the air-liquid interface. This *ex vivo* raft model of tissue culture is based on work of Joseph Leighton [196, 197]. Leighton's three-

dimensional histoculture method, which utilized a sponge matrix, was further developed in the 1980s by Robert Hoffman and his colleagues for anticancer drug studies [198]. Our laboratory first benchmarked this method for HIV studies using cultures of human tonsils and lymph nodes [199].

This model has many advantages (Tab. 1.6), the first of which is the preservation of the tissue architecture for 2–3 weeks. Certain functions of the lymphoid tissue are also maintained *ex vivo*, including the ability to release a spectrum of cytokines similar to those released by *in vivo* tissue, and the ability of tissue challenged with recall antigens (tetanus or diphtheria toxoids) to respond by producing specific antibodies [200]. *Ex vivo* tissues support HIV replication without the exogenous stimulation that is necessary for productive HIV infection in isolated lymphocytes. Also, these tissues support replication of other human viruses including HHV-6 [189, 201], HHV-7 [193], CMV [202], HSV-2 [203], vaccinia [204], measles virus [205, 206], and West Nile virus [207], as well as non-viral human pathogens such as the bacterium *Borrelia burgdorferi* [208] and the parasite *Toxoplasma gondii* [209]. Some of these viral and non-viral pathogens are commonly associated with HIV-1, thus allowing the simultaneous study of HIV co-pathogenesis.

Recently this *ex vivo* tissue system was extended to include cultures of cervico-vaginal and rectosigmoid tissues. Since these tissues serve as the first gateway for HIV-1 sexual transmission, preserving the specific mucosal cell phenotypes and functions are critical for understanding early events in HIV-1 transmission. Moreover, cervico-vaginal, rectosigmoidal, and other *ex vivo* tissues can be used to evaluate potential microbicides in pre-clinical tests.

Advantages	Disadvantages
• Preservation of tissue cytoarchitecture, including major lymphocyte subtypes and follicular-dendritic cell network	• Tissues start to deteriorate after 3 weeks in culture
• *In vivo*-like spectrum of cytokine release	• Difficulty in monitoring cells beyond the depth of confocal microscopy (unless cells are isolated for analysis)
• Continuous expression of HIV coreceptors over 2 weeks in culture	• The system does not reflect the effects of *in vivo* systemic factors
• Support of HIV replication without exogenous stimulation or activation	• Labor: multiple blocks of tissue are required for every experimental condition to overcome tissue heterogeneity
• '*In vivo* pattern of replication': HIV replicates in both activated and nonactivated cells	• Donor-to-donor variability
• Other tested human viruses, bacteria and parasites readily infect tissues, allowing the study of their pathogenesis and interactions	
• Viral movement in the tissue can be followed in real time by confocal microscopy	

Table 1.6. Advantages and disadvantages of human tissue ex vivo model for the study of human pathogens. (From Grivel & Margolis, 2009 [210]).

1.6.2.1 Cervico-vaginal models to study HIV-1 infection

Cervical explant cultures were first developed by Fink et al. for the study of epithelium metaplasia *in vitro* [211]. They consisted of large tissue explants cultured on a thin slab of agarose-gelled serum-free Eagle's Basal Medium on top of a stainless steel supporting grid. This culture system was successfully adapted to the study of HSV-2 and HSV-1 infection *in vitro* and was shown to support the replication of these two human herpesviruses [212]. O'Brien et al. modified this method for the study of the production of glycoprotein from normal and malignant cervical explants of smaller size cultured either fully immersed in serum-free culture medium or maintained at the air-liquid interface, supported only by a stainless steel grid mesh [213]. On the basis of glycoprotein production, the authors concluded that the grid technique was superior to the immersion culture. It was this grid technique which was adapted to the study of HIV-1 infection in human cervical explants by Palacio et al. [214]. In this method, cervical explants (3×3×2 mm) were infected by immersion in viral stocks, washed twice to remove unadsorbed virus, and then supported on a stainless steel mesh at the air-liquid interface. In contrast to some NHP studies, where the first cells productively infected by HIV-1 are CD4$^+$ T cells (see section 1.5.1), in this *ex vivo* model the majority of viral antigen was detected in sub-epithelial cells expressing markers of macrophages, isolated from tissues infected with HIV-1 R5 variants. This can be explained considering that infection of explants by soaking in cell-free virus suspension gives the virus full access to both epithelial and subepithelial cells, thus mimicking viral penetration through a damaged epithelium. To study the ability of HIV to cross a continuous layer of genital epithelium, explants have to be polarized in an Ussing chamber mechanically sealed with rubber O rings [160]. In such a system, the tightness of the seal is a critical issue and has to be constantly controlled, as monitored for example from the lack of permeability to a small tracer molecule, such as inulin. Such experiments demonstrated that intact epithelium is impervious to cell-free and to cell-associated HIV-1 penetration. This result was at variance with results obtained in a polarized and sealed cervical explant model, in which a rapid penetration of HIV through the epithelial layer was reported [158]. In the latter model, circular explants obtained by punch biopsies were cultured, epithelium pointing upward, on the upper chamber of a transwell and sealed with agarose. Production or transfer of virus was detected from the infection of indicator cell lines placed in the lower chamber, mimicking the basolateral submucosa. An alternative way to maintain cervico-vaginal explants is to culture them on rafts rather than immersed in culture medium [147]. Essentially, this is an adaptation to cervico-vaginal tissues of the collagen raft culture model initially developed for tonsils in our laboratory [199]. This adaptation consisted in immersing explants in viral stocks, transferring them on top of collagen sponges, and culturing them at the air-liquid interface. Aside from their culture methods, this and the models described above differ by the way in which HIV-1 infection is detected. A productive infection in the immersion models, whether they involve explants containing the whole mucosal layer or only epithelial sheets [119], relies on the infection and viral amplification by the cells that populate the explants. In the polarized models, HIV-1 infection depends the transfer of virus to indicator cell lines.

While each of these systems has its intrinsic limitations, they have all proven to be valuable tools for studying several aspects of HIV-1 infection in the lower female

genital tract. These models have been widely used as platforms to test potential microbicides [159, 215-218] and viral fitness [219]. Obviously, none of these models fully reproduces the complex phenomenon of HIV-1 transmission, and explant models suffer from the limitation that the tissue is disconnected from the body and its supply of cells, especially of immune cells, which may be recruited to the genital tract upon infection and fuel the initial pool of infected cells *in vivo*. However, we consider this model adequate for the study and characterization of the very early events in HIV-1 infection.

1.6.2.1.1 Optimization of the raft-based histoculture

In spite of their peculiar advantages, neither of the two main culture methods (polarized or immersion/raft) addressed the nature of the HIV-infected cells by use of polychromatic flow cytometry in the absence of cell activation. In an attempt to improve these models, we have developed an explant culture model built on the collagen sponge raft culture system (Fig. 1.6). In this model, small blocks of cervical tissue mucosa are infected by immersion in viral stock for 2 hours, washed extensively, and cultured on top of collagen sponge gel in RPMI supplemented with 10% FBS in the absence of cellular activators (see section 2.4.2). To account for tissue variation due to sampling, at least 16 explants are used per experimental condition. Donor-matched explant cultures containing potent antiviral agents are used to control for viral adsorption during infection and subsequent desorption during culture.

To apply the power of polychromatic flow cytometry to characterize the cells infected in explants, it is required to prepare a single-cell suspension of tissue cells. Although mechanical digestion has been used by other groups [156], it is our experience that such a treatment results in poor cell recovery because of cell death during tissue processing. Instead, we have developed an enzymatic digestion protocol, which spares most of the cell surface markers studied [210]. The first application of this new method of cervical explant culture and analysis was reported by Saba et al. [143]. This study established that the majority of cervical T cells are effector memory cells. Moreover, as *in vivo*, in spite of the broad expression of CXCR4, explants preferentially supported replication of R5 rather than X4 HIV-1 variants. Because this model does not involve the use of cellular activators, it was possible to characterize the cells isolated from infected explants: R5 HIV-1 infection occurred preferentially in activated $CD38^+$ $CD4^+$ T cells and was followed by activation of bystander $CD4^+$ T cells. The same culture model was employed in the present work to address the effect of seminal interleukin-7 on HIV-1 transmission to cervico-vaginal tissue explants.

Uterus

Cervix

Vagina

Endocervix

Ectocervix
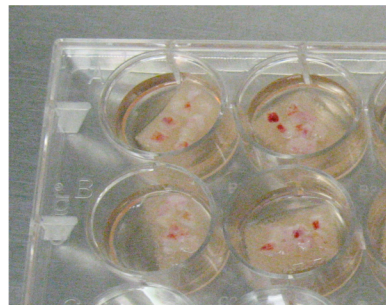
Cervico-vaginal tissue

Muscular tissue
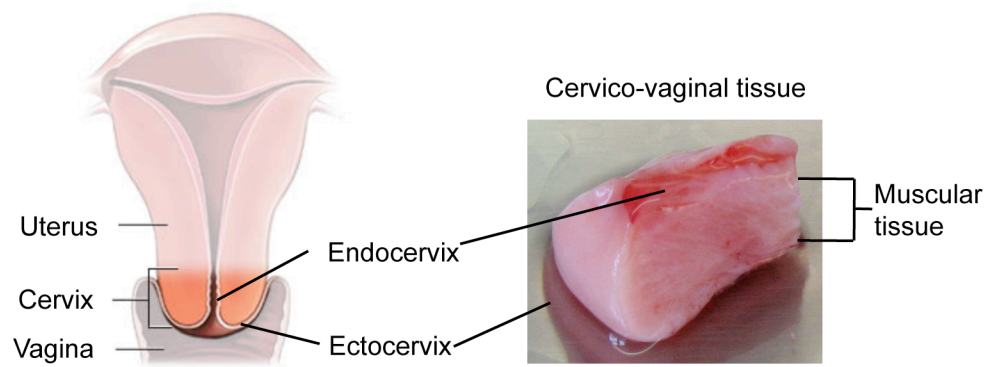
Strips of mucosa
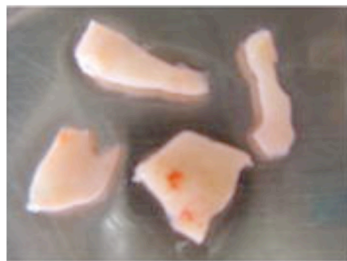
Mucosal tissue blocks

Tissue blocks on top of collagen gels in a 12-well plate

Figure 1.6 Preparation of cervico-vaginal tissue for histoculture. Note the difference in color between ectocervix and endocervix, which both appear shiner compared to muscular tissue due to the presence of a layer of mucus covering the epithelium.

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2. MATERIALS AND METHODS

2.1 Ethics statement and source of clinical samples

The protocol was approved by the Institutional Review Board of both the National Institutes of Health and the All India Institute of Medical Sciences (AIIMS), New Delhi, India. The study participants were recruited in AIIMS between September 2008 and November 2009 (Table 2.1). After 2–5 days of sexual abstinence, semen samples were obtained by masturbation into a sterile container and processed within 1 hour of ejaculation. Semen was centrifuged at 800×*g* for 10 min, and the resulting supernatant (seminal plasma) was aliquoted and stored at -80 °C until use. 84 therapy-naïve HIV-infected patients were enrolled in the study; one patient was excluded from further analysis because he was infected with HIV-2. Due to the social and cultural stigma for HIV-1 infection in India few of the enrolled patients missed follow up visits, thus sufficient material to measure HIV-1 blood plasma load was available from 74 individuals; paired HIV-1 load and CD4$^+$ T cell counts were available from 70 individuals. Semen was collected from 50 HIV-1-infected individuals; paired blood and seminal plasma samples were available from 43 of these. 33 HIV-1-uninfected individuals, among healthy volunteers referred to the AIIMS fertility clinic, were enrolled in the study as HIV-1-uninfected controls. Blood and semen were collected from 27 and 28 individuals respectively; paired blood and seminal plasma samples were available from 22 HIV-1-uninfected controls.

Tonsils from routine tonsillectomies were obtained from the Children's Hospital (Washington, DC). Cervico-vaginal tissues were obtained from routine hysterectomy through the National Disease Research Interchange (NDRI, Philadelphia, PA). All tissues were obtained according to an Institutional Review Board-approved protocol.

		HIV-1 + (n=83)	HIV-1 – (n=33)
		Number (frequency %)	
Age	< 30 years	23 (27)	14 (42)
	> 30 years	60 (73)	19 (58)
Marital status	unmarried	19 (23)	0
	married/long term consensual relation	64 (77)	33 (100)
Employment status	unemployed	9 (11)	0
	self-employed	44 (53)	14 (42)
	full-time wage earner	30 (36)	19 (58)
Mode of HIV-1 acquisition	heterosexual intercourse	70 (84.3)	NA
	injection drug use/transfusion related	5 (6)	NA
	males who have sex with males	6 (7.2	NA
	unknown	2 (2.5)	NA

Table 2.1 Socio-demographic characteristics of the enrolled HIV-1-infected and HIV-uninfected individuals. NA = not applicable.

2.2 Nucleic acid extraction and real-time PCR

Nucleic acids were extracted from seminal and blood plasma with the NucliSENS Biomerieux EasyMag 2.0 instrument (BioMerieux, Durham, NC) according to the manufacturer's instructions. 200 µL of sample were placed in a disposable vessel and then loaded onto the extractor instrument. After 10 min of intial lysis incubation, 150 µL of magnetic silica was added to each sample. The samples were then processed according to the manufacturer's instructions. Purified nucleic acid were eluted and stored at -80°C until further use.

To measure the viral loads of human herpesviruses (HHV) we employed a real-time PCR TaqMan assay using the sets of primers and probes listed in table 2.2.

Target gene	Forward primer	Reverse primer	Probe (FAM)
HSV-2 UL27	cgcatcaagaccacctcctc	gctcgcaccacgcga	cggcgatgcgccccag
EBV EBNA	gactgtgtgcagctttgacgat	cagcccccttccaccataggt	cctccctggtttcc
CMV UL83	tcgcgcccgaagagg	cggccggattgtggatt	caccgaacgaggattccgacaacgt
HHV-6 UL67	cgctaggttgaggatgatcga	caaagccaaattatccagagcg	cccgaaggaataacgctc
HHV-7 UL67	agcggtacctgtaaaatcatcca	aacagaaacgccacctcgat	gagaacatcgctctaactggatca
HHV-8 ORF26	gtccagacgatatgtgcgc	actccaaaatatcggccgg	ttggtggtatatagatcaagttc

Table 2.2 Primers and probes used in the quantification of human herpesvirus target genes by real-time PCR.

Amplifications were performed in 25 µl reaction mixture containing 1X Fast TaqMan PCR master mix, each primer at 300 nM, probe at 200 nM and template DNA.

Reverse transcription for the quantification of HIV-1 RNA load in blood and seminal plasma was performed with the RevertAid reverse Transcriptase kit (Fermentas, Glen Burnie, MD) according to the manufacturer's instructions. Reverse transcription was performed in 20µl reaction mixture containing 1X Reaction buffer, 1 mM dNTPs, 20U of RNAse inhibitor, 2.5 µM random hexamers, 200 U of MMuLV Reverse Transcriptase and template RNA. Each reaction was performed for 5 min at 25°C, 60 min at 42°C and inactivation step for 10 min at 70°C using an Eppendorf Mastercycler instrument (Eppendorf, Hauppauge NY). A SYBR green real-time PCR assay was then used to quantify HIV-1 RNA load. The amplifications were conducted using a set of primers (forward primer: tgtgtgcccgtctgttgtgt; reverse primer: gagtcctgcgtcgagagagc) amplifying 100 base pair of a highly conserved region of HIV-1gag previously described for the quantification of HIV-1 load of different group M HIV-1 subtypes including subtype C [220].

Reference standard curves were generated using serially diluted plasmids containing the target genes or a commercially available quantitated viral DNA (Advanced Biotechnologies, Columbia, MD). Aliquots were prepared once by

dilution of DNA in distilled water and were stored at -20°C. Water and DNA extracted from HIV-1-uninfected and HHV-free cell line (MT4) were included for each of the amplifications as negative controls. Following activation of DNA polymerase at 95°C for 10 min, 40 cycles of amplification (denaturation step 95°C for 15 sec; annealing-extension step 60°C for 1 min) were performed with an ABI 7500 Fast Real-Time PCR sequence detector (Applied Biosystems, Foster City, CA). For HIV-1, a dissociation profile was obtained by ramping from 60°C to 95°C. An accurate analysis of the melting temperature curve of the generated amplicons was conducted for all the amplifications to rule out any non-specific interference. Data were analyzed using sequence detection software (Applied Biosystems SDS v1.3.1). For purposes of statistical analysis samples with undetectable HIV-1 RNA or herpesvirus DNA were arbitrary considered to contain 3 copies/mL.

2.3 Cytokine quantification

Using a multiplex bead array we measured the levels of the following 21 cytokines: interleukin (IL)-1α, IL-1β, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-15, IL-16, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), regulated on activation normally T-cell expressed and secreted (RANTES; CCL5), monocyte chemotactic protein-1 (MCP-1; CCL2), eotaxin (CCL11), interferon (IFN)-γ, monokine induced by IFN-γ (MIG; CXCL9), IFN-γ-inducible protein 10 (IP-10; CXCL10), stromal derived factor (SDF)-1β (CXCL12b), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β.

2.3.1 Coupling of antibodies to beads

Individual carboxylated polystyrene Luminex bead sets (Itachi Mirabio, Alameda, CA) were coupled to cytokine-specific capture monoclonal antibodies using a two-step corbodiimide coupling procedure. 1 mL of beads at the concentration of 12.5 beads/mL was transferred in a 1.5 mL protein low binding tube (Eppendorf, Hauppauge, NY) and centrifugated at 8000xg for 2 minutes. Pelletted beads were washed once with cell culture grade water by vortex and sonication for 30 seconds and then resuspended in 160 μL of 100 mM monobasic sodium phosphate (pH=6.2). Beads were activated fro 20 minutes at room temperature (RT) by addition of 20 μL of 50 mg/mL solution of EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Thermo Fisher Scientific, Rockford, IL) in water and 20 μL of a freshly prepared 50 mg/mL solution of Sulfo-NHS (N-hydroxysulfosuccinimide, Thermo Fisher Scientific) in water. Activated beads were washed twice with PBS (pH 7.4) and resuspended in 500 μL of PBS. 200 μg of cytokine-specific capture antibody diluted in PBS were added to the bead suspension. After bringing total volume to 1 mL with PBS, beads were incubated for 2 hours by mixing by rotation at RT and overnight at 4 °C. Beads were washed twice with 500 μL of PBS-TBN (PBS, 0.1% BSA, 0.02% Tween-20, 0.05% Azide, pH 7.4) and resuspended in 500 μL of PBS-TBN. Beads were enumerated using an automated hemocytometer (Cellometer, Nexcelom Bioscience, Lawrence, MA) and presence of antibodies coupled to the bead surface was verified using an anti-mouse IgG R-Phycoerythrin (RPE)-conjugated antibody (Invitrogen, Carlsbad, CA). Conjugated beads were kept at 4°C until use. All capture monoclonal antibodies were purchased from R&D (Minneapolis, MN).

2.3.2 Bead-based array assay

Biotinylated polyclonal antibodies were used at twice the concentrations recommended for a classical ELISA (according to the manufacturer). All assay procedures were performed in assay buffer containing PBS supplemented with 0.1% normal mouse serum (Gemini Bioproducts, West Sacramento, CA), 0.1% goat serum (Gemini Bioproducts), 0.05% TWEEN-20, and 20 mM Tris-HCl (pH 7.4). The assays were run using 2000 beads of each bead set per well in a total volume of 50 μL. A total of 50 μL of each sample was added to the well and incubated overnight at 4°C in a Millipore Multiscreen plate (Millipore, Billerica, MA). The liquid was then aspirated using a Vacuum Manifold (Millipore), and the plates were washed twice with a solution of 0.05% TWEEN-20, and 20 mM Tris-HCl in PBS (pH 7.4). The beads were then resuspended in 50 μL of assay buffer containing biotinylated polyclonal antibodies specific for the measured cytokines for 30 minutes at room temperature. The plates were washed twice with washing buffer using the vacuum manifold. The beads were resuspended in 100 μL of assay buffer containing RPE-conjugated streptavidin (Invitrogen) at the concentration of 8 μg/mL and incubated for 30 minutes at RT shaking. The plates were washed twice with washing buffer using the vacuum manifold and beads were resupsended in 100 μL of assay buffer.

All detection byotinilated antibodies and recombinant human cytokines used as standards were purchased from R&D.

The plates were read on a Luminex-100 platform. For each bead set of the 21 tested, a minimum of 100 beads in a volume of 80 μL was recorded in the region-specific gate. The median fluorescence intensity was recorded for each of these beads and was used for analysis with the Bio-Plex Manager software (version 5.0; Bio-Rad, Hercules, CA) using a 5P regression algorithm.

Values that were below the lower limit of detection (LLD) were reported as the midpoint between zero and the LLD (LLDs in pg/mL: IL-1β: 4; IL-2: 12.1; IL-6 and IL-7: 4.7; CXCL8: 16.4; IL-15: 10.3; IL-16: 20.6; CCL3: 39.1; CCL20: 6.2; CCL5: 10; CCL2: 25; CCL11: 7.5; IL-1α, GM-CSF, CCL4, IFN-γ, CXCL9, and CXCL10: 3.4; TNF-α: 2.1; CXCL12b and TGF-β: 6.9).

2.4 Human tissue culture

2.4.1 Preparation of collagen sponge gels

Explant culture medium (ECM) was prepared as follow: RPMI 1640 (Gibco Life Technologies, Grand Island, NY), 15% of fetal bovine serum (Gemini Bioproducts, West Sacramento, CA), 0.1 mM of MEM-nonessential amino acids (Gibco Life Technologies), 2.5 μg/mL fungizone (Gibco Life Technologies), 1 mM MEM–sodium pyruvate (Gibco Life Technologies) and 50 μg/mL of gentamicin (Cellgro-Mediatech, Manassas, VA).

Collagen sponge gels 12-7 mm (adsorbable gelatin sponge; Pharmacia and Upjohn Company, distributed by Pfizer) were hydrated in ECM supplemented with timentin at the concentration of 0.3 mg/mL using a bent flat spatula to press the gel against the bottom of a petri dish. This process is critical to avoid that the presence of air will block the capillaries through which nutrients reach the tissue. For tonsillar tissue, rehydrated collagen sponge gels were cut in 4 equal pieces that were placed into a 6-well plate (1 piece into each well) containing 3 mL of ECM

supplemented with timentin per well. For cervico-vaginal tissue, gels were cut into 6 equal pieces that were placed into a 12-well plate containing 1 ml of ECM supplemented with timentin per well.
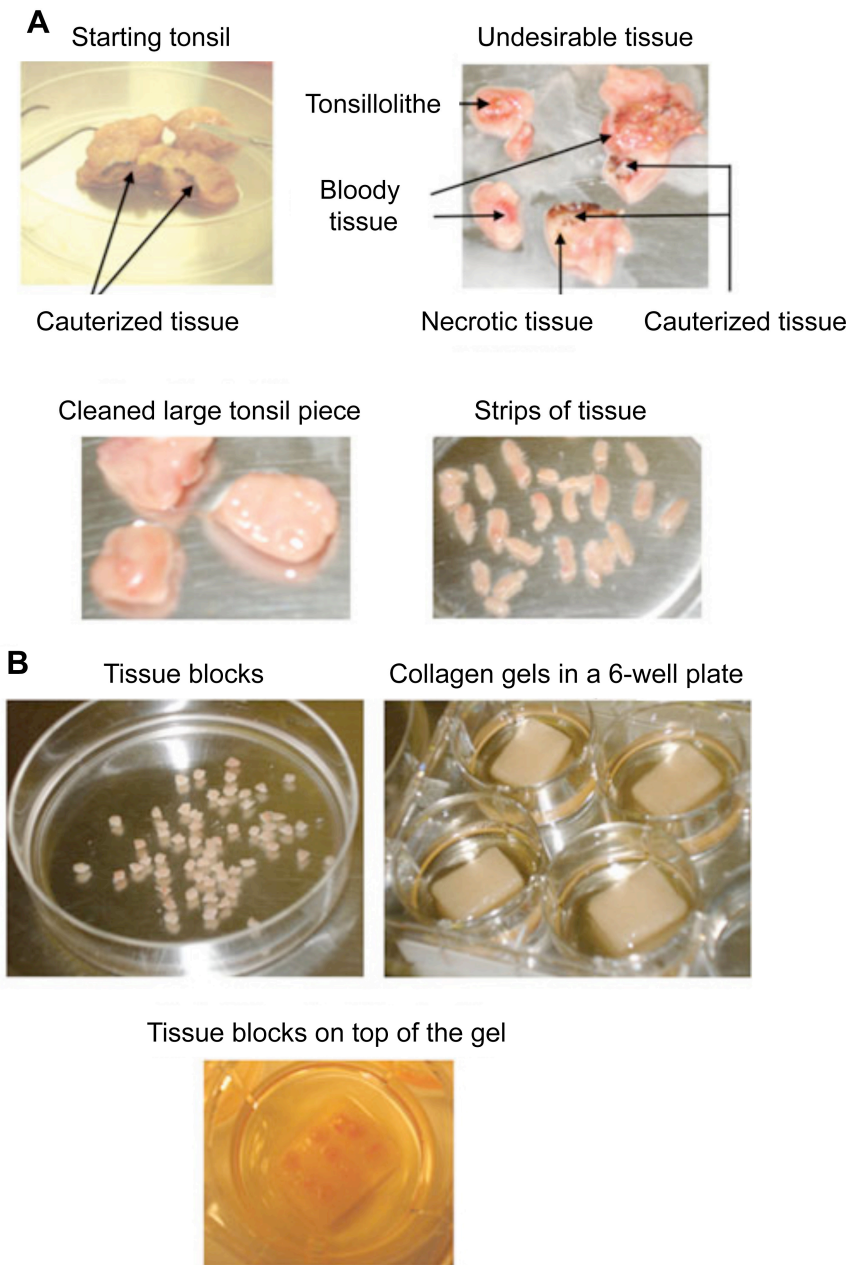
2.4.2 Dissection and *ex vivo* infection of human tissues with HIV-1

To establish long-term tissue culture, it is critical to start the experiment with tissues in good condition, lacking blood clots and necrotic parts [210]. Tonsillar tissue was delivered within 5 hours post-surgery in saline solution without ice cooling during transportation. Cervico-vaginal, which is sturdier and less susceptible to deterioration than tonsillar tissue, was delivered after 24 hours post-surgery, in ice-cold RPMI 1640.

2.4.2.1 Lymphoid tissue

Tonsils were dissected into big pieces using a 10-gauge blade scalpel and forceps. Cauterized tissue, the capsule surrounding the tissue, bloody, inflamed parts, and any parts containing tonsillolithes or necrotic (light brown-green color) were removed (Fig 2.1A). Tonsillar tissue was dissected into approximately 8 mm^3 blocks and placed on collagen sponge gels (9 blocks per piece) at the air-liquid interface (Fig 2.1B). For each experimental condition 18 or 27 blocks were used depending on the experiment. Blocks were maintained in culture overnight at 37°C, 5% CO_2, and high relative humidity (>83%).

The day after, medium was changed using ECM supplemented with timentin. Tissue blocks were infected by application of 6.3 µL of HIV-1$_{BaL}$ or HIV-1$_{LAI.04}$ undiluted or 1:100 diluted in RPMI 1640, on top of each block and maintained at 37°C, 5% CO_2, and high relative humidity.

A
Starting tonsil

Undesirable tissue

Tonsillolithe

Bloody
tissue

Cauterized tissue

Necrotic tissue   Cauterized tissue

Cleaned large tonsil piece

Strips of tissue

B
Tissue blocks

Collagen gels in a 6-well plate

Tissue blocks on top of the gel

*Figure 2.1 Preparation of tonsillar tissue for histoculture. (**A**) Black arrows point to the part of the tissue that should be discarded. (**B**) Tissue before and after being deposed on collagen sponge gel in a 6-well plate.*
(From Grivel & Margolis, 2009 [210]).

2.4.2.2 Cervico-vaginal tissue

In the received cervical samples, the endocervix was recognized as the part with darker color and usually coated by thick mucus (Fig. 1.6). The mucosal epithelium and the underlying stroma of both the ectocervix and the endocervix were separated from the muscular tissue and dissected into approximately 8 mm^3 blocks using a 23-gauge blade scalpel and forceps.

For each experimental condition, 24 cervical blocks were transferred into two 1.5-mL conical tubes (12 blocks per tube), each containing 0.35 mL of viral stock HIV-1$_{BaL}$. After 2 hours of incubation at 37 °C in a thermomixer at 300 rears per minute (rpm), tissue blocks were transferred into a 6-well plate and gently washed three times with 4 mL of PBS. Blocks were placed on top of a collagen sponge gel into a 12-well plate (8 blocks/well). Blocks were maintained in culture at 37°C, 5% CO_2, and high relative humidity (>83%). As control to discriminate productive HIV-1 replication from virus absorption and subsequent release from the tissue, we infected cervico-vaginal blocks with HIV-1$_{BaL}$ in the presence of the HIV reverse transcriptase inhibitor Lamivudine, also known as 3TC, at the concentration of 10 μM. Lamivudine 10 μM was maintained for all the length of tissue culture.

2.4.2.3 Treatment with interleukin-7

Recombinant human interleukin (IL)-7 (Peprotech, Rocky Hill, NJ) was added to culture medium at the concentration of 5 or 25 ng/mL and tonsillar and cervico-vaginal tissue blocks were maintained in culture in the presence or in the absence of IL-7 for 6, 9, or 12 days (depending on the goal of the experiment). Tonsillar tissue blocks were treated with IL-7 immediately after cutting and incubated overnight with IL-7 before HIV-1 infection. IL-7 was added to the viral stock during infection of cervico-vaginal tissue blocks. Culture medium was sampled by pooling the medium from all 2 or 3 wells of same condition and thereafter medium was changed using ECM without timentin every third day after infection.

2.5 Viruses

To infect lymphoid and cervico-vaginal tissue block *ex vivo* we used 2 different prototypic HIV-1 variants: the CCR5-using virus HIV-1$_{BaL}$, and the CXCR4-using virus HIV-1$_{LAI.04}$. Both HIV-1$_{BaL}$ and HIV-1$_{LAI.04}$ viral preparations were obtained from the Virology Quality Assurance Laboratory at Rush University (Chicago, IL). Viral stocks were obtained from the medium of peripheral blood mononuclear cell cultures inoculated with either HIV-1$_{BaL}$ or HIV-1$_{LAI.04}$, originally received from the NIH AIDS Reagent Program. HIV-1 p24$_{gag}$ antigen concentrations were 49 ± 3 and 53 ± 3 ng/mL for HIV-1$_{BaL}$ and HIV-1$_{LAI.04}$ stock, respectively. Viral inocula were adjusted to obtain similar replication levels in lymphoid and cervico-vaginal tissue.

2.6 Flow cytometry

Tissue blocks were harvested and transferred into a 1.5 mL conical tube. Tonsillar and cervico-vaginal tissue blocks were respectively digested for 30 and 45 min with Liberase low Dispase concentration (Roche, Indianapolis, IN) at 8 μg/mL (final concentration) in 1 mL of RPMI1640 (Gibco Life Technologies) containing DNase I (Roche) at 100 μg/mL (final concentration) at 37 °C in a thermomixer at 900 rpm. After digestion, the cell suspension was removed from the tube and filtered with a prewet 100 μm cell-strainer (BD Bioscience, San Jose, CA) placed on top of a 50

mL tube containing 10 mL of PBS. Tissue blocks in the digestion tube were mechanically disrupted with a pestle and washed with PBS, and the cell suspension was transferred in the cell-strainer. Gel foam was disrupted with forceps and transferred in the cell strainer together with culture medium left after sampling. Cell suspension was centrifugated at 250xg for 5 min, and resuspended in 1 mL of PBS containing 2 μL of Live–Dead fixable blue fluorescent reactive dye reagent kit (Invitrogen) and incubated at RT for 20 min, in the dark. Cells were washed with 1 mL of staining buffer (PBS supplemented with 2% normal mouse serum (Gemini Bioproducts)). All washing steps were performed using 1 mL of staining buffer. To characterize tissue lymphocytes, cells were stained with the monoclonal antibodies anti-CD3 Qdot 655, anti-CD4 Qdot 605, and anti-CD8 Pacific Blue (Invitrogen) in a final volume of 200 μL, at RT for 20 mins, in the dark. After surface staining, cells were washed and then permeabilized with a Fix & Perm kit according to manufacturer instructions (Invitrogen). Cells were stained with an anti-HIV-1 p24$_{gag}$ monoclonal antibody (KC57) conjugated with fluorescein isothiocyanate (FITC) (Beckman Coulter, Indianapolis IN), anti-Bcl-2 phycoerythrine (PE) (BD Bioscience), and anti-APO2.7 PE-Cyanin5 (Beckman Coulter) in a final volume of 200 μL, at RT for 20 mins, in the dark. Cells were washed and then fixed with 200 μL of 2% paraformaldehyde solution in PBS at RT for 20 mins, in the dark. Cells were washed and resuspended in 200 μL of staining buffer. Data were acquired on an LSRII flow cytometer (BD Biosciences) equipped with 355-, 407-, 488-, 532-, and 638-nm laser lines using DIVA 6.1.2 software (BD Biosciences), and analyzed with the software FlowJo version 9.4.10 (Tree Star, Ashland, OR). We identified lymphocytes according to their light-scattering properties. We quantified cell depletion by weighting tonsillar blocks prior to digest and by adding 100 μL of AccuCheck counting beads (Invitrogen) to cell suspension before acquiring. At least 10000 events were acquired in the bead-corresponding gate.

2.7 Dynamic immunofluorescent cytometric bead assay for HIV-1 p24$_{gag}$ quantification

We evaluated productive HIV-1 infection from measurements of HIV-1 p24$_{gag}$ antigen in medium of tonsillar and cervico-vaginal tissue cultures, using a dynamic immunofluorescent cytometric bead assay.

Carboxylated magnetic Luminex beads region 42 (Itachi Mirabio) were coupled to a high-affinity anti-p24$_{gag}$ monoclonal antibody (ImmunoDiagnostics Inc, Woburn, MA) using a two-step corbodiimide coupling procedure (described in section 2.3.1). All assay procedures were performed in assay buffer containing PBS supplemented with 0.1% bovine serum albumin, 0.05% TWEEN-20, and 20 mM Tris-HCl (pH 7.4). The assays were performed using 2000 beads per well in a total volume of 50 μL. Culture medium samples were lysed by incubation with a Triton X 100 solution 1% (final concentration) for 1 hour at 37 °C. 50 μL of lysed samples were added to the bead solution in a flat-bottom 96-well microplate (NUNC Thermo Scientific) and were incubated for 1 hour at RT shaking and at 4 °C overnight. The plates were washed twice with 200 μL of PBS supplemented with 0.05% TWEEN-20 and 20 mM Tris-HCl (pH 7.4) using a microplate wash instrument (Bio-Plex Pro Wash Statio, Bio-Rad). 100 μL of the RD1 PE-labeled anti-HIV-1 p24$_{gag}$ KC57

antibody (Beckman Coulter) diluted in assay buffer to the concentration of 0.5 µg/mL were added to the bead solution and incubated for 30 minutes at RT shaking. The plates were washed twice with 200 µL of washing buffer and beads were resuspended in 100 µL of assay buffer. $p24_{gag}$ standard (Alliance p24 Kit standard) were purchased from PerkinElmer (Waltham, MA).

The plates were read on a Luminex-100 platform. A minimum of 100 beads in a volume of 80 µL was recorded in the region-specific gate. The median fluorescence intensity was recorded for each of these beads and was used for analysis with the Bio-Plex Manager software (version 5.0; Bio-Rad) using a 5P regression algorithm.

2.8 Statistical analysis

We compared continuous variables between groups using the Mann-Whitney test. The Kruskall-Wallis Test with the Dunn Multiple Comparison Test was used to test for differences among more than two independent groups. We compared categorical variables using Fisher`s Exact and and the chi-square test. We assessed linear association of continuous variables using the non-parametric Spearman`s rank correlation coefficient (ρ). McNemar's chi-squared test was used to determine whether the number of correlations differed by HIV-1 status. Multivariate manova analysis of the subsets of cytokines in blood and semen of HIV-1-infected and HIV-1-uninfected men that resulted in a measured value in all tested samples was performed to exclude a possible effect on the correlations by the cytokines that were below the lower limit of detection.

To account for inter-tissue donor variability, we calculated the ratios of the values of HIV-1 $p24_{gag}$ concentrations in culture media, numbers and percentages of $CD8^-$ $p24^+$ cells, percentages of $CD8^-$ $APO2.7^+$ cells, and Bcl-2 MFI, between IL-7-treated and IL-7-untreated donor-matched tissues. For $CD4^+$ T cell depletion, we calculated the difference in the percentages of $CD3^+$ $CD8^-$ cells between IL-7-treated and IL-7-untreated donor-matched tissues. Statistical differences from 0 were evaluated after natural logarithm (log_n) transformation of the ratio or difference values using the One Sample t test. All hypothesis tests were two-tailed, and a *p*-value of <0.05 defined statistical significance.