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REVIEW



# The importance of semen leukocytes in HIV-1 transmission and the development of prevention strategies

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**ABSTRACT**

HIV-1 sexual transmission occurs mostly through contaminated semen, which is a complex mixture of soluble factors with immunoregulatory functions and cells. It is well established that semen cells from HIV-1-infected men are able to produce the virus and that are harnessed to efficiently interact with mucosal barriers exposed during sexual intercourse. Several cofactors contribute to semen infectivity and may enhance the risk of HIV-1 transmission to a partner by increasing local HIV-1 replication in the male genital tract, thereby increasing the number of HIV-1-infected cells and the local HIV-1 shedding in semen. The introduction of combination antiretroviral therapy has improved the life expectancy of HIV-1 infected individuals; however, there is evidence that systemic viral suppression does not always reflect full viral suppression in the seminal compartment. This review focus on the role semen leukocytes play in HIV-1 transmission and discusses implications of the increased resistance of cell-mediated transmission to immune-based prevention strategies.

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## Introduction

Of the estimated 1.7 million new human immunodeficiency virus type 1 (HIV-1) infections worldwide in 2018, the overwhelming majority occurred by sexual transmission (www.unaids.org). Exposure of the vaginal and rectal mucosa to infected semen accounts for most transmission events.[1] In spite of this evidence, a poor understanding of the mechanism regulating immunity at mucosal sites has hampered the development of effective prevention strategies. Transmission of HIV-1 is inefficient relative to that of many other sexually transmitted disease (STD) pathogens and appears to vary by anatomical site. Several covariates, such as concomitant STDs and acute and advanced HIV-1 disease stage, have been associated with elevated titers of HIV-1 in genital secretions and enhanced HIV-1 transmission.[2]

Semen is a complex mixture of cells and molecules with immunoregulatory functions, acting not only as a carrier of the virus but directly modulating the virus itself and the immune response of the recipient's mucosa. The virus is present in semen in three forms: cell-free virions, infected leukocytes, and spermatozoa-associated virions. Although the role of spermatozoa has been a matter of debate, as it is generally accepted that motile spermatozoa are not productively infected,[3] the virus in the form of free particles or infected cells appears instead to play an important role in transmission. However, the relative contribution of each form of the virus has not been fully explored, nor the various factors that may potentially affect semen-mediated transmission.

Here, we discuss the composition of semen in healthy subjects and during untreated and treated HIV-1 infection and the importance of infected leukocytes in initiating infection. Moreover, we review the antiviral immune response that takes place in the male genital tract (MGT) and broad neutralizing antibodies (bNAbs)-based prevention strategies to block transmission mediated by semen leukocytes.

## Semen composition in healthy conditions

### *Seminal plasma*

Semen is a very rich biological fluid, of which the primary function is to ensure the reproduction of the species. Approximately 95% to 98% of the total volume is represented by the acellular fraction, called seminal plasma (SP). This fraction of the ejaculate contains various bioactive substances originating from the testis, epididymis, and accessory glands,[4,5] including immunomodulatory, proinflammatory, and growth factors that can contribute to successful implantation in healthy couples.[6] This protein-rich fraction contains 25 to 55 mg/mL of protein, including enzymes, such as proteases, esterase, and phosphatases, as well as prostaglandin E (PGE), fibronectin, polyamine, and proteins that play a role in the immune system, such as complement molecules and immunoglobulins.[5] Semen immunoglobulins are derived from local production by plasma cells in the genital tissues and systemic circulation.[7] SP also has a strong bacteriostatic and bactericidal effect due to the presence of a variety of innate immune defense mediators, including zinc, lysozyme, transferrin, and transglutaminase.[8] In addition to its role in the protection, transport, and survival of spermatozoa, SP is able

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to modulate the immune response of the female reproductive tract (FRT) for fertilization and embryo implantation[9] and contains various signaling molecules that temporally modulate FRT status.[5]

Moreover, a wide array of cytokines in SP constitute a unique environment that is different from that of other mucosa and the blood. Namely, TGF-β (100 µg/mL) and $PGE_2$ (1 – 80 ng/mL) are the main cytokines present in semen.[4,10] Both molecules are effective immunosuppressive cytokines that can suppress leukocyte activation (e.g. NK cells, macrophages, and DCs).[11,12] TGF-β is present in three isoforms (TGF-β1, TGF-β2, and TGF-β3) and can be activated from the latent to the active form by proteases and the acidic pH in the vagina.[13] The cytokine has been demonstrated to be involved in inducing regulatory T-cell (Treg) differentiation and downregulating NK-cell activity, resulting in immune tolerance of the FRT.[10,11] Beta-chemokines, which are able to recruit T cells and macrophages (i.e. RANTES, MIP-1α, and MIP-1ß) are often detected in semen and may enhance the immune defense in the MGT after intercourse.[14,15] Other types of cytokines present in the semen have inflammatory, regulatory, adaptive, and hematopoietic properties. The presence of soluble HLA-G in semen possibly suppresses NK cells from entering the cytotrophoblast.[16] A sperm-coating glycoprotein, CD52 g, may be able to prevent anti-sperm immunity and infertility.[17] SDF-1 may play a role in leukocyte recruitment involved in the immune defense of the vaginal mucosa following insemination.[6] In addition, SP contains MCP-1, IL-8, fractalkine, GMCSF, IL-7, and IL-15, cytokines normally found at inflammatory sites associated with the recruitment, maturation, and proliferation of immune cells, including monocytes, T-cells, B-cells, DCs, and NK cells.[18–20] In healthy subjects, the concentration of these cytokines is five-fold higher in semen than plasma.[21]

## Semen cells

According to the World Health Organization (WHO), semen from healthy men contains at least 20 million spermatozoa per milliliter[22] and 50% of them show forward motility.[23] In addition to spermatozoa, the cellular fraction of semen includes immature germ cells, epithelial cells, and leukocytes, which together account for less than 15% of semen cells.[23]

Immature germ cells are the major population of non-spermatozoan cells (NSC). They have been categorized as spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. The number of immature germ cells are higher in men who have less than six million spermatozoa per milliliter. Two types of epithelial cells are found in semen: squamous epithelial cells, originating from the excretory duct, possibly indicators of bacterial infection or inflammation, and epithelial cells, arising from seminal vesicles and associated with inflammation of the seminal vesicles.[23]

Leukocytes are normally present in semen, where they represent approximately 13% of the NSC. They are probably involved in the elimination of abnormal and degenerating spermatozoa. Among leukocytes, granulocytes (or polymorphonuclear cells (PMNs)) are by far the most abundant cells, as they are estimated to represent between 50% and 60% of the total population. They are followed by monocytes/macrophages, which represent 20% to 30% of semen leucocytes. Cytotoxic CD4 and CD8 T lymphocytes each account for approximately 5% of leukocytes.[24] Several studies have reported the minute presence of a B cell population[25,26] as well as dendritic cells (DCs) in the sperm of macaques.[27] In humans, the presence of this population is more anecdotal because it concerns almost exclusively men suffering from chronic inflammation.[28] NK cells are usually not abundant in MGT tissues or semen.[15,29]

The semen leukocyte concentration varies significantly between individuals but should not exceed $1x10^6$/mL as recommended by the WHO.[22] Otherwise, this condition is called "leukocytospermia." This condition occurs often during infection or genital inflammation, is mostly asymptomatic, and affects 5% to 10% of the healthy male population.[15] Its prevalence can be as high as 24% in men with HIV-1 infection.[30,31] The higher leucocyte concentration in leukocytospermic semen affects all populations, namely granulocytes, monocytes/macrophages, and T cells.[32] Such an increase in the number of seminal leukocytes is likely due to the exacerbated release of these cells from the epithelium, alteration of the integrity of the epithelial barrier, or attraction of these cells to the site of inflammation.[33] Although the origin of the leukocytes is uncertain, it has been reported that the epididymis and rete testis are the sources for lymphocytes and macrophages in normal semen, whereas the prostate and seminal vesicles are the sources of PMNs. However, in leukocytospermia, the increased number of leukocytes is associated with genital tract inflammation and their origin is possibly the prostate.[34–36]

A limited number of studies have investigated the phenotype of semen leukocytes. A few studies in humans and a previous study by our group in macaques reported the expression of activation markers on CD4 T lymphocytes, such as the IL-2 receptor (CD25), CD69, as an early activation marker, and HLA-DR, as a late activation marker.[26,31,37,38] The expression of various other surface markers, such as CD103, has also been found on CD4 cells, but is more heterogeneous, suggesting that only a fraction of the proliferating lymphocytes have a classical mucosal profile.[33] CD103 is a marker expressed by almost all intraepithelial lymphocytes (95%), whereas it is present on less than 2% of blood cells. Moreover, in macaques, CD4 T cells express the HIV-1 co-receptors CCR5 and CXCR4.[37] In general, naive and memory populations among CD4 T lymphocytes can be identified by their expression profiles ($CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$, respectively). In sperm, most CD4 T cells have a memory phenotype.[37,39] These proportions are different from those found in the blood, as lymphocytes with a naive phenotype constitute approximately half of the cell population. This means that these lymphocyte populations will not have the same ability to respond to antigens and, from the point of view of HIV-1 infection, they do not exhibit the same sensitivity. Memory lymphocytes are more susceptible to infection than naive lymphocytes.[40] Similarly to CD4T cells, CD8T cells also exhibit an activated phenotype, demonstrated by the expression of CD69, HLA-DR, and the TIA-1

activation marker, a granule-associated protein found in cytotoxic CD8 lymphocytes.[33,41]

The phenotype of monocytes/macrophages and DCs has so far been poorly documented. DCs in human sperm have an immature phenotype ($CD80^{-}CD86^{+}$ CD83low $CCR6^{+}$ $CCR7^{-}CD14^{+}$).[28] Although monocytes and macrophages represent the second most abundant leukocyte population after granulocytes, no study in humans has finely characterized them, whereas such characterization has been performed in cynomolgus macaques.[37] Semen macrophages have a phenotype very similar to that of macrophages resident in the human female genital tract, urethra, and foreskin. They constitute a heterogeneous population, with varying levels of CD163, CD14, and CD11b expression. Most are $CD11b^{+}CD163^{bright}$, a profile typical of activated cells. Like T lymphocytes, macrophages also express CD4 and most are $CCR5^{+}$, which may account for the predominance of mucosal R5 virus transmission in HIV-1 transmission. This phenotype may also favor cell-mediated transmission: as semen cells express CXCR3, they should be able to migrate into tissues, such as the cervicovaginal mucosa, and produce CCL5, CCL3, and CXCL10. Moreover, semen T cells and macrophages in macaques express LFA-1 and/or Mac-1 molecules, which are involved in the establishment of the virological synapse and leukocyte adhesion to epithelial cells and transmigration.

## Presence of HIV-1 in semen: cell-free virions and infected cells

Conflicting results have been reported concerning the viral load of HIV-1 in semen and blood. Several studies have reported that viral loads are higher in blood than semen, with generally, but not always, a correlation between the amount of HIV-1 in the two.[42] Race, HIV-1 subtype, CMV replication in the semen, inflammation, and degree of T-cell activation have all been reported to be associated with the amount of HIV-1 RNA and DNA in semen.[43–47] HIV-1 is present in semen as both free virions and infected cells and there is strong evidence for a role of cell-associated (CA) HIV in transmission. The major source of seminal HIV-1/SIV infected leukocytes is T cells and macrophages, with the prevalence of HIV proviral DNA in seminal leukocytes ranging from 21% to 75%.[30] Provirus has been detected more frequently in T cells than macrophages.[33,48] Several lines of evidence suggest that the viral strains and infected cells present in semen originate, at least in part, from the MGT. During the acute phase of infection, the sequences of viral RNA (corresponding to free virus) and DNA (corresponding to infected leucocytes) in semen are highly similar to those of virus present in the blood (review in[35]). However, during the chronic phase, genetic differences between HIV strains in the blood and sperm emerge and free viral particles present in the semen constitute a population that is distinct from that found in the blood.[34,49] This indicates the existence of independent local viral replication, as well as the restricted exchange of virions and infected cells between the two compartments, allowing the parallel evolution of various virus populations within the body. In addition, the sequences of viral DNA and RNA in infected sperm cells may differ from those of free virions, suggesting a different origin of the virions and infected cells.[35] Whittney et al.[50] reported that the viruses in blood and semen were similar during early infection in SIV-infected rhesus macaques but then became distinguishable after the peak of viremia, indicating that anatomical compartmentalization occurred at an early time point. Anderson et al.[30] proposed a number of non-exclusive and variable mechanisms that would allow the contamination of sperm by HIV over time, namely: (i) direct importation of virus from the blood, (ii) clonal amplification of viral blood strains in infected cells infiltrating the MCT, and (iii) local replication in resident cells in the MGT, leading to distinct viral evolution.

Infected cells migrate into semen from male genital tissues. Therefore, seminal-cell profiles provide important information concerning the numbers and types of HIV-host cells in the MGT. MGT organs that could release HIV-1 virions and infected leukocytes are mainly the accessory glands, including the epididymis, prostate, and seminal vesicles (for a comprehensive review see[35]). Analysis of the HIV RNA from male genital fluids has shown that the prostate and epididymis are the main source of the virus.[51] The prostate and seminal vesicles are also the main source of seminal plasma,[52] seminal vesicles accounting for 60% and the prostate 30%.[36] Additionally, they are more susceptible to infection than other accessory glands (reviewed by[52]). Although the testes are unlikely to be the main origin of the virus, this cannot be excluded. The testes are the least HIV-1-infected area relative to the other components of the MGT, due to the blood-testis barrier.[53] According to the availability of susceptible cells in the testes, the infected testes leukocytes possibly migrate across the epithelium of the rete testis to the seminal lumen, where the virus may then be released via Sertoli cells.[35] A model of SIV-infected macaques showed that testes can be productively infected during primary infection and asymptomatic chronic infection.[53] The infected cells of the testes were macrophages and T cells, as reported for men[34,54] and macaques.[55] Moreover, the testes act as a viral sanctuary, due to limited exposure to drugs because of the blood barrier and drug efflux pumps (such as ABC transporter).[56,57] Consequently, the testes must be taken into consideration for effective HIV-1 therapies.

Leukocytes in the semen of HIV-1-infected men (1.0E+05 cells per milliliter of semen) are generally present in lower numbers than in uninfected men (2.4E+05 cells per milliliter). Semen cells in HIV-positive subjects and SIV-positive macaques have a profile typical of resident mucosal cells,[48,58,59] a phenotype that makes them particularly well equipped to replicate the virus. The MGT is also populated with memory mucosal T cells,[29,58] which are targeted by SIV during acute infection in male macaques.[53] Infection induces a modification of the dynamics and activation state of semen cells, including CD4 and CD8 T cells and macrophages. During infection, the number of macrophages remained stable but the number of CD4 and CD8T cells was significantly reduced in both HIV-1-positive subjects and SIV-infected macaques.[38,48,58] These findings are consistent with those of clinical studies that have documented the depletion of CD4T cells at mucosal sites during early HIV-1 infection, such as the gastrointestinal mucosa and the exocervix of HIV-positive women relative to uninfected women.[60,61] A similar effect was observed following SIV infection of macaques.[62] These findings suggest that genital T-cell dependent immune defense functions may be impaired in HIV-infected

subjects. As a consequence, HIV-positive individuals may be more vulnerable to genital infections, some of which are co-factors for HIV transmission.[63]

An increase in the number of semen $CD69^+$ CD4T lymphocytes, which have an activated phenotype, has been observed in SIV-infected macaques. Moreover, most of the CD4T cells and macrophages in semen express the integrin LFA-1, and an increase in the number of macrophages positive for Mac-1 is observed during infection, suggesting that the virus may modify the adhesion capacity of the cells that it infects. These integrins, indeed, play an important role in leukocyte adhesion to epithelial cells and transmigration, promoting cell-cell contact and facilitating HIV-1 replication.[64,65]

## Evidence of HIV-1 infection mediated by infected semen cells

While several studies based on *in vivo* and *in vitro* models have demonstrated that cell-to-cell transmission is more potent for transmission of the infection than cell-free virus,[66–68] CA virus has been largely overlooked. There is still very little comparative data between transmission by infected cells versus that with free virus in humans and their specific contribution is still debated. Using a mathematical model, it has been estimated that cell-to-cell transmission is 1.4 times more effective than free virus transmission and contributes to 60% of new viral infections.[69]

Several studies have sought to determine the source of the transmitted virus by analyzing the viral RNA and DNA sequences, both in donor genital secretions and the blood of newly infected individuals. These studies have shown that the virus found in the blood of newly infected individuals was in some cases closer in sequence to the viral DNA found in the infected cells of the donor's genital secretions and, in other cases, closer to the viral RNA derived from the free viral particles.[67,70,71] The simplest interpretation of these observations is that the source of the virus may vary from one transmission to another, and that both free virus and infected cells play a role in the transmission of HIV-1.

In humans, *in vivo* inoculation of HIV-1-sized colloidal particles and leukocytes showed that they co-localized after several hours in the sigmoid colon or vagina, depending on whether inoculation was rectal or vaginal, respectively.[72] Despite their similar migratory capacity, *in vivo* macaque studies have shown that cell-to-cell transmission is the primary means of vaginal and colorectal transmission of SIV.[73,74] Indeed, repeated rectal exposure to low amounts (92 $TCID_{50}$) of SIV-infected PBMCs transmitted infection to three out of five macaques following two challenges, whereas similar low doses of cell-free SIV did not transmit infection to none of the four animals over four challenges. Moreover, our group has demonstrated that the vaginal inoculation of infected leukocytes can establish systemic infection, in the absence of any mucosal abrasion. Cynomolgus macaques treated with Depo-Provera were intravaginally inoculated with SIVmac251 infected splenocytes labeled with CFSE. Strikingly, the labeled cells were detected in the tissue of the vagina and iliac LNs after 21 hours of inoculation and in axillary LNs after 45 hours of inoculation by in situ hybridization, indicating rapid dissemination of the infected cells.[74] These data indicate that CA virus transmission can establish infection rectally and vaginally, and might be more infectious at this site of exposure than free virus. There is no up-to-date report on transmission initiated via the mucosa by semen cells, which would be more physiologically relevant. These data indicate that CA virus transmission can establish infection rectally and vaginally, and might be more infectious at this site of exposure than free virus. This lack of information is mostly due to technical constraints in purifying semen cells. In addition to experiments in non-human primates of semen cell-mediated transmission models, attempts to decipher mechanisms of transmission mediated by semen leukocytes will benefit from complementary *in vitro* assays.

CD4 + T cells sorted from semen of SIV-infected macaques at all stages of the disease, transmitted infection when co-cultured *in vitro* with permissive cell lines, demonstrating their considerable capacity to produce infectious SIV.[48] *In vitro*, HIV-1 transcytosis through various epithelial cell lines (I407, HT-29, Caco- 2, HEC-1, ME-180) is much more efficient when initiated by infected cells than by free virus particles.[73,75] The observation of transcytosis of free virus requires an inoculum (in units of p24) 100 to 1,000 times greater than that with infected cells[76] to permit a sufficient number of viral particles to cross the barrier to generate a new infection. Infected cells also show a greater ability to induce infection following transmigration through an epithelial barrier than free virus, as demonstrated by Van Herrewege et al.[77] Only one study demonstrated that labeled viable cells from semen bind to and penetrate the ectocervical epithelium. However, entrapment of cells into the mucus layer hampered their binding to endocervical explants.[78]

In conclusion, it is now well established that HIV-1 transmission by infected cells is more effective in initiating a new infection than cell-free virus using *in vitro, ex vivo*, and *in vivo* models and can be 10 to 1,000 times more effective, depending on the model used.[79,80] Studies addressing prevention strategies should take into account this mode of HIV-1 transmission.

## Effect of the antiretroviral therapy on semen infectivity

HIV-1 transmission during unprotected sexual intercourse is associated with the presence of the virus in genital fluids, and the efficacy of antiretroviral therapy (ART) in preventing new infection is based on their ability to reduce HIV-1 viral load in these fluids.

During the early stage of infection, semen containing high levels of HIV-1 RNA has been shown to be potentially infectious in parallel with leukocytospermia and elevated inflammation markers, leading to leukocyte recruitment.[30,37,52] During the chronic phase of infection, a lower risk of HIV-1 transmission has been observed due to a decrease in not only the blood viral load but also the seminal viral load. However, HIV-1 persistence in the semen did not directly affect the number of CD4 or CD8T cells,[81–83] although there may have been an intermittent effect that was unrelated to plasma viral load.[84–86] The level of persistent virus in semen may be

influenced by co-infection with sexually transmitted diseases, such as cytomegalovirus (CMV), chancroid, syphilis, gonorrhea, or Chlamydia.[87–89] Large regions of the membrane protein on CMV and human T-lymphotropic virus type I (HTLV-I) are similar to CD4. This resemblance may contribute to the higher susceptibility of CMV and HTLV-1 infected leukocytes to HIV.[90]

The use of safe sexual practices, along with antiretroviral preexposure (and to a lesser extent postexposure) prophylaxis (PrEP) for HIV-1–seronegative at-risk individuals and ART for systemic viral suppression for people already infected with HIV-1, has been proven to diminish the forward transmission of HIV-1. After HAART treatment, seminal CD4T-cell counts were brought back to the same level as those of non-infected individuals and thus could improve acquired immune function in the genital tract.[38] This is consistent with what has been described for the blood and other mucosal sites, such as the gastrointestinal tract.[91–93]

The likelihood of detecting HIV-RNA in the semen of infected men has been shown to be extremely low in cases of prolonged, efficient, highly active antiretroviral therapy (HAART).[94–96] Thus, natural conception may be considered as a safe option in HIV-1 discordant couples, based on the very low probability of sexual transmission of HIV. However, the MGT is a separate reservoir for HIV-1 and may contribute to HIV-1 shedding in seminal fluid, even in patients receiving HAART. Indeed, systemic viral suppression has not always reflected full viral suppression in the seminal compartment, possibly due to semen being refractory to the effects of ART.[97–99] The shedding of both virions and infected cells continues to be detectable for months to years after starting ART.[100–102] A small proportion of HIV-1–infected men (<10%) achieve viral suppression in their blood but continue to shed HIV-1 episodically in their semen, albeit at levels that are very low (<1000 HIV-1 RNA copies/mL of seminal plasma in 80% of shedding episodes).[103] Such low-level viral shedding in semen may be below the threshold necessary for sexual transmission; however, it is not known to what extent such low-level shedding in semen contributes to the residual risk of HIV-1 transmission that persists. Thus, the ability of antiretroviral (ARV) drugs to penetrate the MGT is a key factor for achieving HIV-1 suppression in seminal fluid and preventing sexual transmission of the virus.

## Immune responses in the male genital tract following HIV-1/SIV infection: implications for transmission

HIV-1 infection has an effect on several physical and cellular parameters of semen. These effects are mostly detected during the chronic phase of infection, in which not only spermatozoa are affected (reduced motility, lower number of spermatozoa, and/or increased abnormal morphology) but also physical characteristics (decreased ejaculated semen volume, increased seminal pH, and an increased number of round cells).[3,104-107] Some alterations of semen may result from ART, which may affect several metabolic and endocrine functions of the testes and MGT, but current data are contradictory.[108] Frapsauce et al.[109] showed little or no influence of nucleoside reverse transcriptase inhibitors (NRTI), protease inhibitors (IP), and nevirapine (NVP) on semen parameters. By contrast, Savasi et al.[110] showed that the median values of all semen parameters were significantly lower among HIV-1 infected patients than the WHO reference group in a retrospective case–control study of 770 HIV-1 patients under stable HAART. In this study, only age and viral load negatively affected progressive motility and semen morphology, whereas no associations were detected in terms of the type of HAART or duration.

The levels of several immunomodulatory mediators, including cytokines (IL-1α, IL-7, IL-8, MIP-3α, MCP-1, and MIG, IP-10) and chemokines (SDFβ1 and TGF-β), are higher in semen than blood, not only in healthy men but also in HIV-1 infected men,[15,21,111,112] reflecting a persistent and primed state of immune activation conducive to HIV-1 infection. The acute phase of the infection is characterized by a higher level of pro-inflammatory cytokines and chemokines than that found in non-infected or chronic HIV-1-infected subjects (for review see[113]). The overexpression of pro-inflammatory cytokines/chemokines in the seminal plasma of infected men alters the cytokine network and may impair the ability of the immune system to respond to HIV-1 infection.[21,112] A correlation between pro-inflammatory cytokine levels and viral load in semen has been reported in several studies. Higher levels of inflammatory cytokines, such as IL-1β,[114] RANTES,[115] IL-6, IL-8, IFNγ,[116]and IL-17,[117] are associated with increased HIV-1 shedding in the genital tract, increasing the risk of transmission to sexual partners.[21,112] In addition, this cytokine network evolves dynamically according to the stage of viral infection, as described by Vanpouille et al.[118] These variations in cytokine concentration may have numerous consequences, as, for example, it has been shown that high concentrations of pro-inflammatory cytokines promote the expansion and activation of the immune cells of the exposed mucosa. For example, endometrial epithelial cells exposed to SP from acutely HIV-infected men produced higher levels of pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α), which increase HIV-1 replication in CD4T lymphocytes.[119] Consequently, the modulation of semen factors may have an effect on viral propagation during the sexual transmission of HIV-1 in the FRT. Genital inflammation, defined as a specific profile of inflammatory cytokines, has been identified as a significant risk factor for increased T-cell activation and HIV target cell recruitment in women.[1,5] Several studies have confirmed that the cytokine and chemokine seminal plasma milieu supports active viral replication through the ongoing activation of target CD4T cells in situ.[120–122]

Functional T lymphocytes have been isolated from the semen of HIV-negative and HIV-positive men.[33] HIV-specific cytotoxic CD8T lymphocytes (CTLs) have been cloned from the semen of HIV-infected men, providing evidence for an active antiviral cellular immune response in the MGT.[33] In a study conducted by Politch et al.,[38] men in a highly advanced stage of HIV infection showed reduced seminal CD8T-lymphocyte concentrations, suggesting that HIV infection impairs antiviral cellular immune defense mechanisms in the MGT. Indeed, virus-specific T cells in the semen do not control replication of the virus in either

HIV-1-infected subjects or SIV-infected macaques.[116] On the contrary, the CD8T-cell response in the blood during acute HIV-1 or SIV infection increases following the increase in viral load and there is an inverse correlation between viral load and the CD8T-cell response during primary infection.[123–125] Future studies should analyze responses to a broader range of HIV-1/SIV proteins to understand the breadth of T-cell immunity in male genital tissue.

Humoral immunity in semen is likely to be important in HIV-1/SIV transmission. HIV-1 and SIV-specific antibodies are present in abundant quantities and high frequencies in the semen of HIV-1-positive men and SIV-positive macaques at various stages of the disease, although the titers are generally lower in semen than blood.[41,126,127] A positive association was observed between genital tract inflammation and high titers of seminal IgA and IgG anti-HIV-1 antibodies,[127] which may reflect either increased transudation of serum Ig into the seminal fluid, such as that reported for men with bacterial prostatitis,[127] or increased local production. Western-blot analysis of seminal plasma HIV-1 antibodies has shown antibodies directed against numerous antigens, including the gp160 envelope protein.[127] Recently Pillay et al.[128] reported that genital-tract inflammation influenced the antibody subclasses and HIV-1-specific antibody titers in the seminal fluid of non-HIV-infected and HIV-infected men. Local cytokines/chemokines were associated with the mucosal-specific Ig subclasses, with higher quantities of IgG1, IgG3, and IgM in HIV-infected men, suggesting that HIV-1 infection likely drives differential IgG subclasses/isotype and functional responses. The elevated mucosal level of the detected Ig subclasses likely affects specific antibody function and contributes to local viral control. Indeed, IgG subclasses show remarkable differences in complement activation, phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and Fc-Receptor binding, with the general order of activating capacity being IgG3 > IgG1 ≫ IgG2 > IgG4.[129] Although the authors did not perform functional assays, other studies demonstrated the superior polyfunctionality of circulating IgG1 and IgG3 in elite controllers relative to then of viremic subjects.[130,131] It is yet to be determined whether IgG and IgM purified from the semen of HIV-positive men show higher polyfunctionality than that from uninfected subjects.

Quantitative analysis of HIV-specific Ig isotypes in semen has revealed a predominance of IgG over IgM and IgA antibodies.[132–134] A large study conducted by Mestecky et al.,[135] which evaluated HIV-1-1-specific antibody responses in various mucosal secretions, including semen, concluded that the IgA response to HIV-1/SIV is surprisingly low. Despite an elevation in total serum IgA levels, anti-HIV IgA levels can be 100-fold lower than anti-HIV IgG levels in patients during the earliest stages of HIV-1 infection (Fiebig I–VI).[136] These IgA antibodies were directed against the gp41 of the envelope and were induced in the mucosal fluids of approximately 87% of patients, including in seminal plasma. However, shortly after induction, their levels rapidly declined.[136] Anti-gp120 IgA appeared later in both the systemic and mucosal compartments. Paradoxically, individuals with elevated total IgA levels typically have the poorest anti-HIV IgA and IgG responses.[135] The mechanism responsible for the relatively low HIV-1-specific IgA response relative to the HIV-1-specific IgG response in mucosal fluids during HIV-1 infection is not well understood. The observed ratio could reflect contributions from plasma transudate or be a result of mechanisms that cause defects in mucosal class switching, such as HIV-1 Nef-mediated inhibition of class switching to IgA.[46] Indeed, there is no correlation between serum total IgA (IgA derived from bone marrow plasma cells) and mucosal IgA concentrations (derived from mucosal plasma cells), highlighting the compartmentalization between systemic and mucosal immunity.[137]

There is a narrow window of vulnerability after virus exposure that may allow Abs with antiviral function to inhibit HIV-1 at mucosal surfaces.[138] Preexisting HIV-1-specific mucosal Abs present at the time of transmission could block HIV-1 acquisition. The mechanisms by which Abs can inhibit HIV-1 movement across the mucosal barrier include direct virus neutralization, viral aggregation, inhibition of transcytosis, intra-epithelial neutralization, phagocytosis, inhibition through mucus, and Fc receptor-mediated neutralization (Ab-dependent cellular cytotoxicity) (reviewed in[139,140]).

Fc-mediated antibody functions have been mostly overlooked in semen. Parsons et al.[141] demonstrated that HIV-1-specific antibodies in SP can mediate ADCC responses *in vitro*. In macaques, the presence of FcγRIIIa dimeric protein-binding semen Abs (used as a surrogate of ADCC function) appears to be associated with local viral shedding.[41] These observations suggest that Abs with the potential to mediate ADCC may modulate semen infectivity and viral transmission. It is tempting to speculate that the presence of ADCC Abs in semen may, at least in part, explain the relatively low rate of transmission during sexual intercourse.

A few studies have analyzed the presence of broad neutralizing antibodies (bNAbs) in semen. Neutralizing HIV-specific IgA are present in the semen and vaginal washes from HIV-1 exposed seronegative sex-workers.[142–145] Moreover, several studies have shown that neutralizing IgG can prevent the infection o macaques following intravenous or vaginal inoculation with simian human immunodeficiency virus (SHIV).[146–149] The presence of high titers of potentially HIV-neutralizing antibodies in the seminal plasma of HIV-positive men and the fact that cell-free HIV in semen may be associated with immune complexes could contribute to the relatively low sexual transmission rate of HIV-1.[88] On the other hand, env-specific IgG present in semen may instead facilitate mucosal transmission of HIV-1. Indeed, a proportion of HIV-1 virions in semen may be coated with IgG and form an immune complex that can cross the mucosal epithelium. For all these reasons, the presence of Abs in genital secretions, such as semen, should be considered in the design of prevention strategies, as it could impede attempts to provide immune-based prophylactics and/or vaccines.

## Challenges and opportunities in using broad neutralizing antibodies to prevent HIV-1 transmission mediated by semen leukocytes

In recent years, bNAbs have received growing attention as valuable tools for HIV-1 prevention and treatment. As the virus is present in semen as both free particles and infected cells, bNAbs, either induced by vaccination or passively infused, should target both forms of the virus.

HIV-1 transmission mediated by infected leukocytes is likely to play a predominant role in infecting individuals[150] and may represent a mechanism through which the virus can evade antibody-based immunity.[151–154] In this scenario, semen leukocytes may act as Trojan horses, protecting cell-associated virus from host immune defenses.[30] However, most *in vitro* neutralization assays and *in vivo* protection experiments have been performed using cell-free virus inocula and there is, as yet, no indication that bNAbs can prevent transmission mediated by semen leukocytes. Previous studies have been conducted using either cell-lines, primary DCs, or peripheral blood mononuclear cells (PBMCs).

*In vitro* studies to evaluate the potency of bNAbs to block CA viral transmission have produced conflicting results,[152–160] possibly due to the different experimental systems used by the various laboratories.[158] Differences have been observed across virus strains and antibody epitopes and substantial variability can be attributable to whether the assay system used acutely transfected or chronically infected donor cells, cell lines, or primary cells or lab-adapted strains or transmitter/founder viruses.[158,161] Despite observed divergences, there is general agreement that bNAbs exhibit reduced efficacy against CA viral transmission, shown by the much higher concentrations or bNAb combinations required than those needed to inhibit cell-free viral transmission. This may be due to several possible mechanisms, such as steric hindrance at the virological synapse, the increased multiplicity of infection (MOI) observed in CA viral transmission, the different conformation of the viral envelope during cell-free and CA viral transmission (possibly affecting certain epitopes more than others, as well as differences between genetically diverse env), or the stability of viral envelope-Ab complexes (for a recent review see[162]). Finally, the exposition of certain neutralizing epitopes may be limited if membrane fusion occurs within endosomal compartments in the target cell.[163]

Experiments performed using "first-generation bNAbs," such as 2F5, 4E10, b12, and 257-D, produced conflicting results and no clear pattern could be determined.[164–166] As for cell-free viral inhibition, the development of "second-generation bNAbs" has permitted a more comprehensive examination of the mode of cell-to-cell virus inhibition by bNAbs. A study by Abela et al.[153] showed that the targeted epitope may influence the efficacy of a given Ab and that anti-CD4bs Abs lose efficacy during CA viral transmission. The relative resistance to neutralization in intercellular assay systems has been confirmed by other studies,[152,154,157] but in one, Abs directed against the CD4bs and V3 loop were the most active in inhibiting transmission between T cells.[152] Li et al. showed that a functional motif in gp41 appears to contribute to the loss of potency and magnitude of multiple bNAbs during cell-to-cell transmission.[156] In the case of DC/T cell transmission, Su et al. showed that bNAbs inhibit HIV-1 transfer from primary DCs and pDCs to autologous CD4 T cells.[167,168] Antibody-mediated inhibition via the Fc region has been observed in the transfer of HIV-1 infection from antigen-presenting cells (APCs) to surrounding T cells, which may be related to the FcRs present on the surface of DCs and macrophages. FcR-mediated protection required the binding of FcRs to Abs.[158,167] Interestingly, anti-gp-120 bNAbs appear to not only be more potent than anti-gp41 bNAbs in conferring Fc-mediated protection but are also more efficient in preventing the transmission of infection from either macrophages or DCs to T cells.[155,167] An increasing number of observations has also highlighted the fact that a combination of bNAbs is possibly necessary to efficiently inhibit CA viral transmission. In *in vitro* studies, no single Ab was able to inhibit all tested strains,[157] and a combination of PG9 and VRC01 was more effective during cell-to-cell transmission than single Abs.[154]

Animal studies have shown that HIV-1 infection can be prevented when animals are given either topical or systemic immunoprophylaxis. Although those studies evaluated Ab efficacy against cell-free viral challenge, human clinical trials might provide evidence of Ab protection against both forms of virus. Unformulated b12 given vaginally provides dose-dependent protection of macaques before vaginal challenge with a single high dose of SHIV162P4[146] and provided sterilizing immunity in seven of seven animals when applied at high dose.[169] Serum concentrations of 25–60 μg/ml of b12 protected against 5 to 28 low dose vaginal SHIV challenges in macaques.[170] Intravenous inoculation of 4E10 provided complete protection from rectal transmission in six macaques challenged with SHIV Ba-L.[171] When formulated as a gel, VRC01 protected seven of nine RAG-hu humanized mice, and a multi-Ab gel containing b12, 2F5, 4E10, and 2G12 provided 100% protection.[172] Vaginal application of a gel containing 4E10, 2F5, and 2G12 was shown to be partially protective in a macaque vaginal challenge model against SHIV162P3.[149] Some bNAbs are now being tested in humans for their ability to promote immune control of HIV-1 in infected individuals and potentially to eliminate HIV-infected cells. These include VRC01, VRC07-523, 3BNC117, and N6 (CD4 binding site–targeting antibodies); 10–1074 and PGT121 (V3-glycan – targeting antibodies); PDGM1400 and CAP256-VRC26 (V1/V2-glycan–targeting antibodies); and 10E8 (MPER-targeting antibody) (for a recent review see[173]). The NIAID HIV Vaccine Trials Network (HVTN) and HIV Prevention Trials Network (HPTN) are carrying out the Antibody-Mediated Prevention (AMP) efficacy trials with intravenous administration of VRC01 (NCT 02716675 and NCT02568215). The AMP trials are designed to assess if a single bNAb can prevent HIV-1 acquisition in humans and to determine how much serum antibody is needed for protection. The Ab will have both neutralizing and non-neutralizing activity, so might not be entirely specific as to mechanism of action; however, if successful, it will show that a specific agent or response is effective.

It is important to note that the relevance of *in vitro* studies to the *in vivo* efficacy of the same Ab to inhibit cell-to-cell versus cell-free transmission is understudied. To date, only one study has evaluated the efficacy of bNAbs to inhibit CA viral transmission *in vivo* in macaques. The authors used $SHIV_{162P3}$-infected splenocytes to intravenously challenge pigtail macaques and infused the animals with the anti-V3 bNAb PGT121.[174] Partial protection from infection was observed, along with a delay in peak viremia or delayed viremia was reported for non-protected macaques.[174] The partial efficacy of the PGT121 bNAb against cell-to-cell

transmission *in vivo* highlights the need to identify new Ab candidates against this mode of viral transmission. In this macaque model, a high dose, intravenous challenge was used. Future trials recapitulating intrarectal or intravaginal route of transmission and evaluating topical use of Abs will be of great interest to assess the prophylactic efficacy of bNAbs in NHP models and predict protection in humans.

Finally, bNAbs may mediate Fc effector functions that could block CA HIV-1 transmission. The Fc domain present on Abs is recognized by the FcR receptor on the surface of various immune cell types to trigger the possible mechanism of antibody-mediated inhibition by bNAbs, also inhibiting CA viral transmission. For example, bNAbs can recruit NK cells via ADCC to kill HIV-1-infected cells.[175–177] Moreover, bNAbs can activate antibody-dependent cellular phagocytosis (ADCP) and the complement pathway.[178] ADCC-mediated inhibition of CA viral transmission by bNAbs and non-NAbs relies on the accessibility of the viral envelope protein on the cell surface. There is evidence that bNAbs require Fc-mediated immune responses to obtain optimal protection *in vivo*.[179–181] Although bNAbs may not provide complete neutralization against CA viral transmission, their Fc regions provide an additional mechanism to direct the antibodies against infected cells. Lu et al.[182] showed that bNAbs can eliminate HIV-1-infected cells and trigger Fc-mediated protection in humanized mice. Infusion with either 3BNC117 alone or the combination of 3BNC117 and 10-1074, performed 12 hours before the transfer of infected cells, was able to reduce not only the percentage of infected cells but also the level of CA HIV-1 DNA relative to those in control mice. Similar results were obtained when using CD4T cells infected with isolated primary HIV-1 strains. Furthermore, the clearance of HIV-infected cells *in vivo* possibly depends on the interaction of the FcγR on effector cells and the Fc domain on the 3BNC117 Ab.[182] These observations highlight the important function of the FcγR mechanism mediated by bNAbs to eliminate HIV-1 infected cells *in vivo*.

Overall, several studies have reported a reduced ability of bNAbs to interfere with cell-to-cell transmission but have also demonstrated that it depends on the cell type and the antibody used. Moreover, these studies primarily focused on cells infected in-vitro. Given the diversity of cell lines or even CD4T cells derived from blood and semen T cells, it is of utmost importance to establish more physiologically relevant *in vitro* systems.

More research is needed to understand why certain bNAbs are less efficient against CA infection and to define which *in vitro* model would best predict antibody protection *in vivo*.

## Complementarity of antibodies and cell-mediated immunity for prevention of cell-associated viral transmission

Most vaccine studies in animal models and human clinical trials have not been focused on blocking cell-associated HIV-1 transmission, so the mechanism of protection at the site of infection remains unclear. The protective effect of RV144 was associated with the selective induction of antibodies of the immunoglobulin G3 (IgG3) subclass, which mediates multiple functions (i.e., ADCC, ADCP, and antibody-mediated release of cytokines/chemokines) that are effective against infected cells.[183,184] However, humoral immunity alone may be insufficient for protection against the transmission of cell-associated HIV-1,[174] and contribution from cell-mediated immunity might be necessary to augment humoral vaccine efforts. A large body of evidence emphasizes the crucial role of T cells in controlling HIV-1 infection in humans and SIV infection in non-human primates. Studies in humanized mice and non-human primates demonstrate that immunotherapy can facilitate the emergence of potent CD8 + T-cell immunity that can durably suppress virus replication.[185,186] Recently, Niessl et al. demonstrated in HIV-1 infected subjects that bNAb therapy during ART interruption was associated with enhanced HIV-1-specific T cell responses.[187] Although cell-mediated responses normally serve to control established infection, *in vitro* studies clearly show that HIV-specific CD8 + T cells can kill both activated and resting CD4 + T cells before progeny virus is produced.[188–191] This suggests the possibility that these responses may also, if induced in sufficient numbers, be able to eliminate HIV-infected cells as they penetrate the mucosal epithelium and thus before persistent reservoirs are established. Given the advantages and disadvantages of each approach, cellular and humoral HIV vaccine methods will likely be complementary in providing full protection from HIV-1 infection. For instance, vaccine-generated HIV-specific cytotoxic T cells and ADCC responses could cooperate to rapidly clear infected cells. Non-human primate studies suggest that very early infections can in some instances be cleared by passively infused neutralizing antibodies[192] and by the broad T-cell immunity induced by CMV vaccine vectors;[193] however, these approaches have not been assessed for synergistic effects. This is an understudied area and further research is needed to address the potential of combining both arms of the immune system to block transmission mediated by semen cells.

## Concluding remarks

Semen is a complex biological fluid, whose role in HIV-1 transmission is defined by a complex array of factors. Semen carries both cell-free virus and infected cells, the latter ones playing a major, yet still underexplored role in transmission. Conventional antiretroviral therapy has been proven to diminish the forward transmission of HIV-1; however, the MGT may contribute to HIV-1 shedding in seminal fluid, even in patients under HAART. Moreover, bNAbs considered as promising prophylactic agents may not inhibit transmission mediated by semen leukocytes as efficiently as cell-free viral particles, and immune-based protection may be more difficult to achieve. This has major implications for the rational design of vaccine strategies to fight HIV-1. More research is needed, especially in animal models, to elucidate the overall influence of semen and semen cells in the sexual transmission of HIV-1 and to improve the protective efficacy of bNAbs.

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