Role of semen infected leukocytes in HIV mucosal transmission : Experimental model of SIVmac251 infection in Macaca fascicularis

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Role of semen infected leukocytes in HIV mucosal transmission – Experimental model of SIVmac251 infection in *Macaca fascicularis*

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Abbreviations

Abs: Antibodies
AID50: Animal Infectious Dose 50
APC: Antigen-Presenting Cell
ART: AntiRetroviral Therapy
BV: Bacterial Vaginosis
CI: Confidence Interval
CMI: Cell-Mediated Immunity
CTL: Cytotoxic T-Lymphocyte
DC(s): Dendritic Cell(s)
DPI: Days Post-Infection
FGT: Female Genital Tract
FTC: Emtricitabine
GALT: Gut-Associated Lymphoid Tissue
GUDs: Genital Ulcerative Diseases
HEPS: Highly Exposed Persistently Seronegative
HIV: Human Immunodeficiency Virus
HLA: Human Leukocyte Antigen
HSV-2: Herpes Simplex type II
i.e.: That is
IFN: Interferon
LC(s): Langerhans Cell(s)
LN(s): Lymph Node(s)
MALT: Mucosal-Associated Lymphoid Tissue
MGT: Male Genital Tract
MSM: Men who have Sex with Men
NHP: Non Human Primate
NNRTI: Non-nucleoside reverse transcriptase inhibitor
PBMCs: Peripheral Blood Mononuclear Cells
PgE2: Prostaglandin E2
PMN: PolyMorphoNuclear cells
PreP: Pre-Exposure Prophylaxis
PVL: Blood plasma Viral Load
R5 HIV: CCR5-tropic HIV-1 strain

SEVI: Semen-derived Enhancer of Virus Infection

SIV: Simian Immunodeficiency Virus

STD(s): Sexually Transmitted Disease(s)

SVL: Seminal plasma Viral Load

TDF: Tenofovir

vDNA: Viral DNA

VIRIP: Virus Inhibitory Peptide

vRNA: Viral RNA

VS: Virological Synapse

X4 HIV: CXCR4-tropic HIV-1 strain

Foreword

Human Immunodeficiency Virus (HIV) infection can be acquired by IV drug abuse with contaminated equipment and transfusion of contaminated blood products. However, HIV mostly spreads by the mucosal route: sexual transmission is the dominant mode of transmission, responsible for between 85% and 90% of cases of infection worldwide (Quinn, 1996; UNAIDS, 2012). Sexual transmission of HIV-1 among Men who have Sex with Men (MSM) predominates in high income countries like Europe, USA and Australia (Belec, 2007). By contrast, women and girls continue to be affected disproportionately by HIV in sub-Saharan Africa, where approximately 60% of all HIV infections occur, although worldwide, 47% of HIV infected individuals are women (UNAIDS, 2009). These epidemiological data indicate that semen is one of the major sources of HIV-1 transmission.

Since the outbreak of the pandemic 30 years ago, many elegant and rigorous studies have provided insight into the mechanisms of HIV mucosal transmission. Numerous models, *in vitro, ex vivo* and *in vivo* in humanized mice, have been developed. Currently, the infection of macaques with Simian Immunodeficiency Virus (SIV) is recognized as being the most relevant experimental model for studies of HIV infection pathogenesis and mucosal transmission. The mucosal virus target cells as well as the early steps of virus transmission and their underlying mechanisms have been studied in detail.

Semen, like other bodily secretions involved in HIV sexual transmission, contains the virus as two forms: cell-free viral particles and cell-associated virus, mostly in infected leukocytes. Although cell-to-cell HIV transmission has been extensively described as more efficient, rapid and resistant to host immune responses, very few studies have investigated the role *in vivo* of infected leukocytes in virus mucosal transmission. One such study has been recently conducted in our lab, and demonstrated that SIV-infected splenocytes are able to transmit infection to female macaques after vaginal exposure. However, all these studies used immune cells from peripheral blood or lymphoid tissues, such as spleen, and none have investigated the capacity of infected leukocytes in semen to transmit the infection *in vivo*. Indeed, nature, phenotype and infectivity of HIV associated with semen leukocytes may be different from that of HIV from other sources.

Therefore, the objectives of this work were, first, to study of semen leukocytes and their dynamics during SIVmac251 infection in detail, then to investigate seminal factors that may influence semen infectiousness, and finally to test semen leukocyte infectivity *in vitro* and *in vivo*, using a model of mucosal exposure in cynomolgus macaques.

Introduction

I. Semen as a vector of HIV

Although semen is a major, clinically relevant, source of infectious HIV, little is known about the mechanisms by which HIV present in semen may cross mucosal barriers. The available data is from a small number of studies on human material and also from studies in nonhuman primate (NHP) models of HIV and AIDS. Indeed, macaques infected with simian immunodeficiency virus (SIV), and the strain SIVmac251 in particular, are currently considered to be the best models for investigating HIV transmission and infection pathogenesis (Anderson, 2010; Hatziioannou and Evans, 2012; Miller, 1994; Miller *et al.*, 1989; Veazey *et al.*, 2012). The data reported in this chapter has mostly been obtained from infected patients, and NHP models of HIV/AIDS.

A. Viral production in the male genital tract

1) Semen producing organs

Semen is a complex fluid, secreted by various organs of the male genital tract (MGT). It is composed of two fractions: one acellular, the seminal plasma; and one cellular (Le Tortorec and Dejucq-Rainsford, 2010). Seminal plasma is protein rich and is a mixture of secretions from the testes, epididymis, prostate, seminal vesicles and bulbo-urethral glands (Figure 1). The cellular fraction comprises of spermatozoa and round cells, including immature germ cells and leukocytes. Spermatozoa and immature germ cells are produced by the testis, whereas all semen producing organs provide leukocytes.

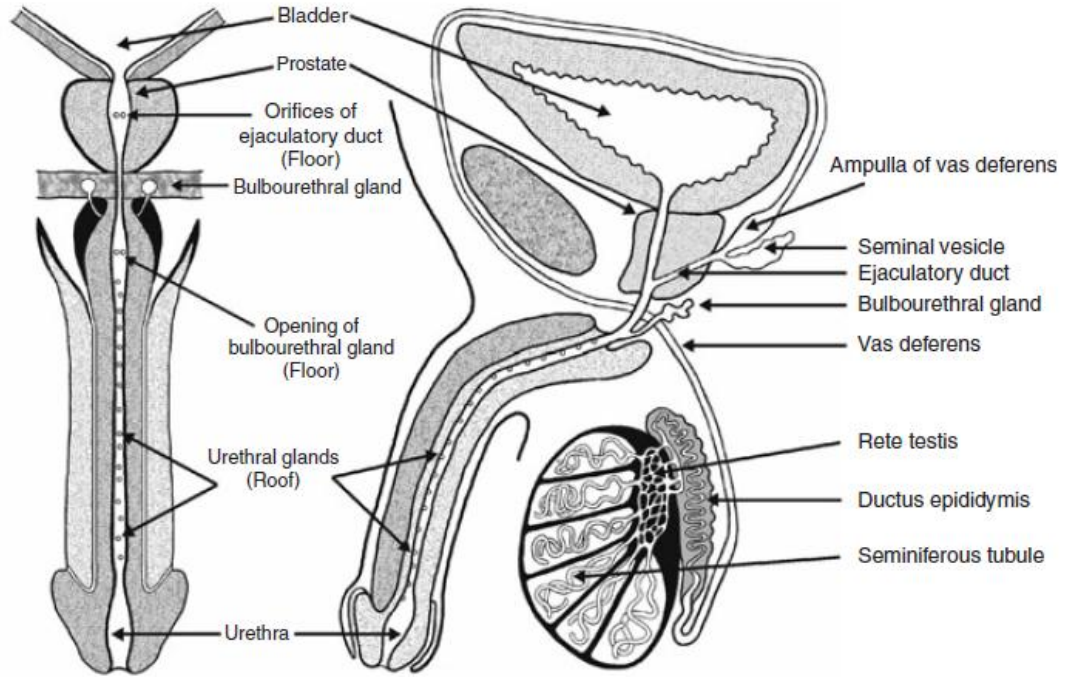

Figure 1 : Anatomy of the male genital tract. Adapted from (Cao and Hendrix, 2008).

2) Distribution of HIV in infected organs

Histopathological observations of human HIV$^+$ patients and SIV-infected macaques clearly demonstrate that the MGT is an site of active viral replication (Dym, 1990; Le Tortorec and Dejucq-Rainsford, 2010; Miller, 1998; Pudney and Anderson, 1991).

Infected cells, positive for either viral proteins (p24, gp120) or viral RNA, have been found throughout the MGT of HIV$^+$ individuals, and in particular in the testes, epididymis, prostate, seminal and bulbo-urethral glands, and urethra (da Silva *et al.*, 1990; Muciaccia *et al.*, 1998; Paranjpe *et al.*, 2002; Pudney and Anderson, 1991; Roulet *et al.*, 2006). Similar findings for SIV$^+$ macaques have been reported (Le Tortorec *et al.*, 2008; Miller *et al.*, 1994b; Shehu-Xhilaga *et al.*, 2007) (Figure 2).

Prostate and seminal vesicles are associated with higher levels of infection than the epididymis and testis. Macrophages and T cells are the principal infected cells. Mononuclear leukocytes are found, isolated or within small lymphoid aggregates, in the *lamina propria* and the epithelium of all semen-producing organs.

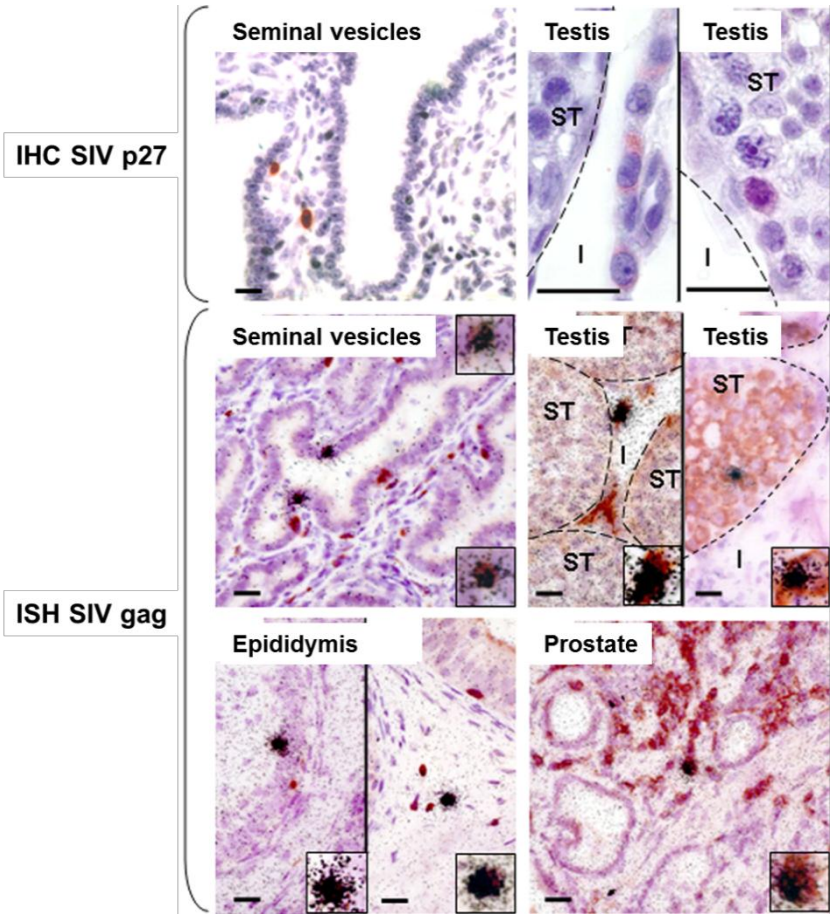

Figure 2 : Localization of SIV-infected cells in the macaque MGT. Adapted from (Le Tortorec *et al.*, 2008). Scale bars: 20 µm.

Large inflammatory infiltrates, mostly consisting of T cells, have been found in the MGT of HIV^+ individuals and SIV-infected macaques, associated with the production of inflammatory cytokines (Le Tortorec *et al.*, 2008) (Figure 3).

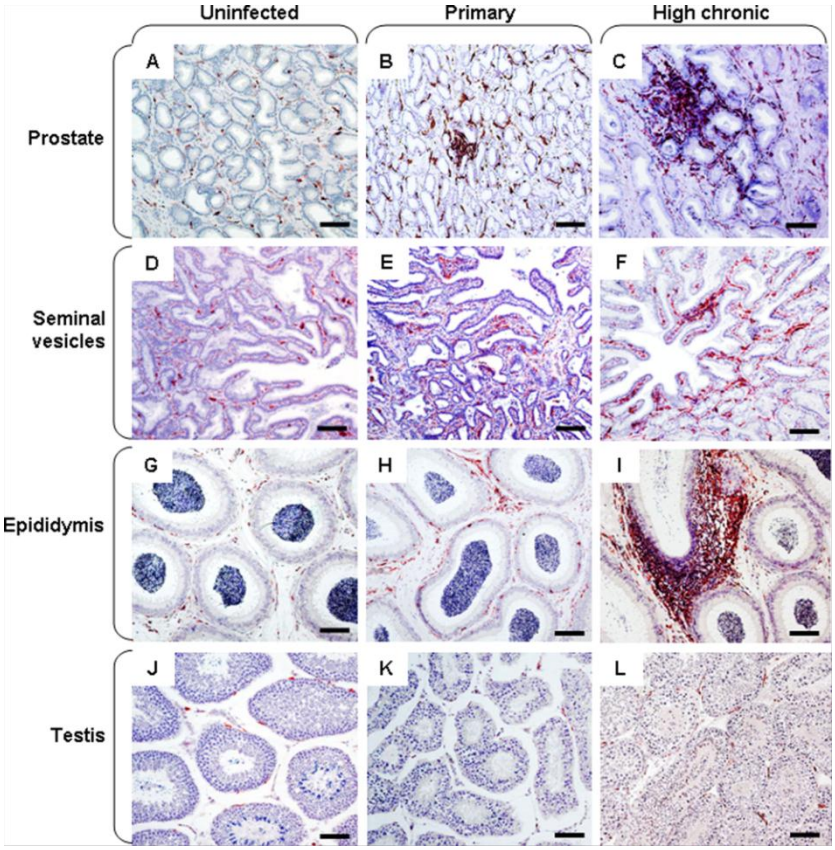

Figure 3 : Inflammatory infiltrates in the MGT of SIV-infected macaques. Immunohistochemical detection of HLA-DR$^+$ cells. Scale bars : 100 µm. Adapted from (Le Tortorec *et al.*, 2008).

Epithelial cells do not appear to be infected *in vivo*. Nuovo *et al.* reported that HIV RNA could be detected by *in situ* hybridization in spermatogonia and their progeny from HIV patients who had died from AIDS (Nuovo *et al.*, 1994), but these observations have not been confirmed by other studies. Two studies with organotypic culture models from human tissues reported that Leydig cells, present in the testis interstitium, are susceptible to particular HIV-2 and SIV strains, but are not infected by HIV-1 (Roulet *et al.*, 2006; Willey *et al.*, 2003). However, SIV have never been reported to infect these cell types *in vivo*.

HIV in the semen may result from excretion by infected cells present either within the mucosa or in the lumen of semen-producing organs, after transmigration through the epithelium (Belec, 2007; Le Tortorec and Dejucq-Rainsford, 2010).

Secretions from the prostate and seminal vesicles make up 30% and 60%, respectively, of the seminal fluid, so these two organs may be the main sources of the virus in semen (Le Tortorec and Dejucq-Rainsford, 2010). This possibility is supported by two observations in HIV$^+$ men. Prostatic massage (“prostate milking”) in men has been shown to increase the HIV viral load in semen significantly (Smith *et al.*, 2004a); and vasectomy does not significantly affect the semen viral load (Anderson *et al.*, 1991; Krieger *et al.*, 1998).

Importantly, HIV DNA has also been detected in pre-ejaculatory fluid, secreted by Littre and Cowper glands during sexual stimulation (Ilaria *et al.*, 1992; Pudney *et al.*, 1992).

3) Nature of HIV in semen

Semen contains HIV-1 / SIV in two forms: cell-free viral particles (vRNA) and cell-associated virus. Several studies describe HIV-1 RNA in seminal plasma in 80-90% of HIV^+ men, and HIV-1 DNA can be isolated from the cellular fraction of semen from 21-75% of such cases (Bagasra *et al.*, 1994; Ball *et al.*, 1999; Dulioust *et al.*, 1998; Mermin *et al.*, 1991; Quayle *et al.*, 1997; Tachet *et al.*, 1999; Xu *et al.*, 1997; Zhang *et al.*, 1998a). The amount of HIV DNA ranges from below the detection threshold to 80,000 copies/ml, and vRNA copy numbers range from undetectable to more than 6 $\log_{10}$ copies/ml (Ball *et al.*, 1999; Krieger *et al.*, 1998; Mayer *et al.*, 1999; Tachet *et al.*, 1999).

The infectiousness of both cell-free and cell-associated viral forms have been evaluated but several independent studies (Anderson *et al.*, 1992; Coombs *et al.*, 1998; Dulioust *et al.*, 1998; Dyer *et al.*, 1996; Krieger *et al.*, 1991a; Krieger *et al.*, 1991b; Krieger *et al.*, 1995; Nunnari *et al.*, 2002; O'Shea *et al.*, 1990; Vernazza *et al.*, 1994; Vernazza *et al.*, 1997a; Vernazza *et al.*, 1997b). The recovery rate of infectious virus from seminal cells seems to be much higher than that from seminal plasma (median 20%, 4-55% versus 5.9%, 3-11% respectively) (Anderson *et al.*, 2010a).

The discrepancy between the relatively high number of vRNA copies in seminal plasma and the low infectiousness of cell-free virus suggests that much of it is replication incompetent or inactivated (Anderson *et al.*, 2010a). However, Thomas *et al.* refuted this notion, suggesting that a low rate of virion-cell interactions, rather than a a large proportion of particles being defective accounts for the low infectivity of semen (Thomas *et al.*, 2007). Also, various seminal factors display anti-HIV activity (see below). Another possible explanation is the putative cytotoxicity of semen in co-cultures used to isolate HIV (Allen and Roberts, 1987; James *et al.*, 1983; Okamoto *et al.*, 2002; Stites and Erickson, 1975).

Semen from healthy men may contain greater than 10^6 leukocytes/ml. Leukocytospermia, an asymptomatic inflammation of the MGT, is found in 5-10% of healthy men (Politch *et al.*, 2007; Tomlinson *et al.*, 1992b; Yanushpolsky *et al.*, 1995). Interestingly, leukocytospermia is more frequent among HIV-infected individuals, where the prevalence may be as high as 24% (Anderson, 2009).

It is generally considered that semen leukocytes are the principal source of cell-associated virus (Anderson *et al.*, 2010a). These cells enter semen from all organs along the MGT. Semen leukocytes include polymorphonuclear cells, macrophages and lymphocytes (Ball *et al.*, 1999; Denny *et al.*, 1995; Gil *et al.*, 1998; Tomlinson *et al.*, 1992a; Wolff and Anderson, 1988a), which are believed to contribute to local immune defense under normal conditions (Anderson, 2005) (Figure 4).

The phenotype of semen leukocytes has been poorly documented. Most of the lymphocytes are T cells, including similar numbers of $CD4^+$ and $CD8^+$ T cells (Olivier *et al.*, 2012). Semen CD4+ T cells are activated, and strongly express CD69 (Sheth *et al.*, 2012) and CD38 (Gianella *et al.*, 2012). In HIV-infected men, semen, like peripheral blood and the gut, is depleted of CD4+ T cells (Gianella *et al.*, 2012; Politch *et al.*, 2009).

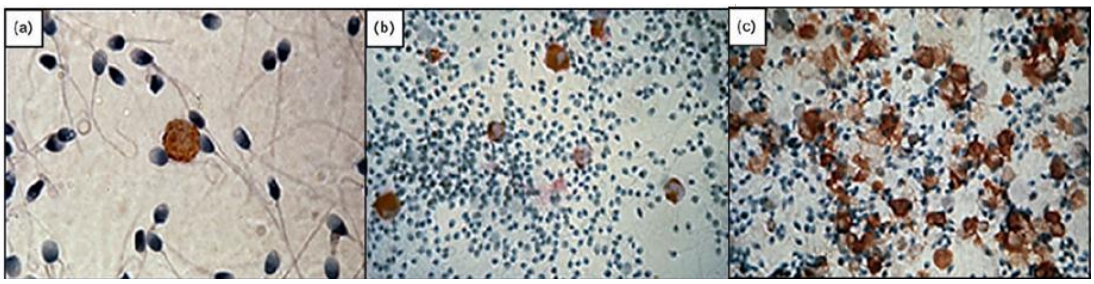

Figure 4 : Immunohistochemical staining of leukocytes present in human semen. (a) $CD4^+$ T cell (b) CD68+ macrophage (c) CD45+ leukocytes from a man with leukocytospermia. Adapted from (Anderson *et al.*, 2010a).

The presence of proviral DNA and vRNA has been documented in semen macrophages and $CD4^+$ T cells. The infectiousness of these cells *in vitro* has been described (Quayle *et al.*, 1997). However, their contribution to HIV transmission *in vivo* remains to be investigated.

Whether or not spermatozoa are infected and can transmit the infection remain controversial (Piomboni and Baccetti, 2000; Pudney *et al.*, 1999; Quayle *et al.*, 1998). Some early studies based on electron microscopy and *in situ* hybridization reported evidence that spermatozoa may contain HIV particles and/or RNA (Baccetti *et al.*, 1991; Bagasra *et al.*, 1994; Dussaix *et al.*, 1993). However, these findings have not been confirmed, despite quantitative PCR studies in isolated spermatozoa (Persico *et al.*, 2006; Pudney *et al.*, 1999; Quayle *et al.*, 1998). Indeed, more than 4,500 inseminations with viable and motile spermatozoa isolated from HIV-infected patients have been performed without any case of contamination of the seronegative partner (Bujan *et al.*, 2004; Marina *et al.*, 1998; Savasi *et al.*, 2008; Semprini, 1993).

Recently, it was reported that spermatozoa can capture HIV virions through adhesion molecules expressed on their surface, and can efficiently transmit infection *in vitro,* through cell-to-cell contact, to dendritic cells (Ceballos *et al.*, 2009). Thus, spermatozoa, even if not productively infected by HIV-1, may act as virion carriers and enhance viral transmission to target cells.

B. Dynamics of viral shedding in semen

HIV-1 shedding in semen has repeatedly been shown to vary with time and between individuals (it can be intermittent, especially during the chronic stage, or continuous) (Bujan *et al.*, 2004; Coombs *et al.*, 1998; Gupta *et al.*, 2000).

1) Seminal viral shedding during HIV/SIV infection

a. vRNA shedding in seminal plasma

Several studies have reported a positive correlation between the vRNA loads in blood and seminal plasma, although seminal plasma viral loads (SVL) are generally lower than in plasma viral loads (PVL) (Chan *et al.*, 2008; Gupta *et al.*, 1997; Kalichman *et al.*, 2008; Tachet *et al.*, 1999) (Figure 5); However, SVL are higher than PVL in up to 30% of HIV-infected men, especially under antiretroviral treatment (ART) (Tachet *et al.*, 1999).

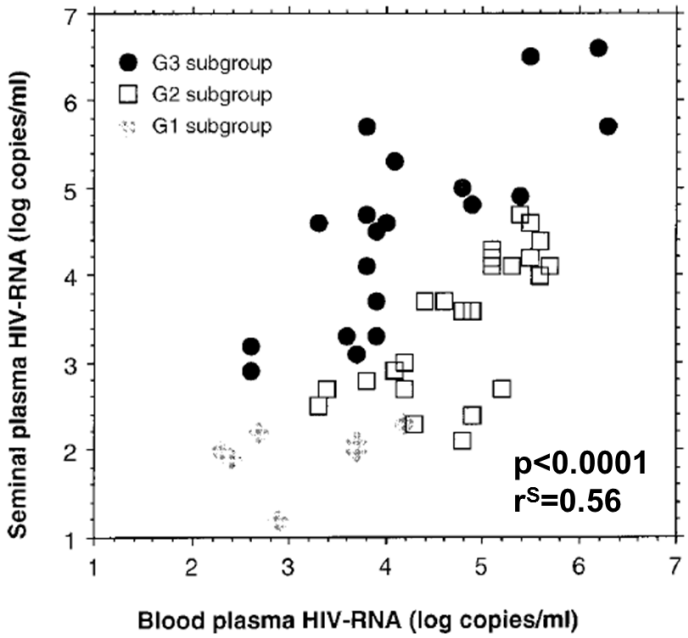

Figure 5 : Spearman correlation between PVL and SVL. G1 subgroup: no detectable SVL. G2 subgroup: PVL greater than SVL. G3 subgroup: SVL greater than PVL. Adapted from (Tachet *et al.*, 1999).

SVL is considered to be a relevant predictive factor of semen infectiousness (Chakraborty *et al.*, 2001). Semen vRNA loads, and thus risk of male-to-female transmission, is maximal during primary and terminal stages of HIV infection, although high SVL are reported in some cases during chronic asymptomatic infection (Cohen, 2007) (Figure 6).

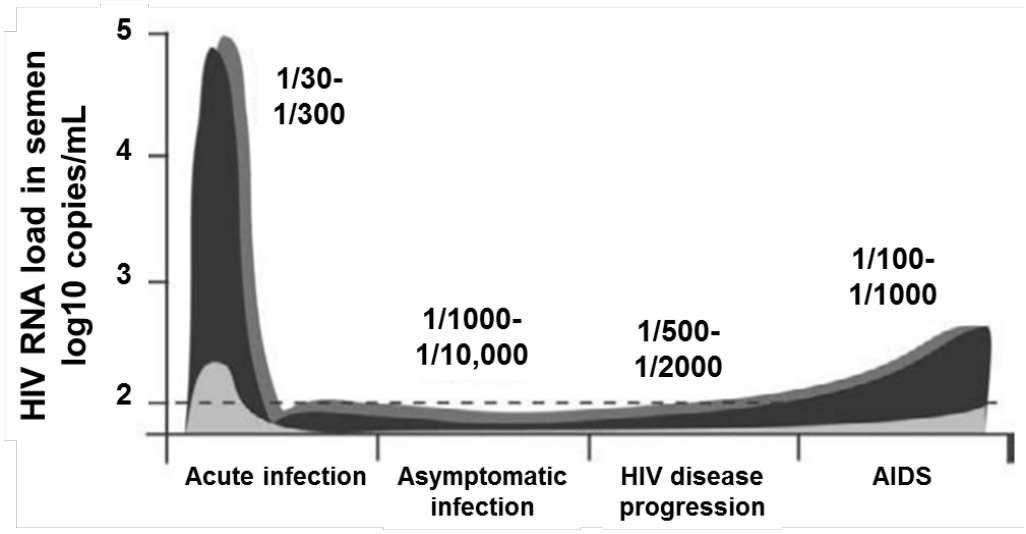

Figure 6 : Semen viral loads and risk per act of male-to-female HIV transmission during HIV infection. Adapted from (Cohen, 2007).

Furthermore, associations have been found between elevated SVL and concomitant STDs, like infection with HSV-2, *N. gonorrheae*, or *C. trachomatis* (Dyer *et al.*, 1998b; Wolff and Anderson, 1988b); such co-infections may therefore be cofactors increasing the risk of transmission of HIV (see Chapter II).

Antiretrovirals have been described to decrease vRNA loads in semen significantly, in parallel reducing the load in blood plasma. This decrease is associated with a theoretically lower risk of HIV sexual transmission (Anderson *et al.*, 1992; Dyer *et al.*, 1996; Gupta *et al.*, 1997; Hamed *et al.*, 1993; Leruez-Ville *et al.*, 2002; Liuzzi *et al.*, 1999; Vernazza *et al.*, 2000). However, viral shedding in semen is not systematically suppressed during ART (Lorello *et al.*, 2009; Marcelin *et al.*, 2008; Politch *et al.*, 2012; Tachet *et al.*, 1999; Taylor *et al.*, 2003; Vernazza, 2001), and shedding rebound is also frequent after treatment interruption, as observed in blood (Liuzzi *et al.*, 2003).

b. Cell-associated viral shedding

High proviral DNA levels in semen have been found to be associated with acute HIV infection (Tindall *et al.*, 1992), lower peripheral $CD4^+$ T cells counts (Xu *et al.*, 1997), leukocytospermia and recent STD (Atkins *et al.*, 1996; Xu *et al.*, 1997), and vasectomy (Krieger *et al.*, 1998).

Tachet el al. reported that, proviral DNA is detectable in 50% of HIV-1 patients under ART, whereas vRNA loads remain undetectable (Tachet *et al.*, 1999). Proviral DNA loads can reach 90 copies / 10^6 cells (Zhang *et al.*, 1998a), and infectious virus can be isolated by co-culture from the semen of 18% of treated individuals (Nunnari *et al.*, 2002).

2) The male genital tract is a distinct compartment for HIV replication

Viral shedding in semen, albeit intermittent, is independent of the viral dynamics in blood, and both cell-free and cell-associated virus is found in semen even in patients under highly active antiretroviral therapy (HAART) with undetectable viral loads in blood. These observations suggest a compartmentalization between MGT and peripheral blood. Consistent with this notion, HIV strains in semen evolve separately from the population in the blood or other anatomical compartments (Byrn and Kiessling, 1998; Coombs *et al.*, 1998; Eron *et al.*, 1998; Ghosn *et al.*, 2004; Gupta *et al.*, 2000; Kroodsma *et al.*, 1994; Pillai *et al.*, 2005; Ping *et al.*, 2000; Vernazza *et al.*, 1994; Zhu *et al.*, 1996); and infected leucocytes in semen produce viral strains that are different from those in blood leucocytes. However, the

HIV strain populations in seminal plasma and cellular components differ from each other (Ghosn *et al.*, 2004; Paranjpe *et al.*, 2002; Zhu *et al.*, 1996).

It is therefore likely that the MGT is a viral compartment distinct from the blood compartment. Viral particles produced locally are under specific selective pressure. The variants from seminal plasma differ from those from cellular components, and consequently seminal lymphocytes and macrophages cannot be the only producers of the viral particles in the seminal fluid: there is presumably another productive source shedding virus into semen, for example the infected cells within the male genital tract (Anderson *et al.*, 2010b). It thus seems likely that semen cell-free and cell-associated virus may originate from different sources within the male genital tract, suggesting that more than one semen-producing organ contributes to HIV production (Le Tortorec and Dejucq-Rainsford, 2010).

3) The male genital tract as a pharmacological sanctuary?

The term sanctuary is typically used to refer to a site into which there is only limited penetration of antiretroviral drugs, and thereby allowing persistent HIV replication (Eisele and Siliciano, 2012).

Several studies indicate that the MGT may constitute a viral sanctuary responsible for HIV shedding in semen (Dejucq-Rainsford and Jegou, 2004; Le Tortorec and Dejucq-Rainsford, 2010). In particular, there appears to be a blood-testes barrier, involving cells expressing significant amounts of proteins contributing to efflux pumps, explaining the poor penetration of several antiretrovirals into the MGT. The antiretroviral agents affected include protease inhibitors like saquinavir, ritonavir and amprenavir (Taylor and Pereira, 2001), and NNRTIs like etravirine (Antoniou *et al.*, 2013).

Therefore, the persistence of viral shedding, whether cell-free or cell-associated virus, in the semen of some patients under ART, is an important issue for the development of new antiretrovirals and strategies for preventing HIV sexual transmission.

However, other antiretrovirals, like efavirenz, maraviroc, raltegravir and darunavir, display a good penetration into the MGT, with concentrations of the free drug (and not the total concentrations, which include the protein-bound molecule) in semen that are comparable to those in blood (Antoniou *et al.*, 2013; Avery *et al.*, 2011). In most HIV patients, HAART successfully suppresses viral shedding in semen such that in these cases, the MGT is not an anatomical site of residual replication (Eisele and Siliciano, 2012).

C. Semen co-factors influencing HIV mucosal transmission

Seminal plasma is a fluid containing numerous different proteins, with an average total protein concentration of 35 to 55 g/l. Pilch *et al.* identified 923 proteins by a high-confidence proteomic analysis using mass spectrometry (Pilch and Mann, 2006). Most are secreted by prostate and seminal vesicles, and display protective activity for spermatozoa and activity promoting the oocyte fertilization process. Many seminal proteins, notably mucins and antimicrobial factors, have been found to have an anti-HIV activity; on the other hand, some seminal plasma components have been reported to facilitate HIV infection.

1) Seminal protein interactions with HIV

a. Semen-derived Enhancer of Virus Infection

Münch *et al.* demonstrated that semen-derived amyloid fibrils enhance HIV infection (Munch *et al.*, 2007). Prostatic acid phosphatase fragments, which are present in large amounts in human semen (Ronnberg *et al.*, 1981), form fibrils that capture HIV virions and promote their attachment to the target cell surface, thereby enhancing the effective infectious viral titer by several orders of magnitude. Semen-derived Enhancer of Virus Infection (SEVI) is a highly cationic peptide, and its positive charges interact directly with the negatively charged surfaces of both target cells and HIV virions, promoting virus attachment and fusion with the cell membrane (Roan *et al.*, 2009).

These observations were made 1) *in vitro* with several HIV-permissive cell lines and primary peripheral blood mononuclear cells (PBMCs), 2) *ex vivo* with tonsillar tissue explants and finally 3) *in vivo* with transgenic rats expressing human CD4 and CCR5 on their $CD4^+$ T cells and macrophages (Munch *et al.*, 2007). SEVI increases HIV infectiousness particularly if only few virions are present in the inoculum. Importantly, SEVI activity seems to be donor-dependent (Kim *et al.*, 2010b).

More recently, the same team identified other cationic peptides in semen which also form amyloid fibrils and thereby enhance HIV infection (Roan *et al.*, 2011): fragments from the semenogelins, which are abundant proteins in semen, constitute together with fibronectin the semen coagulum, a gelatinous structure crucial for fertilization (de Lamirande, 2007; Robert and Gagnon, 1999).

b. Redox activity of seminal plasma

Seminal plasma contains large amounts of polyamines which are oxidized by diamine oxidase, producing radicals that inactivate HIV-1 (Agarwal and Prabakaran, 2005; Klebanoff and Kazazi, 1995). This oxidation process may be increased by contact with vaginal peroxidases, produced *in situ* by the endogenous flora. HIV virions, especially the lipids present within the envelope, are particularly sensitive to oxygen radicals (Stief, 2003).

c. Seminal plasma interferes with HIV-1 attachment to dendritic cells

Sabatté *et al.* described a potent inhibitor in semen of the attachment of HIV-1 particles to DC-SIGN *in vitro* (Sabatte *et al.*, 2007), which is expressed by the mucosal dendritic cells resident in the *lamina propria*. A significant inhibition of HIV-1 capture was observed for both R5-tropic BaL and X4-tropic IIIB HIV strains, using seminal plasma dilutions as high as 1:10. This component has not been identified yet, but was described to have a molecular weight greater than 100 kDa, to be heat stable and resistant to the action of trypsin.

d. Virus opsonization

Semen from HIV-1-infected individuals contains soluble complement components (Bouhlal *et al.*, 2002). Both R5 and X4 strains can activate seminal complement *in vitro* and generate C3 cleavage fragments. Bouhlal *et al.* demonstrated *in vitro* that opsonization of viral particles with complement enhances HIV-1 infection of T cells, monocytes and macrophages, and of the colorectal cell line HT-29.

Antibodies specifically recognizing HIV-1, including neutralizing antibodies, are found in semen from infected men, IgG being more abundant than IgA (Belec *et al.*, 1989a; Mestecky, 2007; Soderlund *et al.*, 2004; Wolff *et al.*, 1992). It remains unclear whether opsonization of seminal HIV-1 particles (Belec *et al.*, 1989a) with antibodies plays a protective role (by neutralization or Antibody-Dependent Cell-mediated Cytotoxicity – ADCC) or facilitates infection by enhancing virus contact with target cells expressing Fc-receptors, such as macrophages and dendritic cells (Lehner *et al.*, 1991).

e. Seminal plasma downregulates surface expression of CD4 on T cells

Balandya *et al.* described the presence of a factor in human seminal plasma that inhibits the surface expression of CD4 on $CD4^+$ T cells, and both activation and proliferation. Moreover, CCR5 is upregulated on seminal plasma-treated T cells, and this may promote the preferential transmission of R5-tropic strains (Balandya *et al.*, 2010).

However, the T cells used in this study were obtained from peripheral blood, and the assays were conducted *in vitro*, with laboratory adapted HIV-1 strains. *In vivo*, no down-regulation of CD4 on semen CD4+ T cells has been observed, and semen T cells have been described as displaying an activated phenotype. Therefore, further work is needed to confirm whether seminal plasma has any such activities on semen $CD4^+$ T cells and HIV infectivity *in vivo*.

2) Impact of seminal plasma on female genital tract

a. Neutralization of vaginal pH

Human semen is weakly basic, with a pH of 7.0 to 8.4 (WHO, 2010). After ejaculation, semen increases the pH of vaginal fluid to neutrality or higher pH within 30 seconds and for up to 2 hours (Fox *et al.*, 1973; Wolters-Everhardt *et al.*, 1986). Moreover, frequent vaginal intercourse without condoms is associated with persistent neutralization of vaginal pH. Because HIV-1 free particles and human leukocytes are sensitive to low pH, they are susceptible to inactivation by vaginal acidity. Therefore, the buffering action of seminal plasma may protect both cell-free and cell-associated virus and enhance HIV infection (Doncel *et al.*, 2011).

b. Immune changes on cervico-vaginal mucosa

Seminal plasma has been described to contain both **immunosuppressive components** (Politch *et al.*, 2007; Robertson *et al.*, 2009), important for human reproduction, and **pro-inflammatory molecules**, which induce an inflammatory reaction within the mucosa of the female genital tract (FGT) (Sabatte *et al.*, 2011). Thus, the effects of semen deposition in the vaginal lumen are complex and contradictory, and may improve or diminish HIV transmission by perturbing the natural equilibrium in FGT (Christopher-Hennings *et al.*, 2008; Doncel *et al.*, 2011; Sabatte *et al.*, 2011).

The immunosuppressive action of seminal plasma facilitates the survival of spermatozoa in the relatively hostile environment of the vagina by inducing a state of non-responsiveness to sperm antigens. Indeed, promoting a tolerogenic response to paternal alloantigens favors maternal acceptance of the conceptus at implantation and avoids allogeneic fetal rejection (Badia *et al.*, 2008; Kelly, 1995, 1997; Politch *et al.*, 2007; Robertson *et al.*, 2009; Robertson *et al.*, 2002; Robertson and Sharkey, 2001).

The mechanisms underlying the suppression of the immune response within the FGT involve, at least in part, the large amounts of TGF-β and prostaglandin E2 (PgE2) in seminal plasma (Sabatte *et al.*, 2011). Semen contains more active TGF-β (~ 1 ng/ml) than do any other body fluids, and huge amount of it is in an inactive or latent form (~ 80 ng/ml). This latent form can be activated on contact with the acidic pH of the vagina and the enzymes in cervico-vaginal secretions (Politch *et al.*, 2007; Robertson *et al.*, 2009; Robertson *et al.*, 2002; Robertson and Sharkey, 2001).

The seminal concentration of PgE2 is also very high, reaching levels of 100 µg/ml (Harizi and Gualde, 2005).

Both PgE2 and TGF-β down-regulate the activation of mucosal immune cells, including neutrophils, natural killer cells and macrophages. Importantly, these cells when activated are involved in the secretion of various antimicrobial molecules. PgE2 and TGF-β induce the differentiation of DCs into a regulatory profile and promote the development of T regulatory responses (Boyce, 2008; Harizi and Gualde, 2005; Wan and Flavell, 2007).

The true effects of the immunosuppressive components of seminal plasma on HIV or other STD infection have not been investigated, but it is reasonable to speculate that they may influence the transmission of pathogens (Kelly, 1995).

Seminal plasma from healthy men contains large amounts of other cytokines and chemokines, including pro-inflammatory molecules: SDF-1α, MCP-1, IL-8 at high concentrations (medians > 1000 pg/ml), and also Il-1α and β, IL-6, IL-13, RANTES and MIP-1β (median concentrations < 150 pg/ml) (Politch *et al.*, 2007). Importantly, leukocytospermia and HIV-1 infection are associated with higher levels of some of these molecules, including RANTES, Il-1β, Il-6, MIP-1β, and IL-13, and also of other factors, such as TNF-α, IL-12 and MIP-1α (Berlier *et al.*, 2006; Politch *et al.*, 2007; Sheth *et al.*, 2005; Storey *et al.*, 1999).

These seminal inflammatory molecules have been found to be associated *in vitro* and *in vivo* with immune inflammatory response induction within the FGT, by:

- **Attracting by chemotaxis immune cells which are targets for HIV**, including macrophages, DCs and T cells (Berlier *et al.*, 2006; Sharkey *et al.*, 2012),
- **Stimulating immune cells activation** and differentiation (Robertson, 2005; Sharkey *et al.*, 2012),
- **Increasing the production of pro-inflammatory molecules** by mucosal cells (Robertson, 2005; Sharkey *et al.*, 2007; Sharkey *et al.*, 2012).

These immune changes within the exposed mucosa may facilitate HIV infection, by increasing the availability of viral target cells in the absence of any pre-existing mucosal inflammation. Moreover, the secretion of chemotactic molecules in response to seminal factors may promote the transmigration of infected semen leukocytes, thereby increasing the risk of HIV transmission.

To conclude this section, it is clear that semen deposition in the vaginal lumen has diverse and in some cases contradictory effects on the resident cells of the mucosa. The related effects of semen on the rectal mucosa have been less studied, but it is reason to suggest that there are many similarities with the effects in the lower FGT. Semen may be more than a simple carrier of HIV: it may also act as a facilitator for transmission of both cell-free and cell-associated virus. Indeed, the infection rate for female macaques vaginally inoculated with human seminal plasma prior to the exposure with cell-free virus was higher than without pre-treatment with SP (Miller *et al.*, 1994a).

In addition, both immunosuppressive and pro-inflammatory mechanisms are induced by semen, which may have consequences both for HIV infection and the local immune responses. (Table 1). This may be also true for anti-HIV specific responses which could be elicited by vaccination.

"Proviral"	"Antiviral"
Inflammation (e.g. prostatitis, urethritis and topical irritants)	
Concurrent infections (viral, bacterial, other STDs or pathogens causing inflammation)	
Cytokines: upregulated: IL-6, IL-8, TNF-α, IFN-γ, IL-1β and IL-10) downregulated: IL-2 and IL-12)	Cytokines (type I IFN)
Hormones (PGE)	Hormones (DHEA and Estrogens)
Growth factors (TGF-β)	Growth factors (TGF-β)
Other proteins (SEVI)	Other proteins and peptides (e.g. SLPI, lactoferrin, protegrins[3], defensins, lysozyme)
Complement	
Antibody receptors[2]	Antibodies (e.g. VN4, IgA antibodies to viral non-structural proteins)
	Enzymes (cystatin C)

Table 1 : Summary of associations of seminal plasma components showing "proviral" and "antiviral" activity by *in vitro* and *in vivo* studies[1]. Adapted from (Christopher-Hennings *et al.*, 2008).

[1] See text for details and references.
[2] Are found in semen, but it is not known whether they have antiviral effects in semen.
[3] Not known whether present in semen, but are in leukocytes which can be found in semen.
[4] Not known whether present in semen, but antibodies are found in semen and virus-neutralizing antibodies are antiviral.
STDs=sexually transmitted diseases, PGE=prostaglandin E, DHEAS=dehydroepiandrosterone sulfate, SLPI-1=secretory leukocyte protease inhibitor 1, VN=virus neutralizing.

II. Mucosal transmission of HIV

There are three potential routes of sexual transmission: genital, rectal and oral. Evaluating the contribution of the oral route to the dissemination of HIV worldwide is difficult, and the available data do not allow the risk of transmission associated with oral sex to be accurately quantified. Since 1987, over 40 cases of contamination by HIV have been attributed to oro-genital transmission (Rothenberg *et al.*, 1998). The few relevant studies conducted to date report contradictory findings. Indeed, some authors describe an increased risk of becoming infected for people reporting oro-genital intercourse (Rothenberg *et al.*, 1998), in particular for receptive oral sex in cases of buccal lesions or cocaine inhalation; others report no association between oral sex and an increased risk of HIV transmission (de Vincenzi, 1994) (Mastro and de Vincenzi, 1996). Overall, the data suggest that oro-genital intercourse is associated with a low transmission rate. Although the risk of HIV transmission *via* oral sex should not be excluded (Belec, 2007), we will focus our work on the two major routes of sexual transmission of HIV: genital and rectal. There is substantial recent data relevant to exposed MGT, and we will also consider the mechanisms of transmission that have been described for the penile route.

A. Diversity in HIV-1 sexual transmission

1) Different routes of HIV sexual transmission

The prevalence of each route of sexual transmission differs substantially between regions of the world (UNAIDS, 2009). Generally, in developed countries, the largest infected populations are Men who have Sex with Men (MSM), mainly exposed through rectal and penile (urethral and foreskin) routes (North America, Central Europe, Australia and New Zealand, Asia, and the Middle East), whereas in developing countries, genital heterosexual transmission is more frequent (Sub-Saharan Africa, Caribbean, and Oceania – except Australia and New Zealand) (Figure 7). Nevertheless, there are exceptions: in several developed countries including China, and countries of Eastern and Central Europe, heterosexual transmission dominates, whereas in developing regions in Latin America and North Africa transmission among MSM predominates.

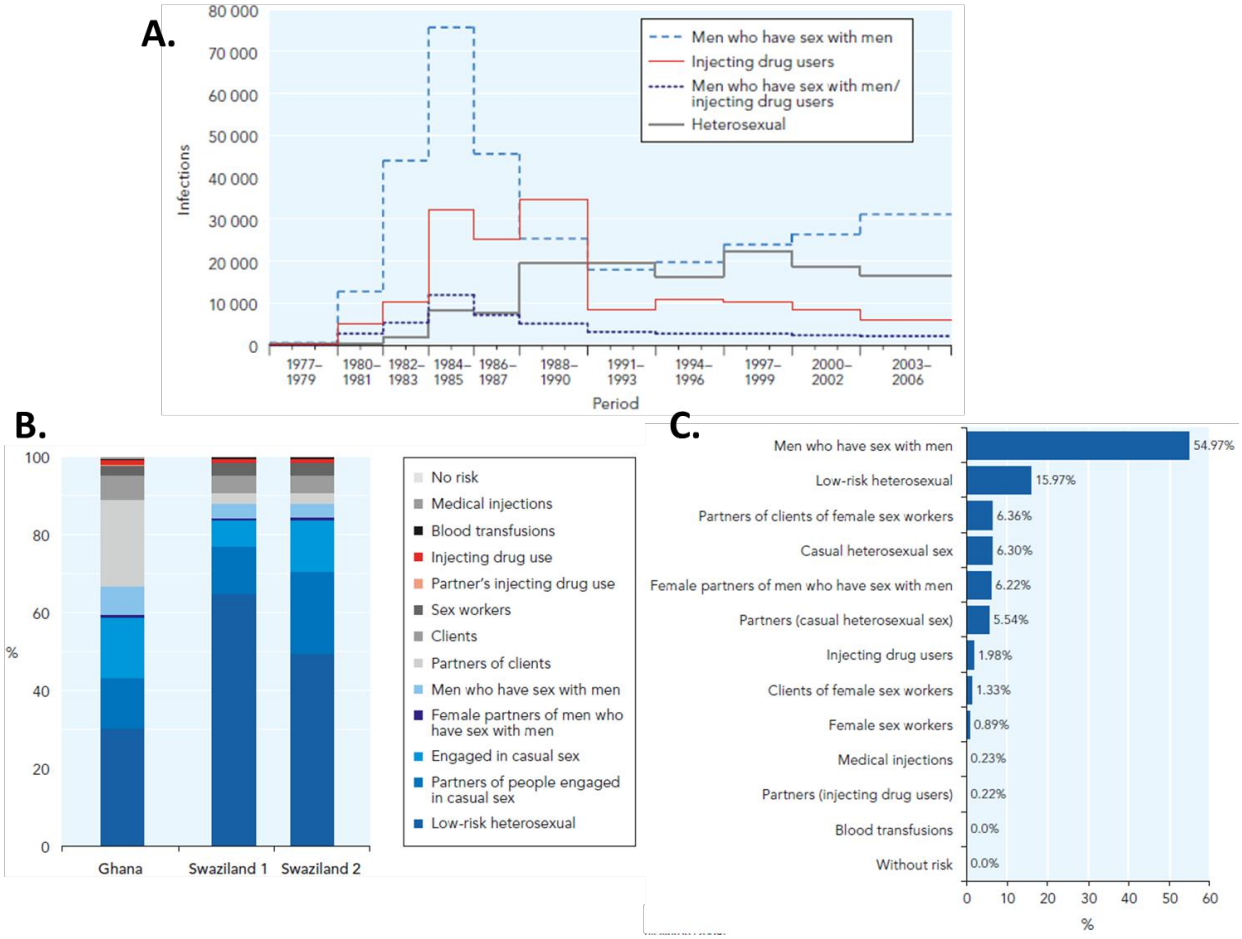

Figure 7 : Distribution of HIV incidence by mode of exposure in North America, Sub-Saharan Africa and Peru. (A) 50 USA States and District of Columbia, 1977-2006. Source: Hall *et al.*, 2008. (B) Ghana and Swaziland, 2008. Source: Bosu *et al.*, 2009 and Mngadi *et al.*, 2009. (C) Peru, estimate for 2010. Source: Alarcon Villaverde, 2009. Adapted from UNAIDS 2009 Report (UNAIDS, 2009).

It is generally believed that the risk of transmission is much higher for rectal than vaginal intercourse (Mastro and de Vincenzi, 1996) (Vittinghoff *et al.*, 1999) (Beyrer *et al.*, 2012). Indeed, a recent systematic review and meta-analysis of the risks of HIV transmission associated with anal sex reported a 1.4% per-act probability (95% CI 0.2-2.5) (Baggaley *et al.*, 2010). This is roughly 18-times larger than estimates for vaginal intercourse (Beyrer *et al.*, 2012). Recently experiments with macaques inoculated vaginally or rectally with the same amount of SIV generally support these epidemiological data (Chenine *et al.*, 2010).

Epidemiological studies indicate that HIV heterosexual transmission is heterogeneous and bidirectional but asymmetric (the male being less frequently infected by an infected female partner than the female by an infected male partner), and that risk of transmission per intercourse is low (Mastro and de Vincenzi, 1996). Boily *et al.* in 2009 published a meta-analysis from a systematic review of 43 publications arising from 25 different study

populations, on the risk of heterosexual HIV infection (Boily *et al.*, 2009): a clear distinction between high and low income countries was highlighted. In the absence of antiretrovirals and excluding sex workers from the analysis, the male-to-female risk was 0.08% per act (95% CI 0.06-0.11) and female-to-male risk was 0.04% per act (95% CI 0.01-0.14) in high income countries; however, the risk was considerably higher in low income countries, with male-to-female risk of 0.30% per act (95% CI 0.14-0.63) and female-to-male risk of 0.38% per act (95% CI 0.01-0.14). Co-factors such as viral load in the infected partner, stage of infection, concurrent sexually transmitted disease (STD) and circumcision strongly affect the risk of transmission, as described below.

The geographical distribution of modes of sexual transmission (heterosexual versus MSM) coincides with the distribution of HIV-1 subtypes of clade M (more than 90% of the worldwide HIV infections) (Simon *et al.*, 1998). Indeed, subtype B predominates in Europe and USA, where homosexual and drug use-related HIV transmission predominates, whereas subtypes A, C, D and E circulate mostly in Africa and Asia where heterosexual transmission is more frequent (Piot, 2002; Walker *et al.*, 2005).

Thus, sexual transmission of HIV is characterized by a diversity of infection routes and, in general, low efficiency. HIV is one of the largest pandemics to affect the human population, with a worldwide distribution and localized high prevalence in sexually active populations. This clearly indicates that other factors must influence virus transmission; many such factors have been described. Understanding how HIV crosses the mucosa and establishes systemic infection is crucial for the design and development of efficient prophylactic strategies.

2) Diversity of sexually exposed mucosa

The genital and gastrointestinal tracts are characterized by a diversity of mucosal types, with various types of epithelial surface, resident immune components and vulnerability to both HIV infection and other pathogens (Figure 8). All mucosal tissues display a common basic architecture: an epithelial layer at the interface with the external environment, which rests on a thin layer of connective tissue named the *lamina propria*, containing many immune cells and capillaries. Under the mucosa layer, the submucosa is a loose connective tissue, with abundant blood and lymphatic vessels, and attaches the mucosa to the *muscularis mucosae*, itself bounded by the serosa.

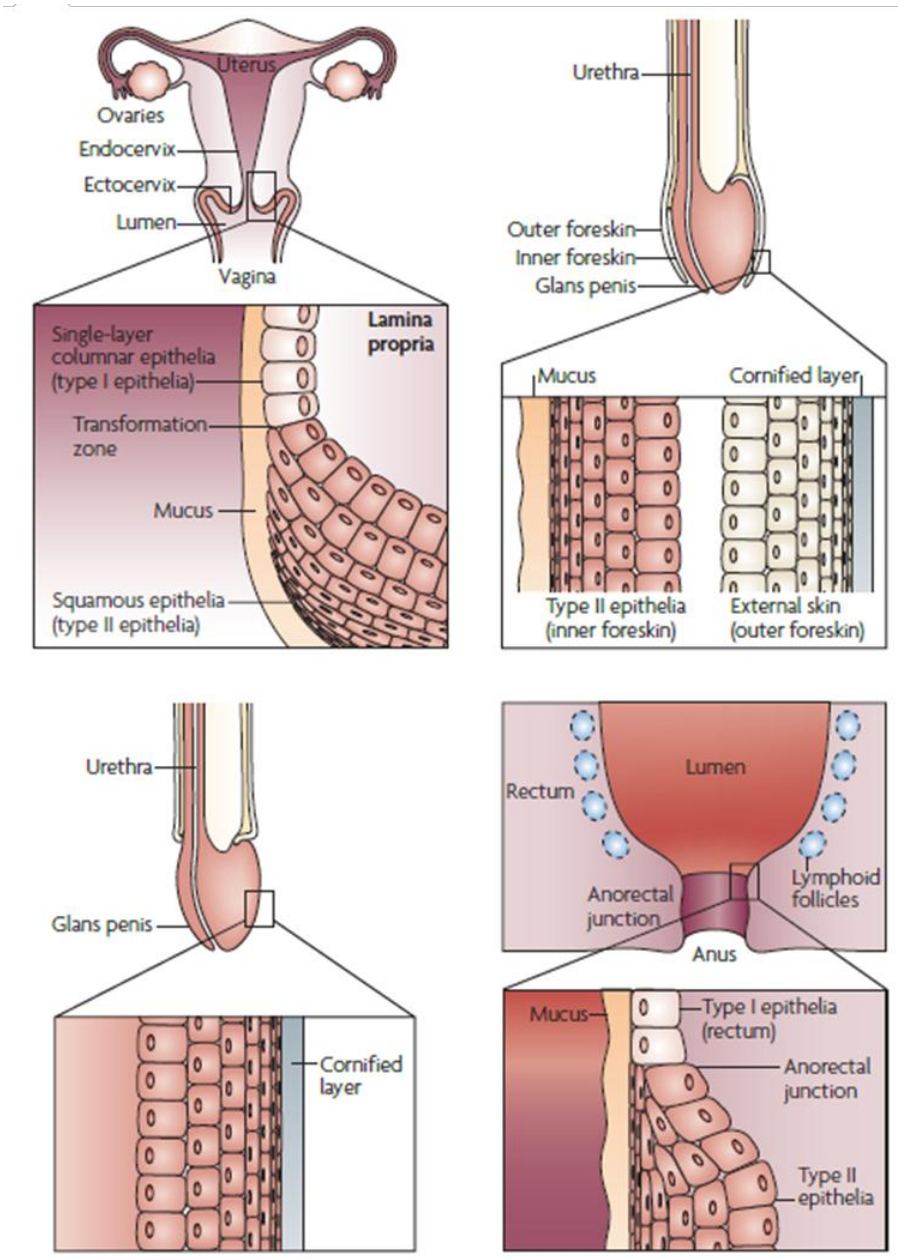

Figure 8 : Different types of sexually exposed mucosa. Adapted from Iwasaki, 2010.

Mucosal surfaces differ from each other by the type of epithelium covering them: single-layer columnar epithelium (type I mucosa) and multilayered squamous, keratinized or not, epithelium (type II) are found in both the MGT and FGT, and in ano-rectal region (Table 2) (Iwasaki, 2010).

Moreover, type I and type II mucosa differ with respect to immune cell composition and the presence (type I) or the absence (type II) of mucosal-associated lymphoid tissues (MALTs), and of IgA transport mechanisms.

Feature	Type I mucosa	Type II mucosa
Tissues	Uterus, endocervix and upper anorectal canal	Vaginal canal, ectocervix, foreskin and lower anorectal canal
Epithelia	Simple columnar epithelium	Stratified squamous (non-keratinized) epithelium
Polymeric immunoglobulin receptor	Present	Absent
Major antibody isotype	Secretory IgA	IgG
Mucosa-associated lymphoid tissue	Present	Absent
Microfold cells	Present	Absent
Langerhans cells	Absent	Present
Mucus source	Goblet cells (in the rectum) and glands in the crypts (in the cervix)	Epithelial cells

Table 2 : Comparison of the genital and ano-rectal mucosae. Adapted from (Iwasaki, 2010).

To understand how HIV and SIV penetrate mucosal tissues, the detailed structure of each type of mucosa needs to be understood.

3) Structure of the different sexually exposed mucosas

c. Female genital tract

The internal FGT is made up of a lower part (introitus, vagina, ectocervix) and an upper part (endocervix, uterus, Fallopian tubes and ovaries) (Figure 9). During sexual intercourse, the exposed area is in the lower FGT, and the upper FGT remains isolated from the external environment.

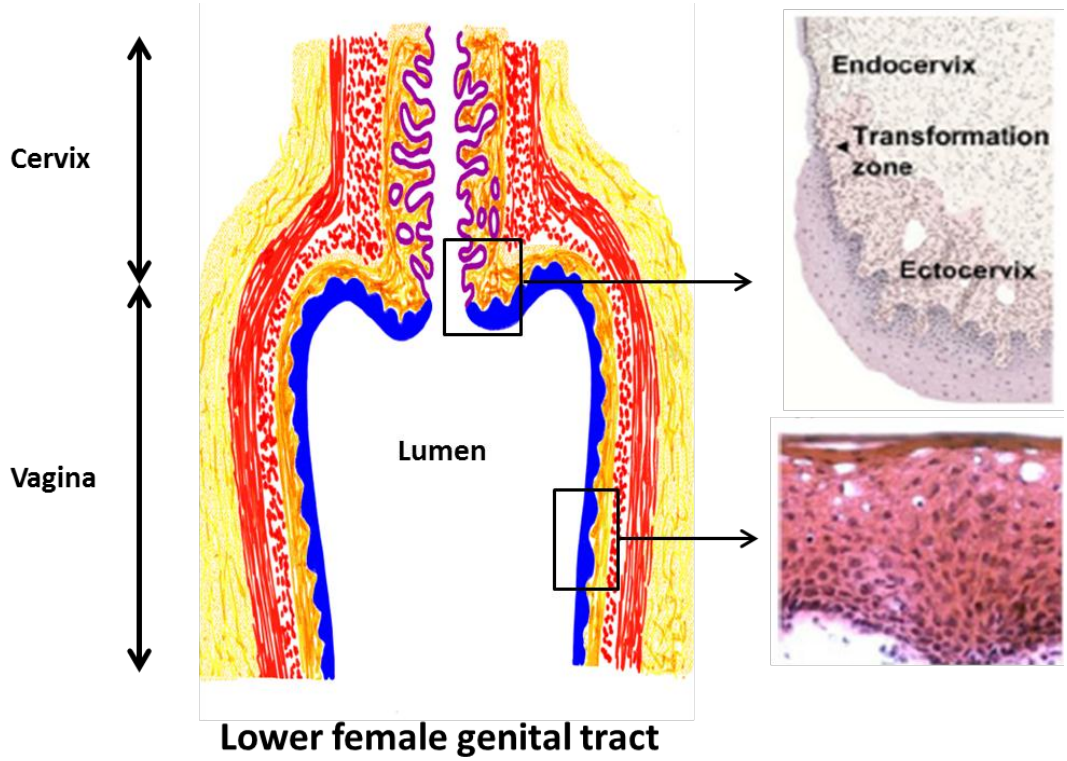

Figure 9 : Anatomy and histology of lower FGT. Adapted from (Dinh *et al.*, 2012b) and (Hladik and McElrath, 2008).

The vaginal and ectocervical mucosa have a multilayered squamous epithelium made up of 20-45 layers of cells (Robboy, 1992), with a mean thickness of 200-300 µm in humans (Ildgruben *et al.*, 2003) and 80-250 µm in macaques (Poonia *et al.*, 2006) (Figure 10). It is composed of four zones: a single layer of basal cells (*stratum basale*), with dividing germinal cells which constantly generate new epithelial cells, and overlying layers of spinous cells (*stratum spinosum*) and granular cells (*stratum granulosum*). As epithelial cells migrate upwards from the *stratum basale*, they become flattened and keratinized, with small pyknotic nuclei, forming the *stratum corneum* (Dinh *et al.*, 2012b; Robboy, 1992). Intercellular desmosomes and amorphous lipoidal material within the *stratum corneum* and *stratum granulosum* restrict passive diffusion of molecules into the deeper layers of the epithelium (Blaskewicz *et al.*, 2011; King, 1983a; Shattock *et al.*, 2000).

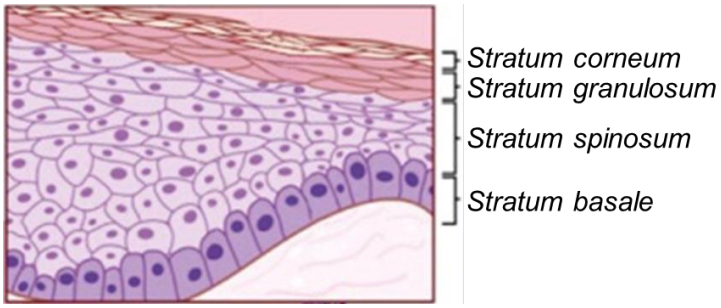

Figure 10 : Structure of a multilayered squamous epithelium. Adapted from (Hearnden *et al.*, 2012).

NHP models, and in particular rhesus and cynomolgus macaques, have, because of the histological similarities with the human lower FGT, been extensively used for the study of HIV cervico-vaginal transmission (Haase, 2005). However, there are several differences between human and macaques. The human cervico-vaginal epithelium has been described as not being keratinized (Pudney *et al.*, 2005) or a displaying a lack of cornification (Dinh *et al.*, 2012b), and several authors report that the macaque cervico-vaginal epithelium is much more keratinized than that in women (Ma *et al.*, 2001; Poonia *et al.*, 2006; Wood, 2008).

There is controversy over the influence of the menstrual cycle on the thickness of the cervico-vaginal mucosa. Several authors report that the vaginal epithelium of women or female macaques is thicker during the follicular phase (Keele and Estes, 2011; King, 1983b; Ma *et al.*, 2001; Wood, 2008), whereas other studies found no such differences (Ildgruben *et al.*, 2003). Poonia *et al.* report that the vaginal epithelium in mature cycling female rhesus macaques was thinner during menses (Poonia *et al.*, 2006).

The area of the cervix that defines the abrupt transition between ecto- and endocervix is referred as the **transformation zone**. The epithelium at this zone has a single layer of columnar cells that covers the **endocervix and uterus** (Figure 3). This thin epithelial barrier is covered with large amounts of cervical mucus, secreted by epithelial cells; its viscosity changes during the menstrual cycle (Lai *et al.*, 2009). The transformation zone and endocervix are generally believed to be the major zones of entry for HIV, because of the thin epithelial surface, the abundance of target cells and the presence of lymphoid aggregates within the submucosa (Alexander, 1990; Moench *et al.*, 2001; Pudney *et al.*, 2005). However, hysterectomized women and female macaques lacking a cervix and uterus, can be infected demonstrating that HIV transmission can occur through the vaginal mucosa (Kell *et al.*, 1992; Miller *et al.*, 1992a).

d. Exposed mucosa of the male genital tract

The nature of the penile mucosal tissues exposed to cervico-vaginal or rectal secretions during sexual intercourse is variable.

In non circumcised men, approximately 70% of the male population worldwide (Organization/UNAIDS, 2007), the foreskin, a loose fold of skin, covers the glans/corona and the urinary meatus (the opening of urethra). During sexual intercourse, foreskin is pulled back to expose the glans. The foreskin is divided into two regions (Figure 4b): the inner foreskin, directly in contact with the glans/corona and delimitating the moist subpreputial cavity, and the outer foreskin. Both zones of the foreskin and the subpreputial cavity covering the glans have a type II keratinized squamous epithelium; the inner foreskin and glans mucosa are described as a "wet mucosal epithelium", whereas the outer foreskin mucosa is a "dry mucosal epithelium" (Anderson *et al.*, 2011; Hladik and McElrath, 2008). It is generally believed that the outer foreskin has a highly keratinized epithelium, similar to skin tissue, whereas the inner foreskin epithelium is thinner and less keratinized (Anderson *et al.*, 2011; Fussell *et al.*, 1988; Ganor and Bomsel, 2011; Iwasaki, 2010; McCoombe and Short, 2006; Patterson *et al.*, 2002). However, recent studies reported either no difference in keratinization between inner and outer foreskin (Dinh *et al.*, 2012a; Dinh *et al.*, 2010; Dinh *et al.*, 2012b), or more keratinization of the inner foreskin than outer foreskin epithelium (Qin *et al.*, 2009).

The **penile urethral mucosa** is also exposed to cervico-vaginal or rectal secretions during sexual intercourse. The meatus is covered by a keratinized stratified squamous epithelium; the zone of transition between the meatus and urethra, the fossa navicularis, has a non keratinized stratified squamous epithelium, and the urethral mucosa is lined with a pseudostratified columnar epithelium (Anderson *et al.*, 2011).

Miller *et al.* reported in 1998 that SIV can be transmitted to male macaques through exposure of intact foreskin and glans (Miller, 1998). Dinh *et al.* described many histological similarities between the penile tissues of macaques and human, making NHP good models for studying the mechanisms of HIV transmission *via* penile inoculation (Dinh *et al.*, 2012b).

e. Structure of ano-rectal mucosa

The anal canal is lined by a type II stratified squamous epithelium. The anorectal junction at the end of the anal canal is an anatomic zone similar to the cervical transformation zone (see above). The rectal mucosa is part of the gastrointestinal tract and shares its type I, single layer columnar epithelium characteristics (Hladik and McElrath, 2008; Iwasaki, 2010; Keele and Estes, 2011) (Figure 11).

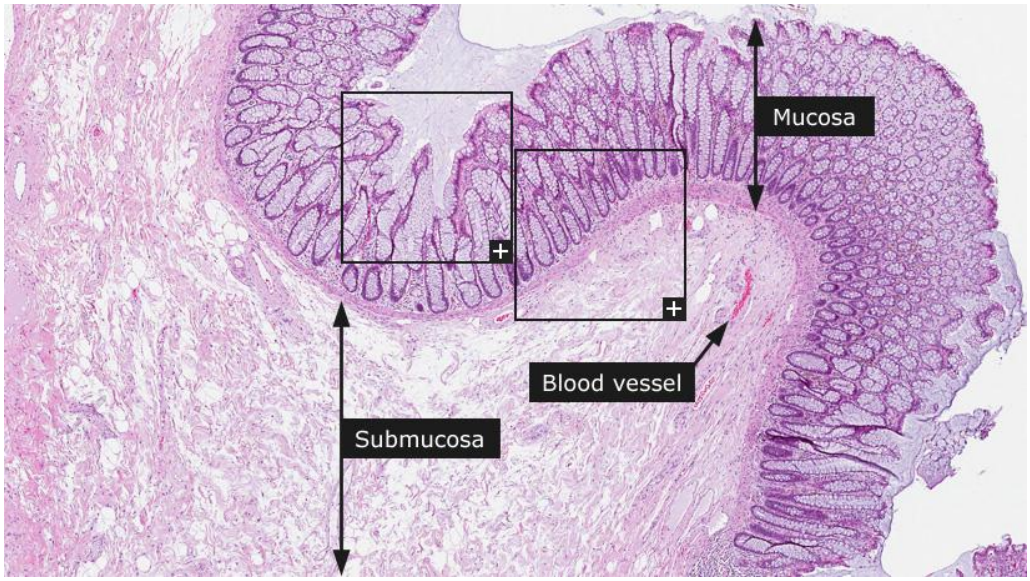

Figure 11 : Structure of rectal mucosa. Adapted from The Human Protein Atlas (www.proteinatlas.org).

NHP models have been used to study HIV rectal transmission. Macaques can be infected by a single, atraumatic rectal inoculation of either SIVmac251 (Couedel-Courteille *et al.*, 1999; Couedel-Courteille *et al.*, 2003; Le Grand, 1993; Pauza *et al.*, 1993) or R5- or X4-tropic SHIV strains (Aubertin *et al.*, 2000; Verrier *et al.*, 2002). The structure of the macaque intestinal mucosa and the organization of the lymphoid system associated to the intestinal tract seem both to be very similar to those in humans (Butor *et al.*, 1996; Vajdy *et al.*, 2000).

B. Mechanisms of HIV-1 mucosal transmission

During sexual intercourse, contaminated genital or rectal secretions from the infected partner, containing HIV in both cell-free and cell-associated forms (Coombs *et al.*, 2003; Pilcher *et al.*, 2004; Quayle *et al.*, 1997), come into contact with the mucosa of the uninfected partner. Like all tissues exposed to the external environment, mucosal tissues are protected by various chemical and physical barriers. Nevertheless, the virus can overcome these barriers and infect target cells in mucosal tissues. I will first describe these barriers, the nature and distribution of the various types of mucosal target cells and then the various mechanisms of crossing the epithelial surface that have been described.

1) Mucosal barriers

The risk of transmission of HIV associated with any single sexual act is low. This indicates that the barriers at the mucosa most often succeed in blocking HIV.

a. Epithelial physical barrier

The most exposed mucosa during sexual intercourse (with the exception of rectal intercourse) have a multilayered stratified epithelium with various degrees of keratinization. An intact mucosa is generally an effective barrier against pathogens and irritants. The efficacy of a thick multilayered epithelium to block, at least partially, HIV has been shown indirectly: female macaques treated with progesterone, which causes substantial thinning of the vaginal epithelium, are very much more susceptible than controls to SIV (Marx *et al.*, 1996).

The uterine and endocervical, rectal and urethral epithelia, each with only a single layer of columnar cells, are considered to be more vulnerable to pathogens, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and HIV (Fu, 1989; Robboy, 2001). The ano-rectal submucosa is highly vascularized by the fragile venous hemorrhoid system (Rouvière, 1978), such that it is a particularly fragile zone, liable to be breached during anal intercourse.

Various cellular structures participate in constituting and maintaining epithelial barriers. I**ntercellular junctions** (ICJ), formed by desmosomes, adherens and tight junctions, contribute to the epithelial architecture by ensuring epithelial cells stick together, without preventing vital exchanges between cells. They also prevent toxins and pathogens from entering the underlying vasculature and immune systems (Anderson *et al.*, 2004;

Guttman and Finlay, 2009; Niessen, 2007) (Figure 12). These junctions are considered to be major actors in the maintenance of epithelial protective barrier function. Importantly, intercellular contacts can be quite loose within a squamous epithelium, but are tight within the columnar epithelium (Belec, 2007).

A recent study compared the expression of molecules involved in the formation of desmosomes (desmogleins 1/2) and adherens junction (E-cadherin) in the stratified epithelia of the MGT and FGT in humans and macaques (Dinh *et al.*, 2012b). They were expressed in all tissues studied, although expression patterns differed: E-cadherin expression was similar in male and female epithelia, desmogleins 1/2 were more abundant in the superficial-most layers of the female than male genital stratified squamous epithelia genital epithelia. Similar differences between male and female macaque tissues were found.

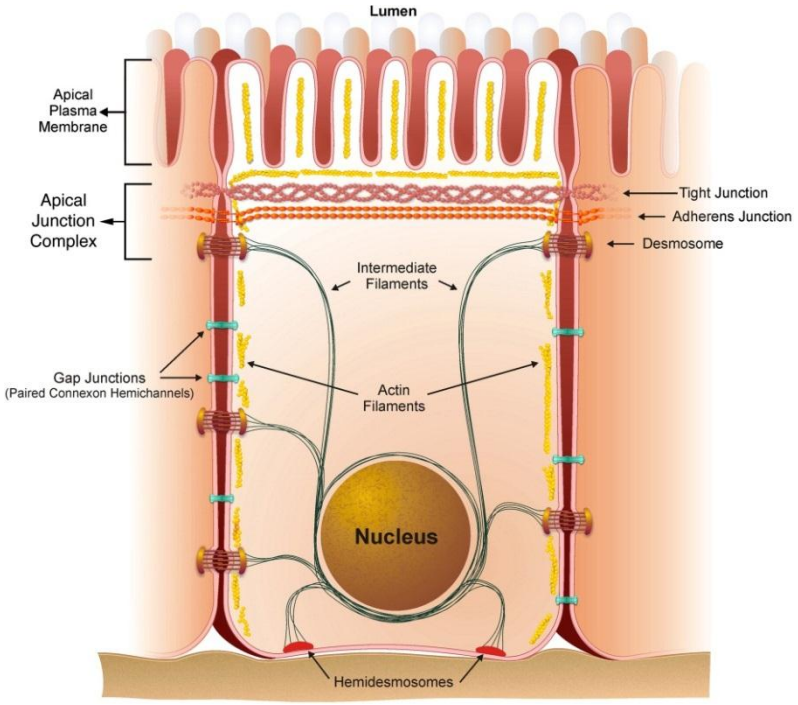

Figure 12 : Structure of intercellular junctions, from the model of intestinal type I epithelium. Adapted from (Guttman and Finlay, 2009).

Cornification of keratinized cells within the *stratum corneum* is thought to play a large, if not the largest, role in epithelial selective barrier function, by retaining moisture and nutrients within the mucosal layer and keeping pathogens out (Candi *et al.*, 2005; Dinh *et al.*, 2012b). Filaggrin and involucrin are proteins central to the establishment of the *stratum corneum*: filaggrin by aggregating keratin filaments and flattening epithelial cells as they differentiate outwards (McGrath and Uitto, 2008); involucrin by acting as a scaffold to which

proteins and lipids bind as they form the outermost layers (Banks-Schlegel and Green, 1981; Candi *et al.*, 2005). Dinh *et al.* compared their expression in female and male GT. Involucrin was found to be similarly abundant in all tissues studied. However, filaggrin was not or was only very weakly expressed in the female *stratum corneum*, whereas strong expression was found in the external male genital epithelia.

b. Mucus

Mucus is a viscous colloid secretion produced by, and covering, mucosal membranes. It is secreted by goblet cells in the type I epithelia and by specialized epithelial cells in the type II epithelia. It is mostly made up of water and mucins that are heavily glycosylated proteins, the main characteristic of which is the ability to form gels. A major function of mucus is to trap microorganisms and pollutants, thereby protecting the underlying epithelium.

HIV-1 is a fragile virus that cannot long survive at ambient or even physiological temperatures outside cells. Therefore, it has been suggested that its infectivity may be significantly decreased if mucus slows viral penetration (Grivel *et al.*, 2011). It has been shown that human and macaque cervico-vaginal mucus efficiently traps free HIV particles, such thattheir mobility is up to 1,000 fold lower than that in water (Lai *et al.*, 2009; Miller *et al.*, 2005)(Tom Hope, unpublished data).

The composition of mucus can differs between localizations and types of the underlying mucosa.

Female genital tract mucus. Over 13 different mucins are secreted in cervico-vaginal mucus, and the pattern depends on the site and stage of the menstrual cycle. MUC 1, 4, 5AC, 5B and 6 are expressed by the endocervical epithelium, MUC 1 and 4 by the ectocervix and the vagina, and MUC 1 and 6 by the endometrium (Gipson *et al.*, 1997; Hickey *et al.*, 2011). The viscosity and composition of cervico-vaginal mucus varies substantially during the menstrual cycle (Hickey *et al.*, 2011). Sex hormones have contradictory effects: in both humans and female macaques, estradiol generally induces a more profuse, watery and alkaline mucus that facilitates passage of sperm during the proliferative stage and ovulation, whereas progesterone induces more viscous and acidic mucus inhibitory to sperm during the luteal stage (Iacobelli *et al.*, 1971; Nasir ud *et al.*, 1982).

Male genital tract mucus. Russo *et al.* recently profiled mucin gene expression in the male genital tract (Russo *et al.*, 2006). RNA for eight mucin genes, including the gel-forming mucin 5AC, was detected in human foreskin tissue, and two mucin proteins, MUC 1 and 4

were detected. The urethral epithelium was found to express MUC 1, 3, 4, 13, 15, 17 and 20, and MUC5AC was detected in urethral glands. Interestingly, MUC 1 binds strongly to DC-SIGN, a transmembrane protein expressed by some of the HIV target cells in mucosal tissues (see below), and thereby prevents viral capture and transfer to CD4+ T cells (Saeland *et al.*, 2009). Seminal MUC6 has been shown to bind DC-SIGN and thus block viral transfer between dendritic cells and $CD4^+$ T cells (Stax *et al.*, 2009).

Ano-rectal mucus. The rectal mucosa produces large amounts of mucus that is continuously deposited into the lumen (Corazziari, 2009; Swidsinski *et al.*, 2007). In particular, MUC, 3, 4, 5B, 11 and 12 are secreted by goblet cells of the colorectal mucosa. This mucus has been described to be an effective barrier, protecting the epithelial surface from direct contact with the intestinal bacterial microflora. Importantly, under inflammatory conditions, breaches develop in this chemical barrier and the mucus layer then fails to prevent bacterial colonization at the epithelial surface (Corazziari, 2009; Swidsinski *et al.*, 2007).

c. Antimicrobial factors

In addition to mucins, mucosal secretions contain different proteins with antimicrobial activity, including against HIV.

Secretory leukocyte protease inhibitor (SLPI) is an extra-cellular component of innate immunity and contributes to inhibiting infection by pathogens and to reducing inflammation. It is found in many bodily secretions, including cervico-vaginal, penile and rectal secretions. This molecule has an anti-HIV activity, notably by preventing the infection of macrophages and T cells (Ma *et al.*, 2004; McNeely *et al.*, 1997). Recently, a study demonstrated that SLPI-pretreated monocytes have the potential to down-modulate human CD4+ T cell proliferation, with implications for immune activation and inflammation (Guerrieri *et al.*, 2011).

Lactoferrin and lysozyme are commonly found in most secretions, and have been described to restrict HIV infectivity by disrupting adsorption and penetration of the virus into CD4+ T cells (Berkhout *et al.*, 2002).

Small human neutrophil peptides, present in mucosal secretions, for example **α- and β-defensins** and **LL-37 cathelicidin**, display anti-HIV activity by disrupting viral particles or altering target cells for infection (Bergman *et al.*, 2007; Chang *et al.*, 2005;

Levinson *et al.*, 2009; Quinones-Mateu *et al.*, 2003). LL-37 cathelicidin also induces α-defensin production by neutrophils (Zheng *et al.*, 2007).

The DMBT1 gene encodes a number of factors expressed at mucosal surfaces. A recombinant fragment of DMBT1 has been shown to bind gp120 and thus agglutinate HIV, resulting in the clearance of the virus (Wu *et al.*, 2006). However, the interaction between DMBT1 and gp140 has been associated with transcytosis of HIV-1 from the apical to the basolateral side of epithelial cells, which may enhance HIV-1 transmission (Stoddard *et al.*, 2009).

Cationic polypeptides present in cervico-vaginal secretions display *in vitro* anti-HIV replication activity (Venkataraman *et al.*, 2005).

Complement components are present in genital secretions, produced locally mostly by epithelial cells (Jensen *et al.*, 1995; Vanderpuye *et al.*, 1992), and are found as native forms. Complement is activated in cascade by genital infection (D'Cruz and Haas, 1990). Interestingly, this component of the innate immune response may paradoxically increase the risk of HIV infection. Opsonized HIV can escape from lysis and highjack this mechanism of defense to make close contact with target cells, especially dendritic cells (Bouhlal *et al.*, 2007; Scofield, 1998).

The menstrual cycle has been reported to influence the expression of several antimicrobial molecules. Indeed, lactoferrin, SLPI and human β-defensin 2 levels in cervico-vaginal secretions decline 10 to 100-fold at midcycle, and then rise toward the end of the menstrual cycle (Wira and Fahey, 2008).

d. IgG and IgA

Natural antibodies, S-IgA, -IgG and -IgM, are secreted in many bodily fluids, including genital and rectal secretions (Dighiero *et al.*, 1982; Guilbert *et al.*, 1982; Quan *et al.*, 1997). They originate both locally and systemically and are poly-specific, recognizing several different epitopes, mostly auto-antigens. However, their poor avidity for antigens limits their efficacy and they do not completely block pathogens. These antibodies form a pre-immune defense system, which is specific to mucosal tissues and pre-exists any contact with infectious agents. It provides permanent vigilance, responding very quickly, if not immediately, to microbial aggression.

In cervico-vaginal secretions, S-IgG are generally more abundant than S-IgA, which are more abundant than S-IgM, although some studies have reported contradictory results (Belec, 2007) (Table 3). Antibody concentrations vary during the menstrual cycle and with aging (Kutteh *et al.*, 1990; Mestecky and Jackson, 1994; Schumacher, 1988). The total concentration of cervical immunoglobulins increases after ovulation, and is highest during the luteal phase (Davis *et al.*, 1983).

In contrast, S-IgA predominate in rectal secretions, and Anderson *et al.* detected more IgA- and IgM-producing than IgG-producing plasma cells in penile mucosal tissue (Anderson *et al.*, 2011). S-IgA and S-IgM are mostly present as polymeric forms, secreted by specialized plasma cells present in the *lamina propria*. These secretory Ig are transported through the epithelial surface to the lumen using the secretory component pIgR, expressed by epithelial cells.

	Genital		Intestinal
	Female	Male	
Dominant Ig isotype	IgG > IgA	IgG > IgA (ejaculate) IgA > IgG (pre-ejaculate)	IgA ⋙ IgG
Levels[a]			
IgG	10–467	16–33 (ejaculate)	1–4
IgA	21–118	11–23 (ejaculate)	143–827
Molecular forms	S-IgA, pIgA, mIgA IgA2 > IgA1	S-IgA, pIgA, mIgA IgA1 > IgA2	S-IgA ⋙ pIgA, mIgA IgA1 >< IgA2
Origin of Ig	Local = systemic	Systemic = local	Local ⋙ systemic
Hormonal regulation	+++[b]	+[b]	−[b]
Antibody-secreting cells			
Main localization	Endocervix	Urethral glands of Litré	Lamina propria
Dominant isotype	IgG ≥ IgA	IgA	IgA ⋙ IgG
Lymphoepithelial inductive sites	−	−	+++
Humoral response to			
Local infections[c]	±	±	+++
Local immunizations[d]	±	±	+++
Systemic immunization[e]	+	++	±
Intranasal immunization[f]	+	?	+
Oral and/or rectal[g]	+	?	+++

Table 3 : Comparative immunological features of the human female and male genital tracts and intestinal tract. Adapted from (Mestecky, 2007).

Three different mechanisms of pathogens elimination by secretory Ig have been described (Figure 13):

- By binding to pathogens present in the lumen, the Ig form immune complexes that are trapped and eliminated by the mucus: this is called the **immune exclusion mechanism** (Belec, 2007).
- The IgA-joining chain-polymeric-immunoglobulin receptor (pIgR) complex mediates **intracellular neutralization of pathogens** that have invaded epithelial cells (Strugnell and Wijburg, 2010).
- Dimeric IgA participate in the **excretion of antigens** from the *lamina propria* by binding and transporting them to the luminal surface of epithelial cells.

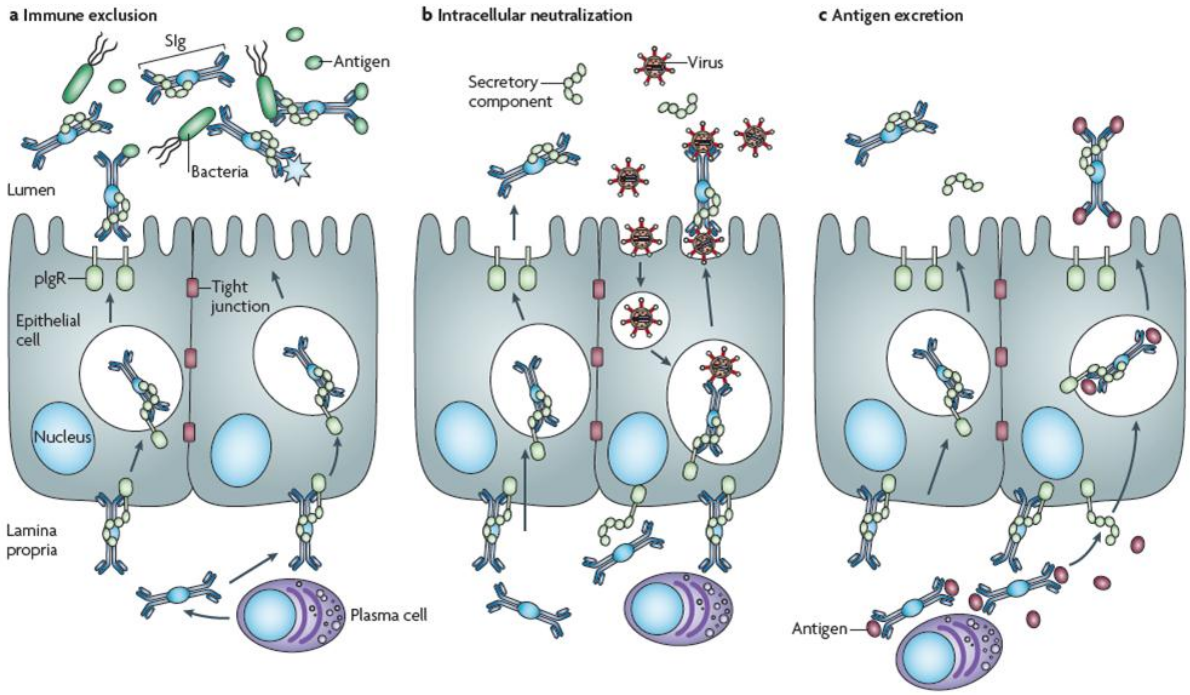

Figure 13 : Protection of the mucosal surface by secretory immunoglobulins through immune exclusion, intracellular neutralization or antigen excretion. Adapted from (Strugnell and Wijburg, 2010).

Finally, the transport of immune complexes through the mucosal epithelium, and thereby the passage of the virus across the epithelial barrier, can be also triggered by the MHC class I–related Fc receptor, FcRn. In humans, FcRn is detected in both fetal and adult intestines and can mediate bidirectional transcytosis of Ig across the intestinal epithelium *in vitro* and *in vivo* (Dickinson *et al.*, 1999; Israel *et al.*, 1997; Shah *et al.*, 2003; Yoshida *et al.*, 2004). FcRn therefore continues to play a significant role beyond the neonatal period, especially in mucosal immune surveillance and adaptive immunity (Kuo *et al.*, 2010).

Ig may significantly affect HIV transmission, as further detailed in chapter I.C.

e. Endogenous flora

Every mucosal tissue has a commensal flora associated with its surface. This flora makes an important contribution to maintaining epithelial homeostasis and integrity (Iwasaki, 2010).

The normal human **vaginal flora** is predominantly composed of *Lactobacillus* species, which carry out key functions for the female host (Antonio *et al.*, 1999; Fredricks *et al.*, 2005; Schlievert *et al.*, 2008). Vaginal hydrogen peroxide-producing lactobacilli inhibit the outgrowth of harmful bacteria, particularly those causing bacterial vaginosis. Moreover, lactobacilli maintain an acidic pH (pH 3.8-4.0) in the cervico-vaginal secretions by producing lactic acid. Cervico-vaginal mucus acidity changes during the menstrual cycle, with a slightly basic pH during the pre-ovulatory phase (7.3 – 8.0), which decreases during ovulation (5.5 – 6.5) (Belec, 2007).

The generally acidic vaginal pH plays a role in protecting against pathogens, including HIV, HSV-2, *Haemophilus ducreyi* and *Chlamydia trachomatis* (Croughan and Behbehani, 1988; Klebanoff and Coombs, 1991; Martin *et al.*, 1999; Sturm and Zanen, 1984). In addition to acidifying the vaginal pH, vaginal lactobacilli produce copious amounts of H_20_2, which is toxic for HIV, as demonstrated *in vitro* by Klebaniff and Coombs (Klebanoff and Coombs, 1991). Olmsted *et al.* demonstrated *in vitro* that low pH immobilizes and kills human leukocytes, thereby also preventing cell-associated HIV vaginal transmission (Olmsted *et al.*, 2005). However, semen, which is basic, buffers vaginal acidity after ejaculation (see chapter II).

The normal rhesus macaques vaginal flora differs from the normal human flora, and shows similarities to the human bacterial vaginosis, with a predominance of species like *Streptococcus*, *Peptoniphilus*, *Gardnerella*-like organisms, and other *Corynebacterium* species over the *Lactobacillus* species (Doyle *et al.*, 1991; Spear *et al.*, 2010). In contrast, the vaginal flora of cynomolgus and pig-tailed macaques is closer to the normal human genital microbiota (Moore and Shattock, 2003; Patton *et al.*, 2001; Patton *et al.*, 1996; Veazey, 2008).

Wira and Fahey reported that the general assumption that the **upper FGT** is sterile is a misconception (Wira and Fahey, 2008). Indeed, this zone is continuously exposed to commensals and pathogens present in the lower FGT. However, there have been no rigorous studies of the commensals present above the cervix. Moreover, this hypothesis

remains controversial. Indeed, Louissaint *et al.* recently described that the distribution within the human FGT of both human lymphocytes and colloid particles (mimicking HIV particles) after vaginal inoculation was similar between the two surrogates and limited to the lower FGT (Louissaint *et al.*, 2012).

A recent study (Price *et al.*, 2010) reported the presence of 42 bacterial families in the **penile microbiota**, with Pseudomonadaceae and Oxalobactericeae being the most abundant irrespective of circumcision status. Circumcision was associated with a significant decrease in the prevalence of anaerobic bacterial families. The moist subpreputial cavity in non circumcised men harbors an anaerobic microflora, similar to that described for bacterial vaginosis, and which has been associated with pro-inflammatory effects on epithelial immune components.

There are extensive descriptions of how the **gut commensal flora** is essential for mucosal homeostasis, by shaping intestinal immune responses (Hooper and Gordon, 2001) and participating in epithelial cell turnover and integrity (Rakoff-Nahoum *et al.*, 2004). More than 1,000 species of bacteria are estimated to live in human gut (Qin *et al.*, 2010).

f. Cytokines and chemokines

The exposure of mucosal epithelial and immune cells to pathogens induces the production of cytokines and chemokines, including type I and II interferons (IFN). These agents have antiviral effects, by modifying both HIV replication, and the infection of other cells (Farrar and Schreiber, 1993; Godaly *et al.*, 1997; Merolla *et al.*, 1995).

SDF-I and RANTES, are ligands of the HIV-1 co-receptor CXCR4 and MIP-1α and –β are ligands of the HIV-1 co-receptor CCR5. These molecules are produced in response of pathogens by many different cell types, including epithelial cells and immune cells like γδ-T cells, cells which are abundant in genital and rectal mucosa. By binding and down-modulating their respective receptors, chemokines can act as competitors for HIV, and block its transmission to target cells (Agace *et al.*, 2000; Cocchi *et al.*, 1995; Lehner *et al.*, 2000; Lehner *et al.*, 1996).

However, pro-inflammatory molecules secreted in response to HIV by mucosal cells may have opposite effects, by attracting virus target cells to the site of infection, and thereby favoring viral replication and propagation (Anderson *et al.*, 2010a; Li *et al.*, 2009).

To conclude, genital and rectal epithelia constitute a physical barrier, and their secretions contain many antimicrobial molecules showing anti-HIV activity. These phenomena undoubtedly play a role in limiting infection events. However, several external factors, such as sex hormones and impregnation during the menstrual cycle, influence many of these barriers. This might lead to “windows of vulnerability”, during which the mucosal shield against pathogens is weakened. Moreover, HIV may highjack some of these natural defenses as infection facilitators, and bypass other factors to transmit infection.

2) Mucosal HIV-1 target cells and associated mechanisms of transmission

Various types of mucosal cells are susceptible to HIV infection, or can be used as vehicles by HIV and efficiently transmit infection to the “true” target cells, mainly $CD4^+$ T cells, macrophages and dendritic cells (DC). There have been many studies on human tissues and animal models that have helped identify these target cells, and thereby elucidate the mechanisms by which HIV can be transmitted.

Mucosal HIV target cells express CD4, the receptor for HIV, CCR5, one of its major co-receptors; the expression of another co-receptor, CXCR4 is more heterogeneous (Patterson *et al.*, 1998; Veazey *et al.*, 2003; Zaitseva *et al.*, 1997). Alternative co-receptors, including CXCR3, CCR4 and CCR6 (Monteiro *et al.*, 2011; Pollakis and Paxton, 2012; Wang *et al.*, 2009) and C-type lectins (Hirbod *et al.*, 2010; Hirbod *et al.*, 2011; Hirbod *et al.*, 2009; Shen *et al.*, 2010), are also expressed by mucosal T cells and antigen-presenting cells. Their localization and relative abundance vary according to the type of mucosa (type I *versus* type II) and the nature of the tissue (genital *versus* intestinal).

a. Epithelial cells

Mucosal epithelial cells are the first cells exposed to the virus. Numerous *in vivo*, *ex vivo* and *in vitro* studies have reported contradictory results about their role in HIV transmission.

It has been described that epithelial cells *in vitro* and *ex vivo* can transport virions from apical to baso-lateral poles via transcytosis (Bomsel, 1997; Bomsel and Alfsen, 2003). This process is triggered by viral attachment and interaction with adhesion molecules on the cell surface: sulfated lactosylceramide (Furuta *et al.*, 1994), galactosylceramide (Dezzutti *et al.*, 2001; Yeaman *et al.*, 2004), syndecans (Bobardt *et al.*, 2007; Wu *et al.*, 2003), glycoprotein

340 (Stoddard *et al.*, 2007), LFA-1 integrin and its receptor ICAM-1 (Alfsen *et al.*, 2005; Bobardt *et al.*, 2007; Bomsel, 1997).

Some genital epithelial cell lines express CCR5 and CXCR4 only weakly (Bobardt *et al.*, 2007). Colorectal epithelial cells express CXCR4, but not CD4, making them theoretically susceptible to CD4-independent infection (Chenine *et al.*, 1998; Delezay *et al.*, 1997). However, *in vivo* observations reveal that genital epithelial cells do not express CD4, or the classical co-receptors for HIV (CCR5 and CXCR4), preventing their involvement in productive infection (Dezzutti *et al.*, 2001; Patterson *et al.*, 1998).

Except for the very few studies describing the presence of infected epithelial cells in the gut of HIV^+ patients (Heise *et al.*, 1991; Kotler *et al.*, 1991), none of the *in vivo* studies in animal models or involving human genital organs in HIV^+ patients, confirmed the data obtained *in vitro* and *ex vivo* (Greenhead *et al.*, 2000; Shattock and Moore, 2003; Spira *et al.*, 1996). The current literature indicates that there is no productive infection of epithelial cells. However, these cells may play a role in HIV transmission through transcytosis mechanisms.

b. Dendritic cells

Mucosal tissues contain various types of dendritic cells (DCs), mostly stromal DCs and Langerhans cells. They are immature and specialized in antigen capture. After having sensed antigens, they migrate to regional lymph nodes (LNs) to cross-present the antigens and initiate immune adaptive responses. Several studies with NHP demonstrate that DCs may play an important role in mucosal transmission and dissemination of HIV (Hu *et al.*, 2000; Pope and Haase, 2003; Shattock and Moore, 2003; Spira *et al.*, 1996). HIV can subvert DC functions to evade immune responses and to gain access to its major target cells: $CD4^+$ T cells (Blanchet *et al.*, 2011).

Stromal DCs are abundant in the *lamina propria* of columnar epithelia (Patterson *et al.*, 1998; Veazey *et al.*, 2003). They express the C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-grabbing non-integrin). These cells can capture HIV virions by interaction with DC-SIGN, internalize the particles without inactivating them and cross-present intact viral particles to other immune cells. Virions can also remain attached to DC-SIGN at the cell surface (Figure 14). In both conditions, DCs are able to transmit infection to target cells via cell-to-cell interaction (***trans*-infection**).

DCs can also be infected by HIV, via the interaction of the virus with CD4 and CCR5, fusion and entry into the cytosol (Cunningham *et al.*, 2010; Piguet and Steinman, 2007; Wang *et al.*, 2007). Infected stromal DCs are found in the *lamina propria* of the vaginal mucosa of macaques inoculated vaginally with SIV (Spira *et al.*, 1996). HIV replication in DCs is generally less productive than that in $CD4^+$ T cells (Kawamura *et al.*, 2003; Pope *et al.*, 1995; Steinman *et al.*, 2003) and the proportion of HIV-infected DCs *in vivo* is often 10- to 100-fold lower (McIlroy *et al.*, 1995). However, infected DCs can efficiently transmit newly produced particles to $CD4^+$ T cells or other target cells (***cis*-infection).**

$CD1a^+$ Langerhans cells (LCs) are present in all stratified squamous epithelia, including those of the ectocervix, vagina (Miller *et al.*, 1992b), penile meatus (Pudney and Anderson, 1995) and foreskin (Anderson *et al.*, 2011; McCoombe and Short, 2006). LCs have a sentinel role, by detecting pathogen invasion and/or damage of the epithelial surface. These DCs respond to danger signals which trigger their migration to the draining LNs where they can function as antigen cross-presenting cells (Romani *et al.*, 2001). LCs have dendrites that extend and retract through the intercellular spaces (Nishibu *et al.*, 2006), and may in some cases reach the epithelial surface (Miller *et al.*, 1992b).

Langerin, a C-type lectin specific to LCs, is able to fix HIV particles on the surface of LCs; the resulting induced internalization leads to Birbeck granules, where the internalized particle is degraded (de Witte *et al.*, 2007). In contrast, mannan, a mannose polymer that binds to C-type lectins including langerin, does not significantly block HIV-1 binding or endocytosis by LCs, suggesting that HIV-1 largely bypasses langerin. This indicates the existence of alternative endocytic pathways, in which the virus is not degraded and therefore permitting virus transmission in *trans* (Hladik *et al.*, 2007) (Figure 14).

In vivo studies using vaginal tissues of HIV^+ patients (Bhoopat *et al.*, 2001) and *ex vivo* experiments with human vaginal explants (Hladik *et al.*, 2007) showed that LCs can harbor HIV/SIV p24 protein and internalize HIV/SIV virions.

Many authors have suggested, based on the natural role of LCs and *in vivo* evidence, that LCs internalize HIV-1 virions in non-lytic endocytic compartments and transport them to NLs for cross-presentation (Ganor *et al.*, 2010; Hladik *et al.*, 2007). This may be a major pathway for HIV transmission to $CD4^+$ T cells *in trans*, independent of productive infection.

The ability of epithelial LCs to produce and release *de novo* HIV-1 virions, and thereby to transmit infection in *cis* remains uncertain. Infected LCs have been detected by *in situ*

hybridization in the vaginal epithelium in SIV$^+$ macaques suggesting a productive infection (Hu *et al.*, 2000; Hu *et al.*, 1998).

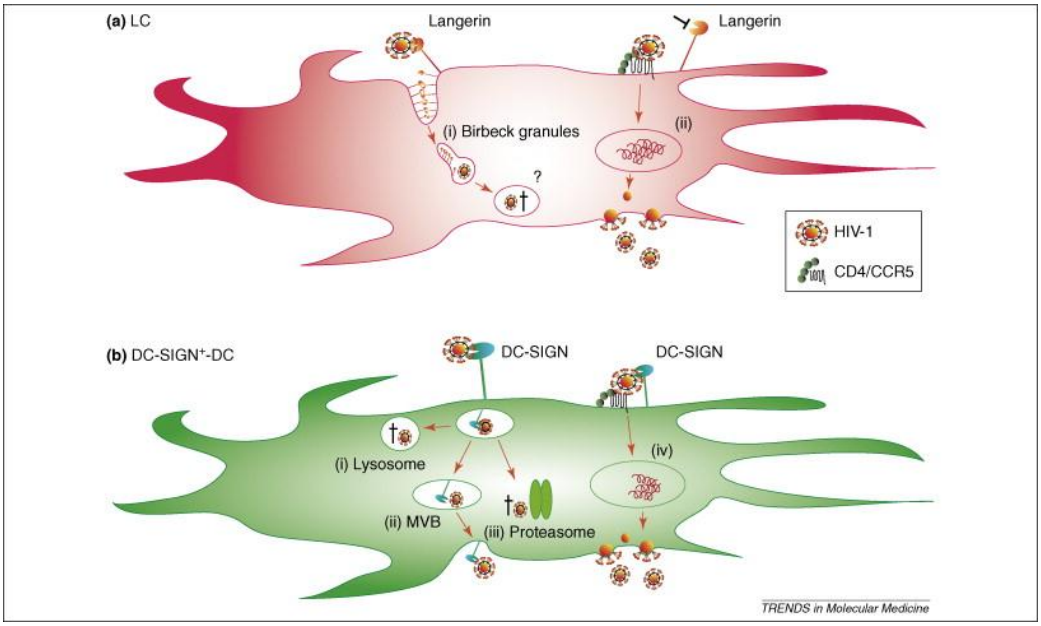

Figure 14 : LCs and DC-SIGN+ DCs interact differently with HIV-1. Adapted from (de Witte *et al.*, 2008).

c. CD4$^+$ T cells

CD4$^+$ T cells are found in all mucosal tissues, mostly in the *lamina propria* beneath both types of mucosal epithelia, but also within squamous epithelia; nevertheless, most intra-epithelial T cells are CD8$^+$ T cells (Anderson *et al.*, 2011; Ganor *et al.*, 2012; McCoombe and Short, 2006; Pudney and Anderson, 1995). The majority of CD4$^+$ T cells are memory T cells displaying high levels of CCR5 expression (Hladik *et al.*, 1999; Hladik *et al.*, 2007; Prakash *et al.*, 2001; Zhang *et al.*, 1998b).

Studies *in vivo* and *ex vivo* studies have demonstrated that intraepithelial and *lamina propria* resident CD4$^+$ T cells can be infected by HIV. NHP studies indicate that CD4$^+$ T cells are the major cell type infected after vaginal inoculation of SIV (Hu *et al.*, 2000; Spira *et al.*, 1996; Veazey *et al.*, 2003; Zhang *et al.*, 1999b). Mucosal CD4$^+$ T cells are rapidly depleted following intravenous inoculation of macaques with SIV, illustrating their high susceptibility to infection *in vivo* (Joag *et al.*, 1997; Picker and Watkins, 2005; Veazey *et al.*, 2003).

d. Macrophages

Macrophages are found in the *lamina propria* of all the tissues, described above as being involved in the sexual transmission of HIV: cervix and vagina (Miller *et al.*, 1992b), foreskin and urethra (Anderson *et al.*, 2011), and ano-rectal mucosa.

In vitro, macrophages can trap intact virions through syndecans or even without specific envelope-receptor interactions through micropinocytosis (Marechal *et al.*, 2001; Saphire *et al.*, 2001). Once captured, HIV-1 can be archived intact for days, and then transmitted to T cells *in trans* (Groot *et al.*, 2008; Sharova *et al.*, 2005). This mechanism may play an important role in virus dissemination. Macrophages express DC-SIGN on their surface, suggesting that DC-SIGN might also favor HIV *trans*-infection by macrophages (Lee *et al.*, 2001; Soilleux *et al.*, 2002). As observed for DCs, infected macrophages can transmit HIV-1 to $CD4^+$ T cells through the virological synapse (Groot *et al.*, 2008).

The presence of infected macrophages has been described in the genital tract and the gut *lamina propria* of macaques infected with SIV and in human explants (Collins *et al.*, 2000; Cummins *et al.*, 2007; Ganor *et al.*, 2012; Greenhead *et al.*, 2000; Heise *et al.*, 1993; Hu *et al.*, 2000).

However, other authors report that infected macrophages were rare (Zhang *et al.*, 1999b), or even undetectable (Hu *et al.*, 2000; Spira *et al.*, 1996) during the first days following infection. Also, macrophages resident in the gut *lamina propria* have been reported to lack CCR5 expression and to be poorly permissive for HIV-1 infection (Meng *et al.*, 2000).

e. Microfold cells

Microfold cells (M cells) cells are intraepithelial cells of the gut mucosa, specialized in sampling intestinal contents and presenting antigens to underlying lymphocytes and macrophages. A variety of pathogens exploit these cells to cross the mucosal barrier (Neutra *et al.*, 1996). Although there is limited *in vitro* evidence suggesting that M cells might contribute to HIV penetration of gut tissue (Amerongen *et al.*, 1991), confirmation *in vivo* for this possibility is still lacking.

3) Modeling HIV mucosal transmission

In normal physiological conditions, the epithelium constitutes a physical barrier that pathogens can not easily cross. Numerous studies *in vitro*, *ex vivo*, and *in vivo*, contribute to our understanding of how HIV successfully bypasses the physical and chemical barriers and

reaches its mucosal target cells. Indeed, various mechanisms of HIV/SIV mucosal transmission have been proposed and they differ according to the site of infection (type I or II mucosa) and the nature of the virus (cell-free or cell-associated virus).

a. Mechanisms by which HIV crosses mucosal barriers

Breaches in the mucosa

During sexual intercourse, even in the absence of any violence, small abrasions or micro-breaches of the epithelium are frequent in vaginal, penile and rectal mucosa (Cold and Taylor, 1999; Norvell *et al.*, 1984). In particular, rectal mucosa is considered to be highly fragile, and presents many microtraumas after anal intercourse (Winkelstein *et al.*, 1987).

Both cell-free and cell-associated viruses may use such breaches to gain access to the underlying *lamina propria* and thereby make contact with resident target cells. In the case of exposure of multilayered squamous epithelia (vagina, foreskin), minor abrasions, which not necessarily involve the whole epithelial thickness, may increase HIV access to LCs dendrites, intraepithelial $CD4^+$ T cells and macrophages (Figure 15).

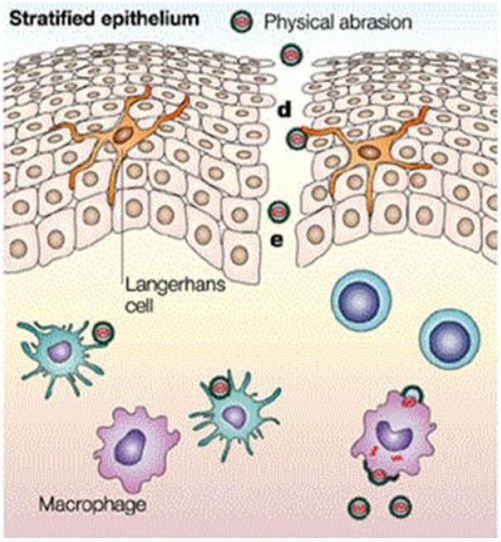

Figure 15 : Transepithelial passage of HIV in the context of physical abrasions. Adapted from (Shattock and Moore, 2003).

However, numerous studies with humanized mice and NHP models clearly demonstrate that infection can follow atraumatic inoculation of either cell-free or cell-associated virus (Berges *et al.*, 2008; Di Fabio *et al.*, 2001; Girard *et al.*, 1998; Le Grand, 1993; Miller, 1994; Miller *et al.*, 2005; Salle *et al.*, 2010): the evidence is that an intact epithelium does not constitute an impenetrable barrier.

Viral particles can break through the epithelial surface *via* transcytosis

Ex vivo experiments on genital pluristratified mucosal explants have led to different conclusions. Some authors state that transcytosis is not a mechanism of HIV transmission through pluristratified epithelia (Bouschbacher *et al.*, 2008; Greenhead *et al.*, 2000), whereas Ganor *et al.* reported HIV-1 budding through epithelial cells of a human foreskin explant (Ganor *et al.*, 2010). HIV-1 transcytosis *in vivo* is more likely to occur across single-layered epithelia.

HIV-1 entry into mucosal epithelial cells is much more efficient when HIV-1 particles bud locally after contact between HIV-1-infected cells and uninfected epithelial cells than by direct entry of cell-free virus into the epithelial cells (Alfsen *et al.*, 2005; Bomsel, 1997; Hubner *et al.*, 2009; Phillips, 1994; Tan and Phillips, 1996) (Figure 16). This mechanism of cell-to-cell interaction is very similar to the “virological synapse” established between an infected cell and another target cell (see below) (Alfsen *et al.*, 2005).

HIV-1 entry into epithelial cells relies on the interaction of both HIV-1 envelope glycoproteins gp120 and gp41 with the epithelial glycosphingolipid receptor galactosyl ceramide (GalCer), organized in lipid raft microdomains (Alfsen *et al.*, 2005; Bomsel, 1997; Harouse *et al.*, 1991). Heparan sulfate proteoglycans (HSPG), like agrin, are expressed at the epithelial apical surface, and act as attachment receptors for HIV-1. The interaction between HSPG and integrins, expressed on the surface of immune cells, triggers transduction of the signal for HIV-1 transcytosis (Alfsen *et al.*, 2005).

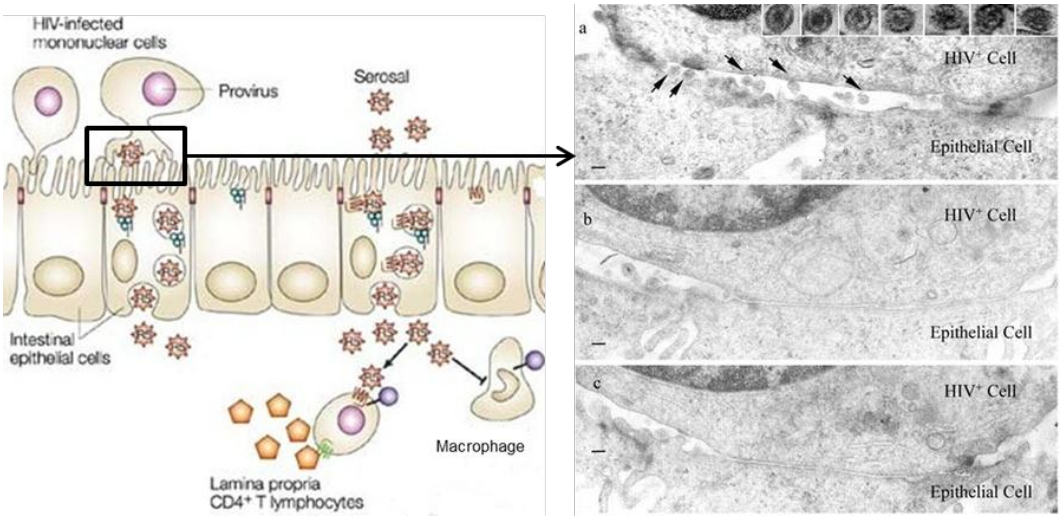

Figure 16 : Transcytosis of HIV-1 through single-layered epithelium. Adapted from (Bomsel and Alfsen, 2003) and (Alfsen *et al.*, 2005).

Infected leukocytes in the inoculum may play a major role in this mechanism of HIV mucosal transmission (Bourinbaiar and Phillips, 1991; Phillips, 1994; Phillips and Bourinbaiar, 1992; Tan *et al.*, 1993). After adhesion to epithelial cells and transcytosis, viral particles gain access to its numerous target cells present in the *lamina propria*: DCs, $CD4^+$ T cells and macrophages (Figure 16).

However, these experiments were conducted *in vitro* using transformed cell lines or *ex vivo* explants, and have not been confirmed *in vivo*.

In addition to their potential role in facilitating HIV particle passage through the mucosal surface, epithelial cells may also contribute to the establishment of a pro-inflammatory state, as a consequence of their interaction with viral particles. Indeed, in a NHP model of vaginal inoculation of SIV particles, endocervical epithelial cells produced inflammatory molecules, including MIP-3α, within the first 1-3 days, and thereby inducing an influx of $CD4^+$ T cells and DCs, including pDCs, directly in contact with the basal pole of the epithelium (Li *et al.*, 2009).

HIV-1 cell-to-cell transmission mediated by infected cells present in the inoculum

HIV-1 particles can move between cells and tissues of the body via fluid-phase diffusion; such diffusion allows HIV to make contact passively with its target cells. However, the extracellular environment may contain molecules that hinder virus movements, such as mucus which is particularly relevant (Boukari *et al.*, 2009; Shukair *et al.*, 2013). The diffusion of virions lowers their concentration and increases the length of time between viral assembly and productive infection, thereby increasing their vulnerability to the host immune response.

In contrast, immune cells constitute a large network of cells in constant interaction with each other and which are highly migratory (Feldmann and Schwartz, 2010; Shieh *et al.*, 1999). There is growing evidence that HIV-1, like other viruses, can hijack these properties and exploit them to spread (Feldmann and Schwartz, 2010; Martin and Sattentau, 2009). Cell-to-cell transfer of HIV-1 is estimated to be 92- to 18,600-fold more efficient than infection by cell-free virus (Chen *et al.*, 2007).

Two models of cell-to-cell transmission have been proposed in the context of HIV-1 sexual infection:

- Uptake of free viral particles or establishment of direct cell-to-cell contacts between infected leukocytes, and virus transfer to the stromal DCs or intraepithelial LCs, leading to virus dissemination in *cis* or in *trans* (see above),

- Establishment of direct cell-to-cell contacts between the infected leukocytes present in the genital or rectal secretions and the intraepithelial $CD4^+$ T cells and macrophages, leading to their infection and a local initiation of viral replication. This model is supported by *in vivo* observations in NHP that the cell type that is predominantly infected during the earliest steps of SIV transmission are $CD4^+$ T cells (Joag *et al.*, 1997; Picker and Watkins, 2005; Veazey *et al.*, 2003).

The most widely described mechanism of cell-to-cell virus spread involves the formation of a "virological synapse" (VS) between an infected cell ($CD4^+$ T cell, macrophage or DC) and a target cell (Arrighi *et al.*, 2004; Garcia *et al.*, 2005; Martin and Sattentau, 2009; Mothes *et al.*, 2010; Turville *et al.*, 2004) (*trans*-infection) (Figure 17). The VS shares many similarities with the immunological synapse, which is formed by cell-to-cell contact between an antigen-presenting cell (APC) and a T cell (Bromley *et al.*, 2001).

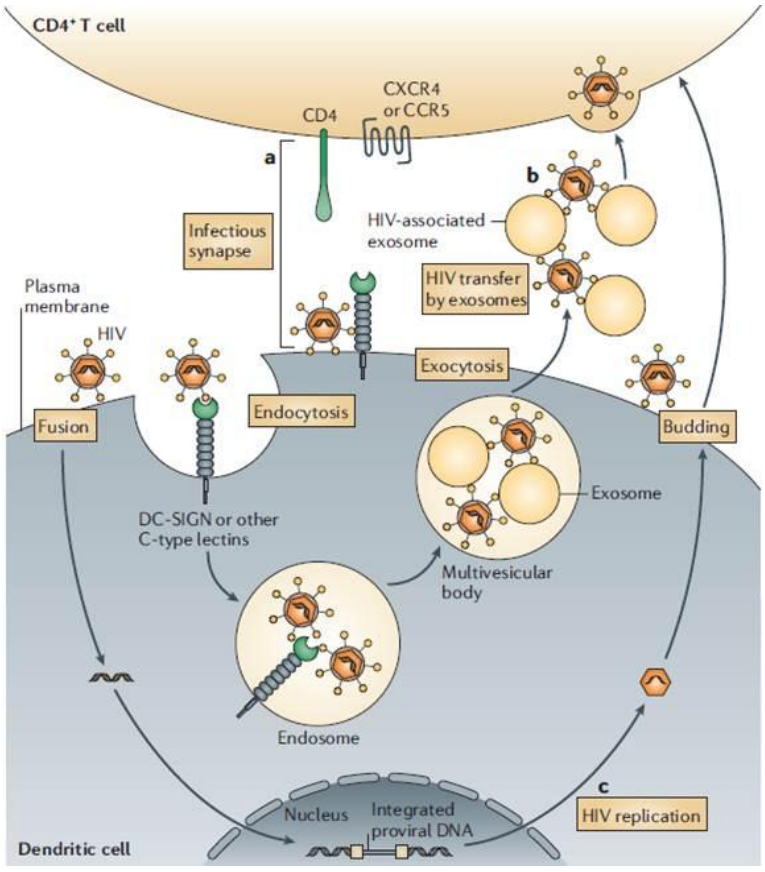

Figure 17 : Transmission of HIV-1 through the virological synapse. a. and b.: *trans*-infection; c.: *cis*-infection. Adapted from (Wu and KewalRamani, 2006).

Viral envelope glycoproteins gp120 and gp41, and the viral receptor CD4 and co-receptor CCR5 or CXCR4, expressed on the cell membrane, participate in the formation of VS. They are recruited and concentrated at the target-effector cell interface in a cytoskeleton-dependent manner (Feldmann and Schwartz, 2010) (Figure 18). Surface molecules including adhesion molecules, like HSPG, integrins and their ligands, and C-type lectins like DC-SIGN stabilize the VS (Groot *et al.*, 2008; Jolly *et al.*, 2007; Martin and Sattentau, 2009).

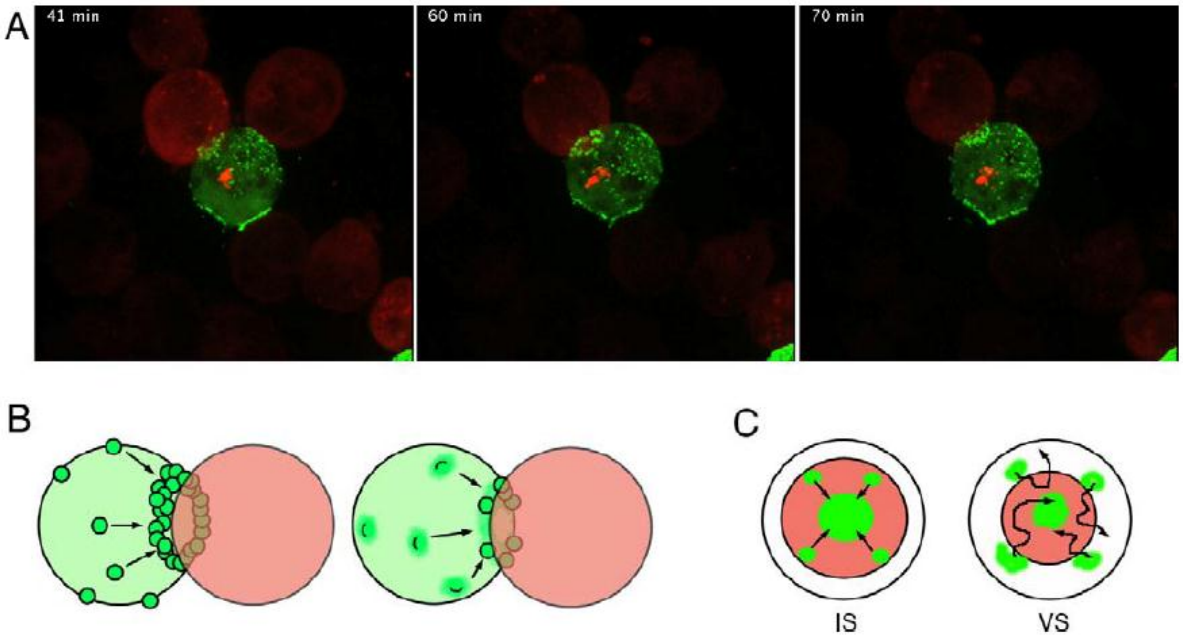

Figure 18 : Polarization of viral budding platforms towards the target cells. (A) Live imaging of Gag-GFP: infected cell (green) expressing centrin-RFP (red dot), conjugated with a target cell expressing actin-RFP (darker red). (B) Models of virological synapse (VS) formation. Mature virions can "surf" on the infected cell surface (left), or viral budding platforms can polarize toward the target (right). (C) Dynamic observation of immunological synapse (IS) and VS reveal that the two structures behave differently. This schematic view of the interface between interacting cells shows intercellular adhesion zones (pSMAC) in red, and mobile elements (TCR clusters in the IS, viral clusters in the VS in green). Preformed surface viral material migrates uncoordinatedly toward the center of the VS (right). Adapted from (Feldmann and Schwartz, 2010).

Sattentau *et al.* reported that extensive membranous connections, termed membranes nanotubes, can form passively when interacting cells separate from each other. Electron microscopy analysis revealed that the point of intercellular nanotube contact is indeed a small-scale virological synapse, implying receptor-dependent and Env-dependent interactions (Sowinski *et al.*, 2008).

HIV can also be transferred from cell to cell via actin-containing membrane extensions, where CD4 and virus co-receptors are concentrated (Majstoravich *et al.*, 2004).

These extensions include microvilli and ruffles, thin plasma membrane extensions supported by actin, and that participate in multiple cellular processes, including transmigration and formation of cell-to-cell contacts (Mattila and Lappalainen, 2008). A mechanism of virions surfing on the outside of these membranes extensions towards the target cell has been proposed (Mattila and Lappalainen, 2008; Sherer *et al.*, 2007).

Polarized spread of this type benefits the virus by channeling the release of newly produced viral particles towards a susceptible cell, in which the viral receptor and co-receptor are clustered at the cell-to-cell junction. Importantly, this mechanism of virus transmission potentially may facilitate immune evasion by:

- Reducing the transit time to target cells (Martin and Sattentau, 2009; Sourisseau *et al.*, 2007),
- Reducing exposure to components of the host immune system, especially neutralizing antibodies (Chen *et al.*, 2007; Martin and Sattentau, 2009).

Role of dendritic cells in virus transmission and dissemination

DC-mediated cell-to-cell transmission of HIV to its "true" target cells —$CD4^+$ T cells and macrophages— may occur at the site of exposure, within the *lamina propria* or the submucosa, or in the draining LNs, after migration of the transmitter DC through the lymphoid system.

Another facet of the contribution of DCs to HIV transmission has been proposed recently. Endocervical plasmacytoid DCs recruited by the epithelial release of MIP-3α (see above) produce diverse molecules with antiviral and chemoattractant activity, like IFN-α, MIP-1α and MIP-1β. This induces the influx of $CCR5^+$ SIV target cells, mostly $CD4^+$ T cells, and thereby promotes SIV transmission and local viral replication (Li *et al.*, 2009). Vaginal application of glycerol monolaurate, a molecule that interferes with this pro-inflammatory signaling pathway, before SIV inoculation provided prevention of infection.

Transmigration of infected leukocytes.

Macrophages and T cells, present in the genital secretions of the donor, can be productively infected by HIV-1. These cells express a variety of adhesion molecules that trigger their adhesion to epithelial cells and their migration across the epithelium in the recipient.

LFA-1 and Mac-1 belong to a class of integrins involved in the trafficking of immune cells (Evans *et al.*, 2009). Upon activation by cytokines in genital secretions, for example SDF-1, the conformation of the integrin receptor changes such that it binds adhesion molecules of the ICAM or JAM families, present in the intercellular zone, allowing the migration of leukocytes across a mono- or pluristratified epithelium (Evans *et al.*, 2009).

Other types of integrins can be involved in this process. αEβ7 is strongly expressed by genital intra-epithelial leukocytes and allows specific adhesion to epithelial cadherin, thereby favoring migration within pluristratified epithelia (Schon *et al.*, 1999; Shacklett *et al.*, 2003; Shieh *et al.*, 1999).

Apical-to-basal migration of leukocytes is induced by a chemokine gradient. All the sexually exposed mucosa have been described to release chemokines that may attract leukocytes from the inoculum: IL-8, SDF-1α, the ligand of CXCR4, and RANTES, MIP-1α and MIP-1β, all ligands of CCR5 (Herold *et al.*, 2006; Maxion and Kelly, 2002; Wira *et al.*, 2005). These factors are secreted by epithelial cells and mucosal resident leukocytes, and they are abundant during inflammation. Thus, any mucosal inflammation is expected to increase the chemotactism of infected leukocytes present in the inoculum and thereby the risk of HIV transmission.

In vitro, HIV-infected mononuclear cells can migrate through and transmit infection to a monolayer of cervical-derived cells (Carreno *et al.*, 2002; Mayer and Anderson, 1995; Tan *et al.*, 1993). Another study described macrophages attached to the epithelial surface of a reconstructed vaginal epithelium model, and penetrating between epithelial cells, by a mechanism involving integrins (Blaskewicz, 2009)., Labeled cells in semen, again *ex vivo*, are able to bind to an ecto-cervical explant and migrate through the epithelium (Maher *et al.*, 2005).

Moreover, several independent studies reported successful vaginal transmission in humanized mice inoculated with cell-associated HIV-1 (Di Fabio *et al.*, 2001; Ibata *et al.*, 1997; Khanna *et al.*, 2002; Zacharopoulos *et al.*, 1997). Systemic infection could be obtained in SCID-humanized mice, treated with Depo-Provera and inoculated vaginally with CFSE-stained mononuclear cells infected with HIV-1 (Di Fabio *et al.*, 2001). Interestingly, $CFSE^+$ donor cells were detected in the vaginal epithelium within 4 hours of inoculation and in the draining LNs after 24h, suggesting that inoculated cells had migrated trough cervico-vaginal epithelium and disseminate very quickly to the secondary lymphoid system.

In NHP, systemic infection can be obtained by inoculation of infected leukocytes, both after ulcerative trauma of the mucosal surface (Weiler *et al.*, 2008), or in the absence of any such trauma (Girard *et al.*, 1998; Miller *et al.*, 1989; Salle *et al.*, 2010).

Recently, our group inoculated Depo-Provera-treated female cynomolgus macaques with CFSE-labeled splenocytes infected with SIVmac251: the findings were similar to those obtained in the humanized mouse model (Salle *et al.*, 2010). As early as 21h post-inoculation, CFSE-labeled cells, mostly T cells, were detected in vaginal tissues and the draining internal ilial LN; these cells were confirmed to be SIV-positive cells by *in situ* hybridization (Figure 19). Forty-five hours after inoculation, $CFSE^+$ cells were also detected in the axillary LN, suggesting rapid dissemination of the inoculated cells through the secondary lymphoid tissue.

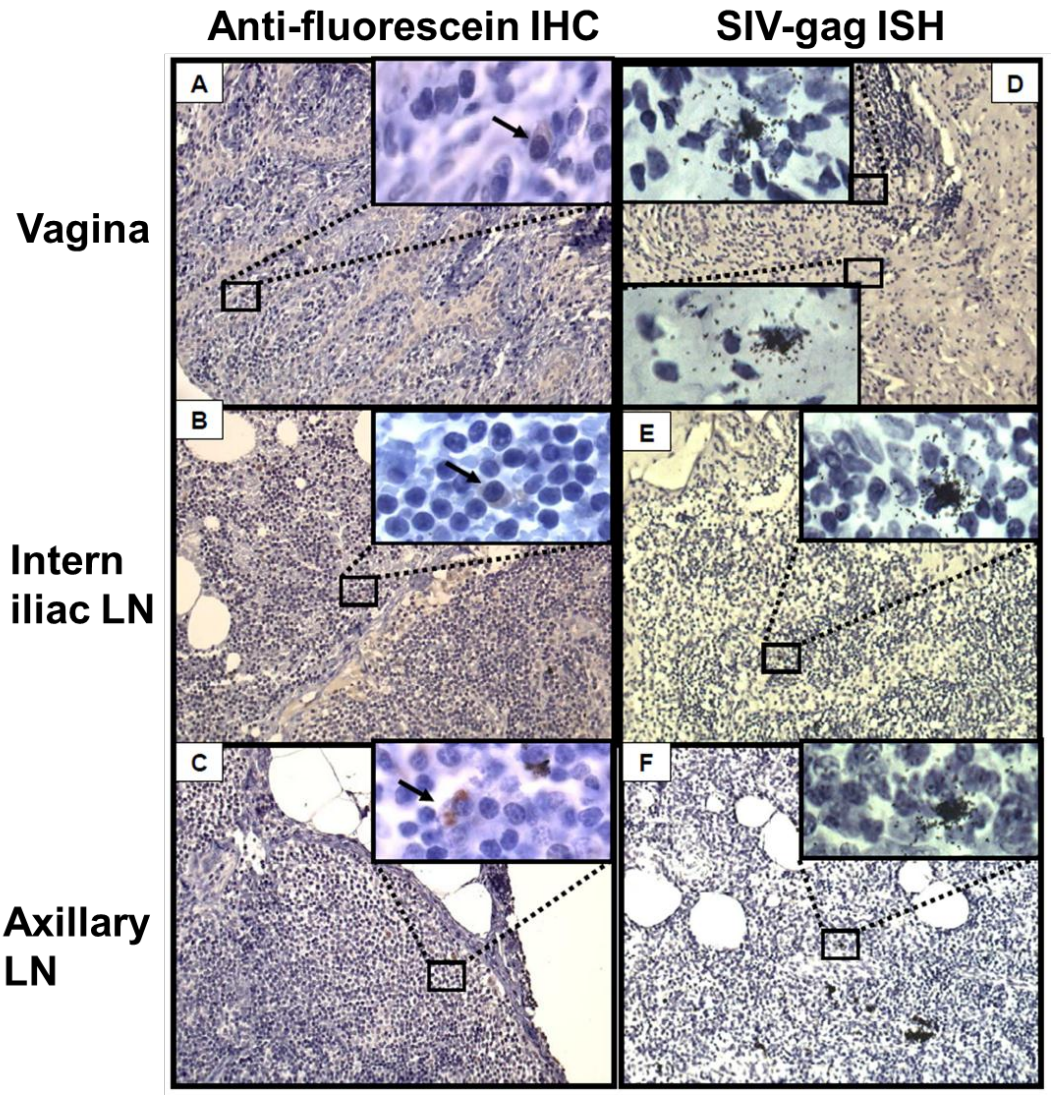

Figure 19 : CFSE-labeled cells and infected cells in tissues after vaginal exposure of SIVmac251-infected splenocytes. (A-C) CFSE-labeled cells revealed by immunohistochemical analysis (*arrows*) in macaque tissues (magnification, x100 and x1000) (D-F) *In situ* hybridization using DNA probes complementary to SIV in the same macaque tissues (magnification, x100 and x630) Tissues were sampled 21h (A, B, D, E) and 45h (C, F) post-inoculation (Salle *et al.*, 2010).

Unprotected heterosexual intercourse induces an allogeneic response in women that is specific for the partner's human leukocyte antigen (HLA) (Peters *et al.*, 2004). This is probably due to exposure to seminal leukocytes and not to sperm, which do not express classical HLA antigens (Anderson *et al.*, 1982). It has been suggested that, because the human vagina is a poor antigen induction site (Kozlowski *et al.*, 2002) and that the allogeneic response was detected in peripheral blood, some seminal leukocytes may cross the cervico-vaginal epithelium and stimulate the immune response directly in the draining LN (Anderson *et al.*, 2010a).

These various observations collectively suggest that transmigration of infected leukocytes may be a relevant pathway of HIV mucosal transmission, across both single-layered and pluristratified epithelia.

To conclude this part, various different mechanisms of HIV mucosal transmission have been proposed and described in diverse models (Figure 20 illustrates these putative mechanisms).

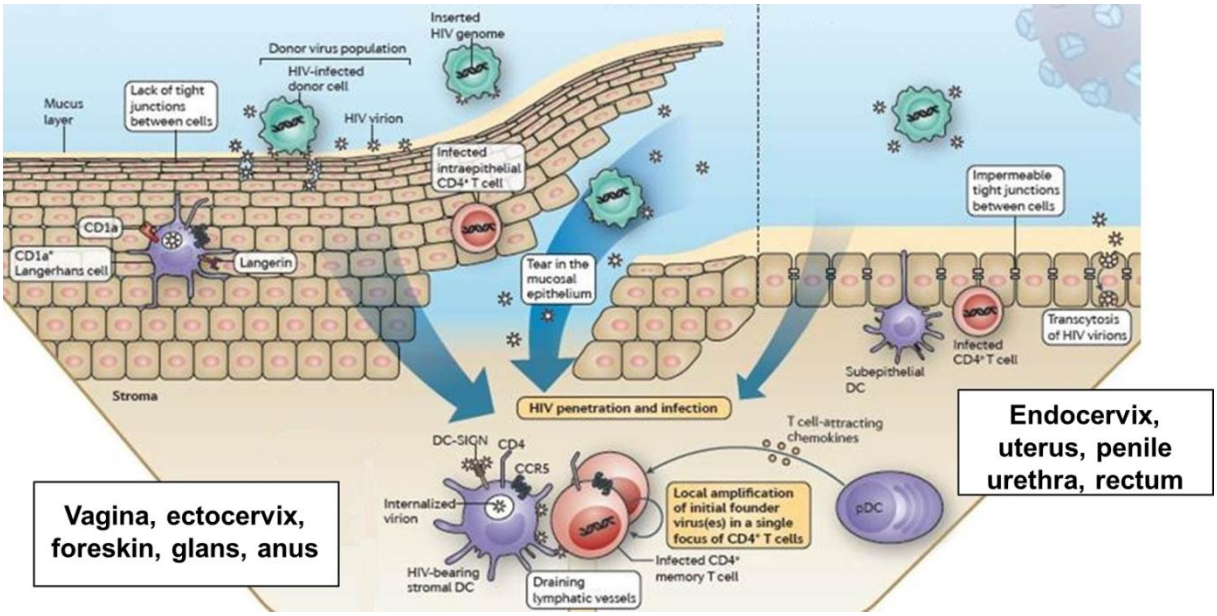

Figure 20 : Schematic representation of putative mechanisms of HIV mucosal transmission, depending on the site of infection and the nature of the virus. Adapted from (Bhardwaj, 2012).

b. Establishment of systemic infection after mucosal exposure

Numerous studies *in vivo* in NHP models have provided insight into how HIV and SIV enter the mucosa following genital or rectal exposure, and then cause systemic infection (Girard *et al.*, 1998; Li *et al.*, 2009; Martinon *et al.*, 2008; Miller *et al.*, 1989; Miller *et al.*, 2005; Salle *et al.*, 2010; Vingert *et al.*, 2003). In particular, studies with rhesus macaques inoculated by the vaginal route, elegantly described very early events of viral pathogenesis, within the first two weeks after infection. However, such access to what happens at the mucosal frontline, more distally at the draining lymph nodes and finally at the systemic level, is clearly not feasible in human HIV-1 patients.

NHP studies on the earliest steps of SIV infection following vaginal inoculation of high dose of virus indicate that local events critical for systemic infection take place very quickly. The virus can cross the mucosal epithelial barrier within hours, initiate local replication within days and establish self-propagating systemic infection, via secondary lymphoid tissue within a week or less (Figure 21) (Hu *et al.*, 2000; Li *et al.*, 2009; Miller *et al.*, 2005; Zhang *et al.*, 1999b).

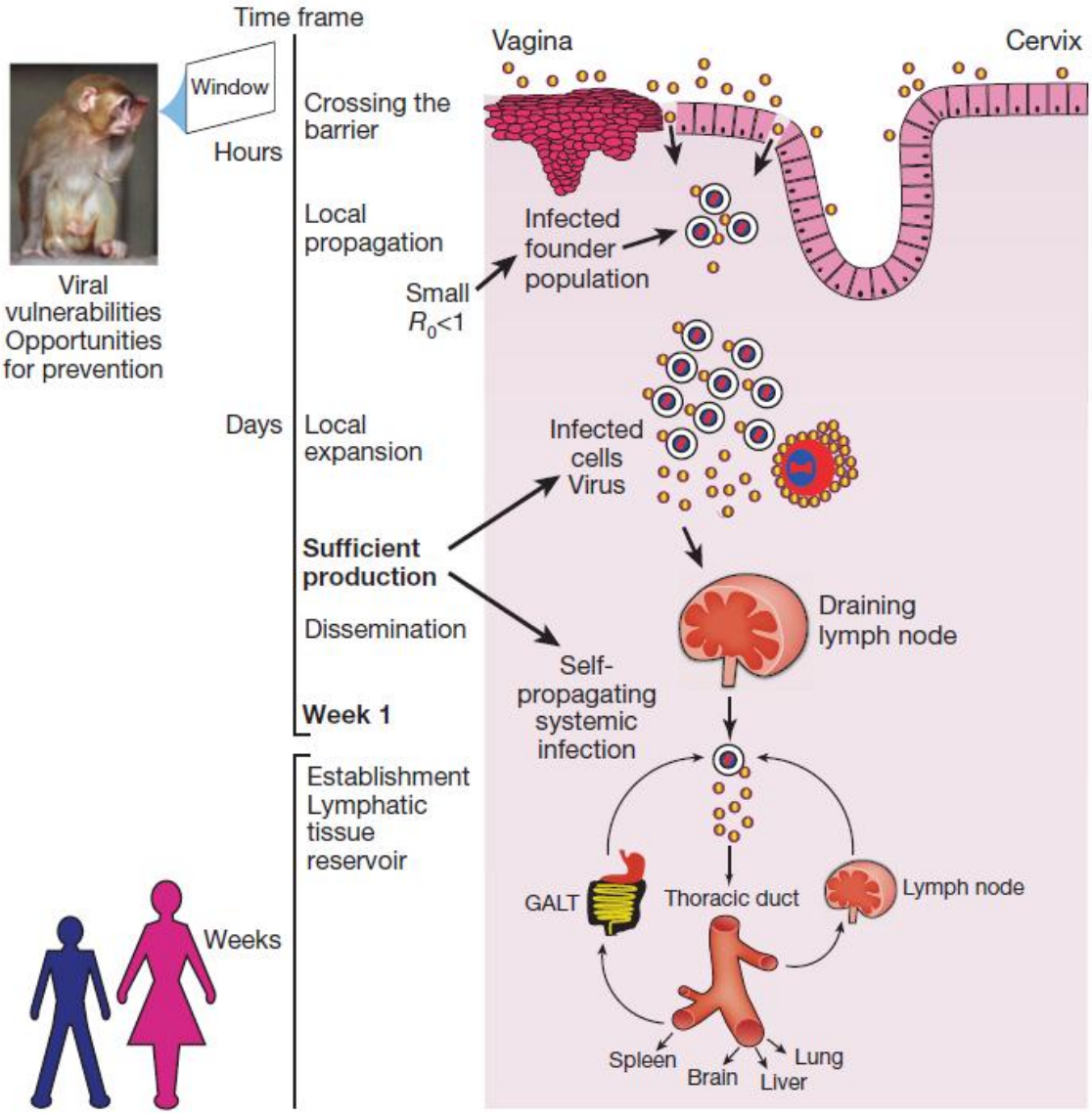

Figure 21 : Time frame, sites and major events during and following vaginal transmission. Adapted from Haase, 2010 (Haase, 2010).

The first 3-4 days after exposure constitute the so-called the **"eclipse phase"**. During this time lapse, viral DNA can be detected in the exposed tissue, meaning that the virus has infected some mucosal cells; however, viral RNA, representing *de novo* produced virions, is not detected at this time (Haase, 2011). This is followed by a rapid local expansion and lymphatic colonization (Figure 21). During the second week of infection, replication accelerates rapidly, taking place mostly in secondary lymphoid organs including the spleen, lymph nodes and gut-associated lymphoid tissue (GALT) (Mannioui *et al.*, 2009; Mehandru *et al.*, 2004; Veazey *et al.*, 1998), where virus has access and close spatial proximity to many target cells. Viral loads in blood and tissues reach to peak levels 10-14 days post-infection (dpi), and thereafter decline towards relatively stable and lower levels by 4 weeks after exposure (Mattapallil *et al.*, 2005; McMichael *et al.*, 2010) (Figure 22).

From this stage, viral reservoirs are established in lymphoid tissues, gut, brain and genital tract, where the virus is present as provirus integrated into the genomes of latently infected cells (mostly resting $CD4^+$ T cells and macrophages) (Blankson *et al.*, 2000; Blankson *et al.*, 2002; Chomont *et al.*, 2009; Chun *et al.*, 1995; Eisele and Siliciano, 2012; Igarashi *et al.*, 2001; Smith *et al.*, 2003; Williams *et al.*, 2001).

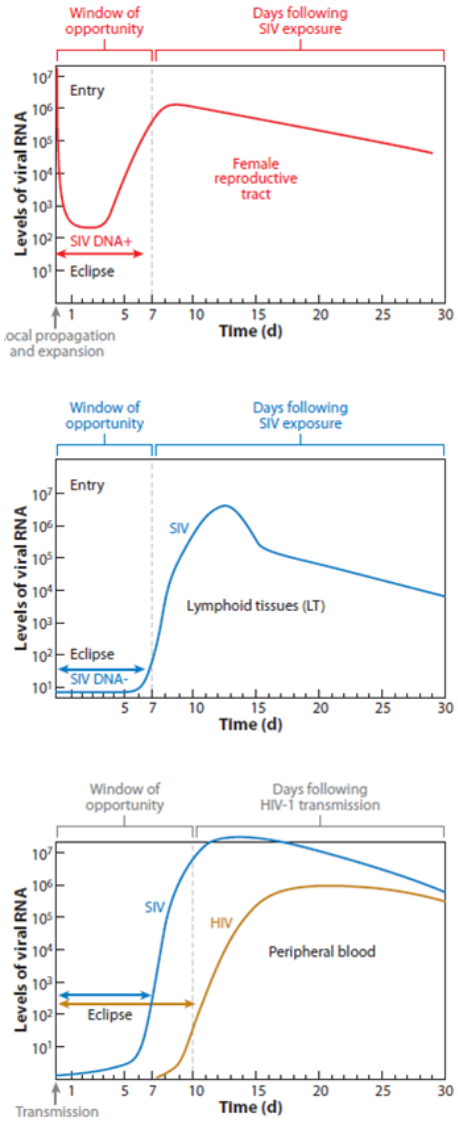

Figure 22 : Pathogenesis of vaginal transmission and early infection, *in vivo* growth curves and eclipses phases. Adapted from Haase, 2011 (Haase, 2011).

The general view is that once systemic infection and viral reservoirs are established, elimination of HIV infection is extremely unlikely, or impossible (Blankson *et al.*, 2002; Chomont *et al.*, 2009; Chun *et al.*, 1998). Even in patients who have been on HAART for several years, proviral DNA can be detected in latently infected cells, and viral rebound can

occur within weeks of treatment interruption (Eisele and Siliciano, 2012; Joos *et al.*, 2008; Lambotte *et al.*, 2002).

Also, the time to eradication of the population of latently infected cells in patients on HAART since the chronic phase of infection has been estimated to be over 60 years (Joos *et al.*, 2008; Siliciano *et al.*, 2003; Strain *et al.*, 2003). In contrast, two longitudinal studies estimated that continuous treatment, initiated during the acute phase of infection and if it totally suppressed viral replication, would be required for 7 to 8 years of to eradicate the latent viral reservoir (Chun *et al.*, 2007; Zhang *et al.*, 1999a).

c. Initial induction of a small founder population of infected cells

In NHP exposed to free virus by the vaginal route, viruses rapidly cross the mucosal barrier and establish a small founder population of infected CD^{4+} T cells (Miller *et al.*, 2005; Zhang *et al.*, 1999b). The founder population, which numbers about only 40-50 cells during the first 3-4 days post-exposure, undergoes a local expansion during the first week after infection and generate sufficient virus and numbers of infected cells to disseminate to the draining lymph node and from there establish systemic infection (Miller *et al.*, 2005). Infected cells are detectable in draining lymph nodes early after infection, 18 to 24h after exposure, suggesting that there are one or several wave(s) of viral spread early after exposure (Hu *et al.*, 2000; Salle *et al.*, 2010). However, it seems that a threshold for a self-propagating infection remains until sufficient quantities of virus and infected cells are produced locally.

An efficient local mucosal immune response, innate or adaptive (in cases of repeated HIV exposure), could theoretically control and even suppress infection at a very early infection time point; indeed, the early period constitutes a **"window of opportunity".** This notion is supported by the observation, made by several authors independently, that vaginal or rectal inoculation of low SIV doses in macaques can lead to transient viremia, without inducing patent systemic infection and seroconversion (Miller *et al.*, 1994a; Milman and Sharma, 1994; Pauza *et al.*, 1994). However, it seems that the cellular immune response against HIV is in most cases too little and too late to prevent chronic infection (Haase, 2010).

NHP studies have lead to the idea that there are only a small number of infection events per sexual transmission; this has not been directly observed in HIV infections, but can reasonably inferred from recent studies characterizing viral genetics of patients early after infection. Single genome amplification, deep sequencing studies and mathematical modeling of the earliest viral strains circulating in blood have indicated that infection is acquired from a single virus genotype in 80% of cases and from two to five viruses in the other 20% (Keele *et al.*, 2008; Li *et al.*, 2010; Salazar-Gonzalez *et al.*, 2009).

d. Selective mucosal transmission of CCR5-tropic viral strains

After sexual exposure, CCR5-tropic HIV-1 strains are selectively transmitted (Keele *et al.*, 2008; Milman and Sharma, 1994; Salazar-Gonzalez *et al.*, 2009). There are very few exceptions to this viral selection, although there is evidence of the transmission of dual-tropic R5X4 strains; these observations suggest the existence of a “gatekeeper”, which selects R5 over X4 HIV (Grivel *et al.*, 2011; Margolis and Shattock, 2006). Grivel, Margolis and Shattock proposed a model for how such a gatekeeper mechanism could select, so near perfectly, HIV R5 variants. This model involves the superposition of different filters, none of them being absolutely impenetrable for X4 strains, but each working with the others in synergy (Figure 23).

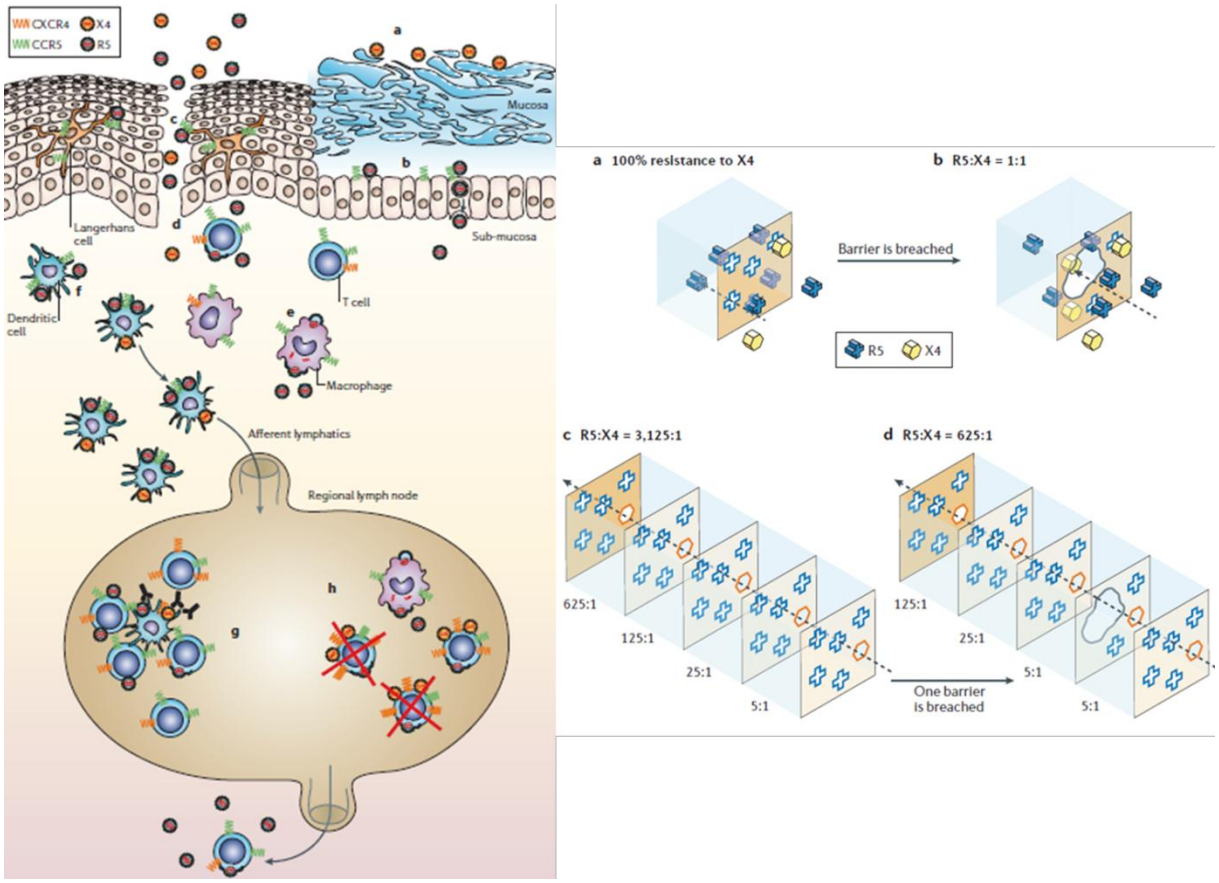

Figure 23 : Model of the selective transmission of R5 strains, as described by Margolis and Shattock. Adapted from (Grivel *et al.*, 2011).

Several filters have been proposed, some in the donor, others in exposed individuals, either at the mucosal barrier or at a systemic level. At least five sequential barriers working together have been described, but there are probably more involved during infection process, and they undoubtedly differ among individuals (Grivel *et al.*, 2011).

First, the presence of both CCR5- and CXCR4-tropic HIV-1 (R5 and X4 HIV-1, respectively) has been reported in semen and cervico-vaginal secretions. However, R5 variants tend to be more abundant than X4 strains in both types of genital secretions (Coombs *et al.*, 2003; Curran and Ball, 2002; Delwart *et al.*, 1998; Pillai *et al.*, 2005; Zhu *et al.*, 1996). X4 HIV-1, associated with accelerated progression to AIDS, only appears late in the course of the disease, and then only in 50% of individuals infected with Clade B virus. Indeed most patients progress to AIDS in the absence of the emergence of X4 strains. These observations suggest a sustained selective pressure against X4 over R5 replication by the donor.

Several factors may influence the selective pressure resulting in the predominance of R5 strains for both penetration and dissemination. One of the first barriers is the mucus layer. In particular, poly-anionic components of mucus can interact and trap positively charged viral proteins more efficiently , such as the gp120 V3 loop of HIV-1 X4-tropic strains (Bergey *et al.*, 1994; Kwong *et al.*, 2000; Margolis and Shattock, 2006). The mucosal epithelia, including the cervico-vaginal epithelium, produce substantial amounts of SDF-1, that binds and down-regulates CXCR4 surface expression on target cells (Agace *et al.*, 2000; Bomsel and David, 2002). Some defensins have also been reported to inactivate X4 strains more potently than R5 strains, although this suggestion remains controversial (Feng *et al.*, 2006; Quinones-Mateu *et al.*, 2003; Seidel *et al.*, 2010; Sun *et al.*, 2005).

After crossing the epithelial barrier, HIV encounters different target cells: mostly memory $CD4^+$ T cells, macrophages and DCs. These cells are characterized by high levels of CCR5 surface expression, with little or no CXCR4 expression (see above) in all sexually exposed mucosa.

Additional post-mucosal gate-keeping mechanisms appear to exist, because R5 strains dominate early stages of infection, even when the transmission occurred by intravenous inoculation (Grivel *et al.*, 2011). Lymphocytes, rather than macrophages and DCs, are the first targets for HIV (Haase, 2010; Miller *et al.*, 1992a; Miller and Shattock, 2003; Zhang *et al.*, 1999b). However, macrophages constitute a large viral reservoir, and together with other APCs, such as dendritic cells, are less susceptible to cytotoxic T cells

(Harouse *et al.*, 2003; Schutten *et al.*, 2001). Thus they survive longer and disseminate R5 strains, with which they are predominantly infected.

It is plausible, or even likely, that other barriers exist, and that there are differences between individuals and according to a variety of factors (age, progression through the menstrual cycle, t sexually transmitted disease, etc.).

To conclude, besides the generally low per-act risk of HIV-1 transmission, the fact that only a few (and often only a single) virions are transmitted after sexual exposure suggests that the mucosal barrier mostly succeeds in stopping penetration by the virus. Nevertheless, the huge extent of HIV-1 pandemic worldwide tends to indicate that co-factors must play a major role in facilitating viral transmission. Understanding these co-factors is crucial for the development of effective prophylactic drugs, vaccines and microbicides.

C. Factors influencing mucosal transmission

Epidemiological studies have helped to identify numerous cofactors that modulate HIV sexual transmission positively or negatively, by increasing or decreasing both the infectivity of the transmitters and the susceptibility of the exposed individual (Belec, 2007; Mayer and Anderson, 1995; Plummer, 1998; Royce *et al.*, 1997). Some cofactors are local, involving the genital and rectal tissues, whereas others depend on extra-genital/rectal features that have indirect effects on the infectious inoculum or on the sexually exposed mucosa.

Here, I will distinguish between factors influencing the infectivity of the transmitter's from those affecting the susceptibility of sexually exposed mucosa.

1) Factors related to the transmitter

a. Viral load and stage of infection

The viral shedding in genital and rectal secretions is a major determinant of the HIV transmission rate (Belec, 2007). HIV-1 is present in these secretions as two forms: cell-free virus, which is quantified by measuring the load of viral RNA (vRNA) in the acellular fraction; and cell-associated virus, quantified as the load of HIV DNA (vDNA) in the cellular fraction (Coombs *et al.*, 1998; Iversen *et al.*, 1998; Kiviat *et al.*, 1998; Zuckerman *et al.*, 2004).

Observations in HIV-1-infected human patients and SIV-infected macaques lead to the following conclusions:

- vRNA loads in blood plasma and mucosal secretions are correlated although the correlation is generally weak (Coombs *et al.*, 1998; Kalichman *et al.*, 2008; Kiviat *et al.*, 1998; Uvin and Caliendo, 1997);
- vRNA loads are generally lower in genital secretions than in blood plasma (Kovacs *et al.*, 1999; Tachet *et al.*, 1999), and rectal viral loads are higher than those in blood plasma and genital secretions (Zuckerman *et al.*, 2004),
- vRNA and vDNA loads in cervico-vaginal and rectal secretions are correlated (Iversen *et al.*, 1998; Kiviat *et al.*, 1998), but those in semen and rectal secretions are not correlated (Krieger *et al.*, 1998; Mayer *et al.*, 1999);
- The variability of viral loads in mucosal secretions is higher than that in peripheral blood (Coombs *et al.*, 1998; Iversen *et al.*, 1998; Tachet *et al.*, 1999),
- Viral loads in mucosal secretions are inversely correlated to peripheral $CD4^+$ T cells counts (Kovacs *et al.*, 1999; Tachet *et al.*, 1999; Xu *et al.*, 1997).

The stage of infection is a major determinant of the risk of transmission: the risk is maximal during primary and late stages of infection, when the viral loads in mucosal secretions are the highest (Hollingsworth *et al.*, 2008; Wawer *et al.*, 2005). Indeed, risk of transmission has been evaluated as 12 to 26- and 7-fold higher during primary and late stages on infection, respectively, than during asymptomatic chronic phase.

b. Concomitant sexually transmitted diseases

Co-infection with other sexually transmitted diseases (STDs), notably Herpes Simplex type II (HSV2), Cytomegalovirus, *N. gonorrheae*, *C. trachomatis* and *T. vaginalis* have been reported to be major factors associated with higher HIV-1 loads in genital and rectal secretion (Dyer *et al.*, 1998a; Kiviat *et al.*, 1998; Kreiss *et al.*, 1994; McClelland *et al.*, 2002; Wolff and Anderson, 1988b). This phenomenon has been explained as a synergistic process, by which the replication of each infectious agent is enhanced by the other. Direct and indirect (like the related mucosal inflammation) mechanisms have been described (Cohen *et al.*, 1997; Ramsey *et al.*, 1995; Sadiq *et al.*, 2005; Sheth *et al.*, 2008; Wald, 2004).

Interestingly, in a systematic review Rottingen *et al.* states that the increase of HIV load related to co-infections of the genital tract is more pronounced in men than in women (Rottingen *et al.*, 2001).

c. Antiretroviral therapy

Antiretroviral therapy (ART) significantly decreases HIV-1 loads in the genital secretions of infected women (Coombs *et al.*, 2001; Cu-Uvin *et al.*, 2000; Kovacs *et al.*, 2001) and men (Anderson *et al.*, 1992; Hamed *et al.*, 1993). Genital vRNA loads decrease quickly after initiation of treatment (Cu-Uvin *et al.*, 2000). Nevertheless, viremia being undetectable is not systematically followed by a similar reduction in genital and rectal secretions of the virus (Anderson, 2009; Kovacs *et al.*, 2001; Krieger *et al.*, 1991b; Politch *et al.*, 2012; Tachet *et al.*, 1999; Zhang *et al.*, 1998a). Indeed, viral loads may be high in the rectal secretions of seropositive patients under ART (Zuckerman *et al.*, 2004).

d. Circumcision

Male circumcision has been associated with a reduction of the risk of transmitting HIV infection. A transversal study of more than 4,400 women in Kenya reported that women whose sexual partner was not circumcised presented a 3-fold higher risk of being infected by HIV-1, independently of other risk factors (Hunter *et al.*, 1994).

The absence of circumcision may raise the risk of transmission by exposing the partner's mucosa to infected foreskin cells (Belec, 2007). Indeed, Miller *et al.* reported the presence of infected mononuclear cells within the foreskin epithelium of SIV-infected macaques (Miller, 1994).

2) Factors related to the exposed individual

a. Genetic factors

The first genetic factor to be described to confer protection against HIV-1 acquisition, and progression toward AIDS, is being homozygous for the allelic deletion CCR5Δ32 (Dean *et al.*, 1996; Liu *et al.*, 1996; Paxton *et al.*, 1996; Samson *et al.*, 1996). This allele is most frequently found in populations in northern Europe, where 1% of individuals are homozygotes (Stephens *et al.*, 1998). The protection is however incomplete, and a small number of cases of HIV infection have been described in people with homozygote CCR5Δ32 deletion; in all such cases, only X4 strains have been isolated from blood plasma (Lama and Planelles, 2007).

Some haplotypes of genes coding for RANTES and DC-SIGN homologs may decrease the risk of HIV acquisition, but the protection conferred is weak, and this is an issue that needs to investigated more extensively (Wichukchinda *et al.*, 2007; Zhao *et al.*, 2004).

b. Anatomic factors

Male circumcision also reduces the risk of acquiring HIV infection. In circumcised men, most of, if not all, the foreskin is removed and the mucosal epithelium covering the glans becomes highly keratinized, like skin. Since 1986 (Fink, 1986), the notion that male circumcision may protect against HIV sexual transmission has been extensively considered. More than forty studies have evaluated the effects of circumcision on the risk of HIV acquisition (Belec, 2007; Siegfried *et al.*, 2005; Wamai *et al.*, 2011; Weiss *et al.*, 2000). It is now generally believed that male circumcision confers protection on the insertive partner against the acquisition of HIV during sexual intercourse. In a meta-analysis of 27 studies, including 21 reporting a reduced risk of HIV among circumcised men, the protection was evaluated to be 42% (34 to 54%) (Weiss *et al.*, 2000). Subsequently large randomized clinical trials demonstrated that circumcision reduces the risk of HIV acquisition in men by 50-60% (Auvert *et al.*, 2005; Gray *et al.*, 2007).

This protective role of male circumcision by the insertive partner may be a consequence of the keratinization of the epithelial surface of the glans in the absence of foreskin (Anderson *et al.*, 2011). Moreover, the absence of the foreskin reduces the transient retention of the partner's genital or rectal secretions which occurs in non circumcised men. Such retention of contaminated fluids probably increases the risk of acquiring HIV.

Cervical ectopy, which consists in the extrusion of endocervical mucosa into the vaginal lumen, has been found to be associated with increased susceptibility to HIV-1 transmission (Kreiss *et al.*, 1994; Moss *et al.*, 1991; Plummer *et al.*, 1991). Indeed, this condition exposes the fragile transformation zone and endocervical mucosa to external aggressions, and results frequently in substantial immune inflammation or ulcerations. This zone is characterized by a single-layer epithelium and the abundance of HIV-1 target cells.

Cervical ectopy is common during pregnancy and in young women; this may explain, at least partially, why young African women are at higher risk of infection than men of the same age (Berkley *et al.*, 1990; Georges-Courbot *et al.*, 1989).

c. Hormonal factors and contraception

The menstrual cycle has been described as modulating women's susceptibility to HIV infection; sex hormones, the levels of which vary substantially during the cycle, regulate both immune function and epithelial structure of the upper and lower genital tract. The consequences of treatment with sex hormones or contraceptives for the cervico-vaginal epithelium are similar in women and female macaques.

During the follicular stage of the cycle, estradiol impregnation stimulates proliferation of the vaginal and ectocervical epithelium and mucin production by endocervical glandular cells. Estrogen injections result in a significant thickening of the epithelium (Smith *et al.*, 2004b; Wood, 2008)

Progesterone production during the secretory stage induces thinning of the vaginal epithelial surface. A treatment based on progesterone (with Medroxyprogesterone, or the synthetic progesterone analog Depo-Provera® for example) led to substantial thinning of epithelium in female macaques (Marx *et al.*, 1996). However, the vaginal epithelium thinning induced by DMPA treatment is slighter in women than in macaques (Mauck *et al.*, 1999; Miller *et al.*, 2000).

This led Smith *et al.* to study ovariectomized rhesus macaques treated with estradiol. They found that they were more resistant than controls against SIV vaginal infection (Smith *et al.*, 2000), whereas NHP during the secretory stage or treated with Depo-Provera (DPMA), were substantially more sensitive to infection (Marx *et al.*, 1996).

A study conducted in women taking three different contraceptives, including injectable progesterone, found that all women taking contraceptives displayed thicker cervico-vaginal epithelium than controls (Ildgruben *et al.*, 2003), contradicting other observations.

DPMA treatment (but not oral contraceptives) has been associated with an increased risk for HIV-1 acquisition in women (Baeten *et al.*, 2007; Heffron *et al.*, 2012; Hel *et al.*, 2010; Morrison *et al.*, 2010; Morrison *et al.*, 2009; Wand and Ramjee, 2012), as it has been demonstrated in NHP models. Different mechanisms have been described.

First, DPMA administration increases epithelial infiltration by leukocytes, including by potential HIV target cells (Chandra *et al.*, 2013; Ildgruben *et al.*, 2003). Progesterone impregnation is also associated with increased activation markers and CCR5 expression *in vivo* and *ex vivo* (Chandra *et al.*, 2013; Patterson *et al.*, 1998).

In parallel, DPMA treatment of human PBMCs inhibits *in vitro* the production of key regulators of both cellular and humoral immunity involved in orchestrating the immune response to invading pathogens, like IFN-α, TNF-α, MIP-1α/β, IL-2 or IL-6 (Huijbregts *et al.*, 2013; Vassiliadou *et al.*, 1999). In women treated with DPMA, peripheral and genital levels of IFN-α were significantly lower (Huijbregts *et al.*, 2013).

However, other studies recently conducted on women under DPMA contraception didn't found any significant link with the risk of HIV acquisition, which remains controversial (Jain, 2012; Morrison *et al.*, 2012). To date, at the population level, the decision to withdraw DMPA from family planning programs in high endemic zones remains not warranted (Jain, 2012), but other contraceptive methods, especially condom use, is recommended to individual DPMA-users presenting a high risk of exposure to HIV (2012).

IgA, IgG and lactoferrin levels in cervico-vaginal secretions decline 10-100 fold at midcycle, and then rise towards the end of the menstrual cycle (Schumacher, 1988). The same study demonstrates that oral contraceptives depress immunoglobulin and lactoferrin levels for the duration of the exposure. The concentrations of other antimicrobial factors, including SLPI, human β-defensin 2 and human neutrophil peptides 1-3, decline significantly at midcycle and remain low for 7-10 days (Keller *et al.*, 2007).

Within the upper genital tract, estradiol impregnation, preparing the FGT for conception, suppresses innate (NK cells, neutrophils and dendritic cells) and cell-mediated (CD8+ T cells) mucosal immunity. This is a consequence of the action of TGF-β and other cytokines and growth factors, which peak midcycle (Casslen *et al.*, 1998; White *et al.*, 1997; Wira and Fahey, 2008).

These observations led Wira *et al.* to suggest the existence of a **"window of vulnerability"** during the menstrual cycle, a consequence of sex hormone impregnation, which lasts for 7-10 days after ovulation. During this period, HIV cervico-vaginal transmission may be facilitated (Wira and Fahey, 2008).

Hormonal contraception, especially progestin-based, has often been found to be associated with an increased risk of HIV transmission. However, there have been numerous epidemiological studies and the results reported are contradictory. However, Baeten *et al.* described various mechanisms by which oral contraception may increase the risk of HIV transmission (Baeten *et al.*, 2007):

- Increasing cervico-vaginal inflammation
- Increasing CCR5 expression in the genital tract
- Increasing the risk of cervical ectopy
- Increasing the risk of acquisition of other STDs
- Modifying immune mucosal responses
- Enhancing viral replication

There is still no solid demonstration that oral contraceptives increase the risk of HIV acquisition in women; therefore, such contraception remains recommended for birth control, but should be associated with condom use for prevention of STD infection (Belec, 2007; Stephenson, 1998). Nevertheless, injectable medroxyprogesterone, which has been linked to an enhanced risk of HIV acquisition (Rehle *et al.*, 1992), should be discouraged in endemic countries, notably in Africa.

d. Perturbation of the endogenous flora

The surface of the vaginal, penile and rectal mucosa is colonized by an endogenous flora, which is mostly protective and contributes to maintaining the physiological equilibrium (see above). Local inflammation is generally absent, unless there is co-infection with a STD. Any perturbation of this microflora may result in an increased risk of HIV acquisition (Martin *et al.*, 1999).

Bacterial vaginosis (BV) has been well documented as an example of a factor potentially associated with an increased risk of HIV acquisition and transmission (Brotman, 2011; Cohen *et al.*, 1995; Martin *et al.*, 1999; Myer *et al.*, 2005). BV is one the most frequent causes of vaginal disorders in sexually active women. It is the perturbation of the vaginal flora balance, with a shift from the naturally predominant Lactobacilli to a majority of anaerobic strains, for example *Gardnerella vaginalis*, *T. vaginalis* and *M. hominis*. Clinically, BV manifests itself by an abundant vaginal discharge, a vaginal pH above 4.5, a strong and characteristic odor and the presence of “clue cells”, which are epithelial cells covered by *T. vaginalis* or other anaerobic bacteria (Amsel *et al.*, 1983; Brotman, 2011).

Different potential mechanisms by which BV may increase the risk of HIV transmission have been described:

- Neutralization of vaginal pH and decreasing H_2O_2 production by *Lactobacilli* (Hillier *et al.*, 1993).
- Modulation of oxidative capacities of mucosal leukocytes and mucins by bacterial sialidases, which allows pathogens to survive longer in the mucus layer (McGregor *et al.*, 1994).
- Activation of HIV-1 replication by LTR activation initiated by bacterial secretions, through the $_{NF}$-κ_B pathway (Al-Harthi *et al.*, 1998).

e. Physical traumatisms and dry sex

Any trauma inflicted on exposed mucosa may increase the risk of HIV acquisition by creating breaches in the epithelium (Belec, 2007).

Some African ethnic groups practice “dry sex” involving the use of irritants or drying agents (Strathdee *et al.*, 1996). These agents, applied vaginally, cause mucosal irritation with inflammatory lesions and even micro-ulcerations in the cervico-vaginal mucosa and also in the penile mucosa. There is still controversy over the consequences of these sexual practices on HIV transmission (Myer *et al.*, 2006; Myer *et al.*, 2005).

f. Sexually transmitted diseases and local inflammation

The effects of STDs on susceptibility to HIV has been extensively documented, from studies that link a history of STD to HIV acquisition (Coombs *et al.*, 2003; Fleming and Wasserheit, 1999; Rottingen *et al.*, 2001), to trials confirming the increased risk of acquiring HIV in the presence of STDs (Cameron *et al.*, 1989; Kassler *et al.*, 1994; Laga *et al.*, 1993; Nelson *et al.*, 1997; Plummer *et al.*, 1991). Fleming *et al.* reported an adjusted-risk ratio (e.g. the estimated risk ratio after confounding factors have been taken into account) for HIV acquisition for a person with a genital ulcerative disease (GUD) is between 2.2 and 11.3, whereas non-ulcerative STDs show adjusted-risk ratios of 3 to 4 (Fleming and Wasserheit, 1999). The pathogens most commonly causing GUD are Herpes Simples Virus type II (HSV-2), *Haemophilus ducreyi* and *Treponema pallidum* (the agent of syphilis). Commonly found pathogens causing non-ulcerative STDs include *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

Several mechanisms are possible for the increased susceptibility to HIV associated with STDs:

- **Mucosal disruption**: breakdown of mucosal integrity makes the main targets for HIV transmission more accessible. Mucosal disruption is common in GUDs (Galvin and Cohen, 2004), and genital ulcers enhance vaginal transmission of cell-associated SIV in macaques (Weiler *et al.*, 2008).

- **Immune changes**: STDs, including infection by *H. ducreyi*, both provoke the influx of leukocytes, including HIV target cells, and activate $CD4^+$ T cells. This may enhance HIV infection, because HIV-1 preferentially infects activated $CD4^+$ T cells (Magro *et al.*, 1996; Van Laer *et al.*, 1995). It is widely believed that any inflammation of the exposed mucosa is associated with an increased risk of HIV acquisition (Trifonova *et al.*, 2007).

The high prevalence of HSV-2 and other STDs, and particularly bacterial vaginitis, in Africa and Asia may explain the high incidence of HIV sexual transmission, and particularly the high female-to-male transmission rate (O'Farrell, 2001).

Finally, the use of irritating vaginal products, like the widely used spermicide Nonxynol-9, can also cause local inflammation and increase the susceptibility to HIV infection in women (Fichorova *et al.*, 2001).

g. HIV-specific antibodies in genital secretions

Several studies report that neutralizing IgG and IgA specific to HIV-1 have been recovered from vaginal secretions of women regularly sexually exposed to HIV (Alexander and Mestecky, 2007; Belec *et al.*, 1995; Hirbod and Broliden, 2007; Lu *et al.*, 1993; Mestecky, 2007; Miyazawa *et al.*, 2009). Also, prospective studies indicate that genital HIV-neutralizing IgA are associated with non-acquisition of HIV (Hirbod *et al.*, 2008; Kaul *et al.*, 2001).

Interestingly, IgA preparations from mucosal secretions of highly exposed persistently seronegative (HEPS) individuals were able to inhibit HIV-1 transcytosis through epithelial cells, suggesting that the presence of such antibodies in mucosal secretions may be a powerful mechanism for protection against the sexual acquisition of HIV-1 infection in these individuals (Devito *et al.*, 2000). In contrast, broadly neutralizing HIV-specific IgG (2F5, 2G1 and IgG1b12) do not inhibit viral particles passing through HEC-1 epithelial cells by transcytosis *in vitro* (Chomont *et al.*, 2008).

Contradictory results have been reported in analogous studies on HEPS individuals. No HIV-specific neutralizing or binding antibodies of any Ig isotype were found in the secretions from various cohorts of individuals (Belec *et al.*, 1989b; Dorrell *et al.*, 2000; Fiore *et al.*, 2000; Skurnick *et al.*, 2002).

In contrast, HIV-specific antibodies are present in the mucosal secretions of infected patients, mostly of IgG and IgA subtypes (Anderson *et al.*, 1998; Belec *et al.*, 1989a, b; Mestecky, 2007; Mestecky *et al.*, 2009; Raux *et al.*, 2000; Wahl *et al.*, 2011). IgA antibodies specific for HIV-1 are present at significantly lower frequencies and levels than IgG antibodies, in all external secretions (Mestecky, 2007). The findings for SIV-infected rhesus macaques are similar (Jackson *et al.*, 1995; Schafer *et al.*, 2002).

Despite the presence of HIV-specific neutralizing antibodies in mucosal secretions, high levels of IgG and/or IgA are generally not associated with lower local viral RNA loads (Wahl *et al.*, 2011). Moreover, HIV-specific IgA are detected more frequently in breast milk of transmitters, suggesting that they not only fail to protect against oral transmission of HIV-1, but that they may indeed enhance transmission (Kuhn *et al.*, 2006).

Thus, the correlates of immune protection against HIV-1 infection have not been clearly established. The results and findings reported for the role in HIV-1 sexual transmission of specific antibodies present either in mucosal secretions of the transmitter or in those of the exposed partner, are contradictory or incomplete or both.

The numerous *in vitro* and *ex vivo* investigations and *in vivo* studies with animal models (Figure 19) have led to the description of many different mechanisms. This diversity of mechanisms potentially involved can be explained by the heterogeneity of the exposed mucosal surfaces and of the nature of the virus. It is probable that several, if not all, of them are indeed relevant to human HIV infection.

Physical and chemical barriers that exist on the mucosal frontline are positively or negatively influenced by many co-factors, and this may explain why HIV infection has became one of the worst pandemics the human species has known, despite the low per-act transmission rate.

The very small number of initial founder viruses in each case suggests that few infectious events are required to establish infection. Moreover, the infection takes one week to become systemic, a period during which the virus is vulnerable and the number of infected cells is low. This constitutes a potential target for prevention strategies, or a “window of opportunity”.

III. Semen cell-associated virus and prevention of mucosal transmission

A. Partial efficiency of the strategies developed so far

The numerous attempts to develop an effective vaccine against HIV have failed. To date, the best protection conferred by a vaccination methodology was 31.2%, with a prime/boost strategy (Thai RV144 trial) (Figure 24). The difficulties and failures encountered by efforts to design a protective vaccine against HIV-1 have lead the scientific community to re-orientate vaccine studies towards several hitherto neglected aspects. In particular, two approaches are being investigated: enhancing innate immunity to achieve a rapid protective response independently of a memory process; and stimulating broad adaptive immune responses, notably neutralizing antibodies (secretory IgA and IgG), ADCC and cytotoxic T lymphocytes (CTL), (Bonsignori *et al.*, 2012; Fuchs *et al.*, 2010; Kim *et al.*, 2010a; Kwong *et al.*, 2012).

Other strategies of prevention, such as the use of microbicides (for example 1% Tenofovir gel) and oral pre- or post-exposure prophylaxis (PrEP), have been developed. The most promising results have come from clinical trials evaluating the efficacy of immediate post-exposure prophylaxis and PrEP: HIV transmission was reduced by as much as 96% (Figure 24). Mathematical models estimate that over the next 10 years, an effective PrEP program might prevent 2.7–3.2 million of the 11 million new HIV-1 infections projected to occur in sub-Saharan Africa (Abbas *et al.*, 2007). An effective PrEP program may also substantially reduce the incidence of HIV transmission in populations at high risk of infection in high income countries, notably the MSM population in the USA (Paltiel *et al.*, 2009). However, this potentially significant public health benefit requires a very high PrEP efficacy and the benefit might be lost or substantially reduced with if the PrEP efficacy is below 50%.

In addition, there are several potential problems with long-term exposure to oral ART: systemic toxicity, the potential emergence of resistant HIV strains, problems of compliance with treatment; non-compliance in particular is likely to greatly reduce efficacy.

Therefore, prevention strategies, like vaccination and the use of microbicides, which could be used on a large scale (especially in low income countries), at low cost and without any risk of long-term toxicity, are still needed.

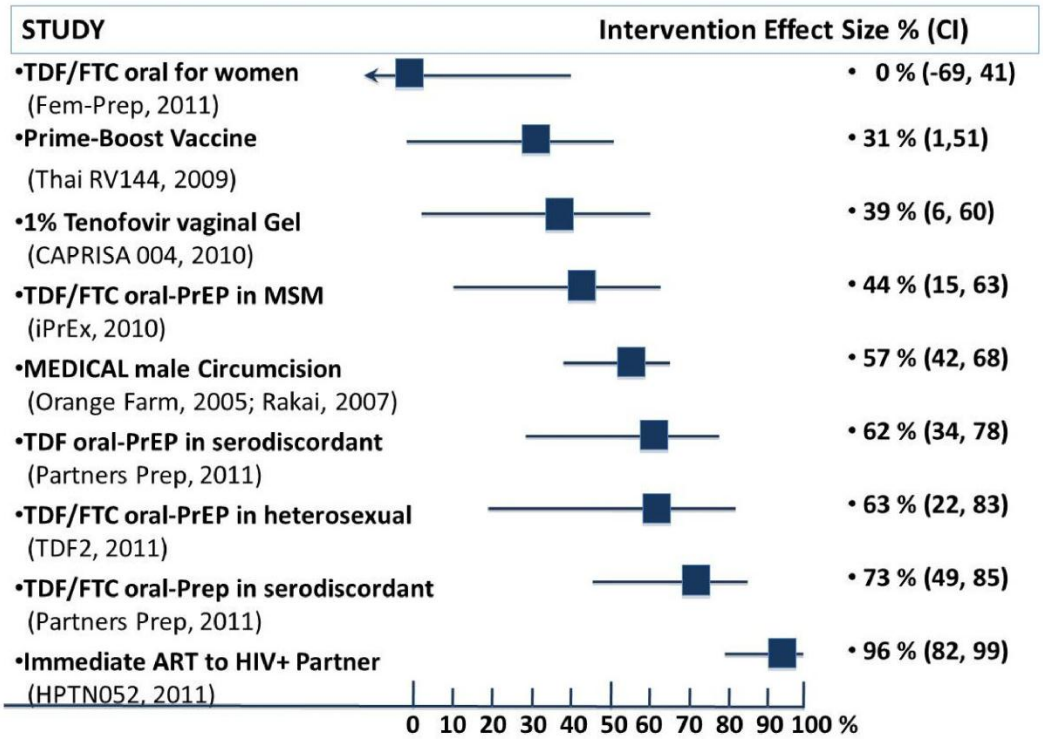

Figure 24 : Synthesis of the efficacy of different strategies developed in prevention of HIV transmission. Adapted from (Ruffin *et al.*, 2012).

Animal models are essential for the process of developing such preventive strategies. Both humanized mice and NHP models have been powerful tools for testing the efficacy of vaccines and microbicides during pre-clinical trials; however, several discrepancies have emerged between the efficacy observed animal models and that reported in human clinical trials. They could be due to the limitations of the various models (differences in the physiology and structure of mucosal tissues, a lower incidence of genital inflammation in animal models than in humans, for example). Indeed, SIVmac251 is genetically more similar to HIV-2 than to HIV-1 strains.

Another possible explanation is that the viral inoculum used in many studies with mice or macaques is most of time incomplete, and includes only cell-free particles produced in culture supernatants.

More generally, the animal models currently used do not reflect many of the factors associated with mucosal transmission of HIV/SIV:

- The complexity of the molecular composition of semen
- The pH environment at the time of exposure
- The specificity of the phenotype and genotype of virus present in semen and produced from the mucosa
- The changes to exposed mucosa with time

- The co-factors, which include local microflora and pathogens
- The immune response to HIV/SIV, including the presence of antibodies in semen which may limit or enhance transmission of opsonized viruses
- The diversity of viral forms, which include both cell-free particles and cell-associated virus

The following section addresses the evidence for the hypothesis that incomplete protection provided by current prevention strategies might be the consequence, in part, of inefficacy against infected cells in the inoculum. If it is confirmed that cell-associated virus is involved in, or even responsible for, HIV transmission, it will become an important issue for the design more efficient prophylactic drugs and approaches.

B. Targeting of cell-associated virus in HIV transmission prevention

1) Efficacy of the prevention strategies developed against cell-associated virus

Some of the prevention strategies developed and tested in animal models and clinical trials may be effective against cell-associated virus.

Clinical trials indicate that **PrEP** is the most effective approach developed so far to reduce HIV incidence, with a protection rate ranging from 44% to 77.9%. The association of TDF and FTC, two inhibitors of the viral reverse transcriptase, successfully blocks the viral lifecycle at an early step. The treatment is administered systemically, so it can reasonably be assumed that most of the HIV target cells in the mucosa are protected from infection by either cell-free or cell-associated virus. As a proof of concept, it has been recently demonstrated *in vitro* that antiretrovirals, like zidovudine and TDF, inhibit viral replication after cell-to-cell transmission (Permanyer *et al.*, 2012).

The second most successful prevention strategy (with the exception of the protection conferred by male circumcision) is the topical application of a **1% tenofovir gel**. However, the protection is limited, with a maximum of 54% in good adherers. These findings differ from those obtained with NHP models and humanized mice, which report high, if not complete protection. One major limitation of these models is that the viral challenge used to test the efficacy of the gel was limited to cell-free virus stocks. So to date, there is no experimental

evidence of whether topical tenofovir application provides significant protection against a complete viral inoculum, containing both free particles and infected cells.

TDF inhibits viral replication before the integration of provirus into the host genome, and is therefore expected to confer protection against infection if two conditions are fulfilled: 1) the bioavailability of the drug is high and uniformly distributed within the exposed mucosal tissue; 2) that no HIV-positive leukocyte succeeds in disseminating rapidly to the draining lymph node, thereby escaping the action of local TDF. However, cell-to-cell virus spread has been demonstrated recently as less sensitive to antiretrovirals like TDF and efavirenz than cell-free virus transmission in an *in vitro* coculture system (Sigal *et al.*, 2011). These observations need to be confirmed *in vivo*.

Various microbicides have been developed, and several have been tested for their anti-cell-associated virus activity in small animals (except in NHP models). First, Di Fabio *et al.* demonstrated that pre-exposure topical application of a gel containing a non-nucleoside reverse transcriptase inhibitor (NNRTI) drug, **TMC120**, was able to fully protect humanized mice against inoculation of the vagina with infected cells (Di Fabio *et al.*, 2003).

Moench *et al.* reported in a Feline Immunodeficiency Virus (FIV)-cat model that the topical application of a gel containing the detergent **Nonoxynol-9** conferred total protection against inoculation with a large number of infected T cells either by the vaginal or rectal route (Moench *et al.*, 1993). N-9 is indeed able to lyse genital leukocytes *in vitro* (Hill and Anderson, 1992).

BufferGel® has been reported to inhibit cell-associated vaginal transmission in pre-treated humanized mice (Olmsted *et al.*, 2005). Indeed, low pH immobilizes and kills leukocytes, thereby preventing them contributing to virus transmission.

Carrageenan has been found to prevent macrophage trafficking from the vaginal cavity to lymph nodes and spleen in mice (Perotti *et al.*, 2003).

However, none of these microbicide candidates have been demonstrated to be effective in human clinical trials.

Recently, Hope *et al.* reported promising results with a synthetic mucin-like polymer system. This system is designed to mimic certain characteristics of the cervical mucus that may be advantageous for microbicide application, without being negatively affected by the diluting and buffering action of seminal plasma (Mahalingam *et al.*, 2011). The two major components, phenylboronic acid (PBA) and salicylhydroxamic acid (SHA), form a transient network at normal vaginal pH. The crosslinking density of this network, and thereby its barrier

properties, increase as the pH increases from the vaginal pH (~ 4.0–5.5) to the seminal pH (~7.5). This mechanism provides a physical barrier that impedes the transport of macrophages, and virions, from semen to the exposed mucosa in *in vitro* and *ex vivo* models (cervico-vaginal explants).

2) Additional prevention strategies targeting cell-associated virus

Two of the various mechanisms by which cell-associated virus could transmit infection after mucosal exposure may counteract the benefits of topical microbicides:

- **Transmigration** of infected leukocytes through the mucosal surface and dissemination to sites that are not reached by the drug,
- Formation of a **virological synapse** between an infected leukocyte and a epithelial resident antigen-presenting cell (Langerhans cell, DC-SIGN$^+$ DC or macrophage), leading to a transfer of virions to the APC through lectins and migration of the APC to the regional lymph node for cross-presentation of the antigen. Also, trancytosis of virions through the epithelium and their uptake by underlying APCs may be triggered by virological synapses between the infected leukocyte and epithelial cells.

Therefore, these putative mechanisms of HIV transmission need to be taken into consideration in the development of microbicide and vaccine approaches. A prophylactic agent could neutralize the infected cells in several ways, either by eliminating or at least blocking the infected cells before they come in contact with the epithelial surface, or by specifically inhibiting their interactions, particularly the virological synapse, with epithelial cells and/or target cells (Figure 25).

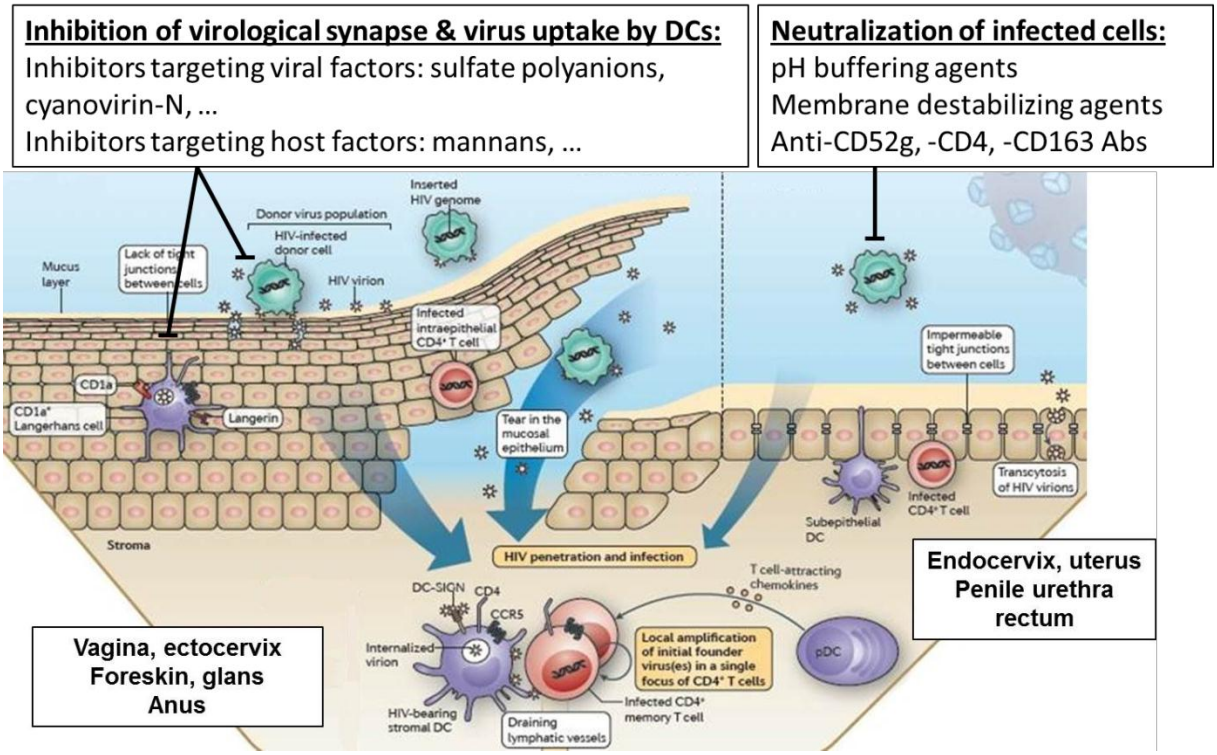

Figure 25 : Potential mechanisms of action targeting cell-associated virus of future microbicide strategies. Adapted from (Bhardwaj, 2012).

a. Neutralization of infected cells

Most of the detergents and membrane-destabilizing agents that have been developed fail to demonstrate effective protection in clinical trials, and indeed N-9 even appeared to have deleterious effects. Similarly, pH-buffering agents, like BufferGel®, have not given encouraging results. More work is needed to develop agents which can inactivate both cell-free and cell-associated virus, without causing any damage to the underlying mucosa, like the synthetic mucin-like polymer system described above (the efficacy of which has not yet been tested *in vivo*).

Good results against free particles, but not cell-associated virus, have been obtained with topical application or infusion of neutralizing antibodies targeting HIV *env* or the CD4 binding site (Abela *et al.*, 2012; Zhong *et al.*, 2013). Similar strategies involving topical application of a cocktail of Abs targeting leukocytes may neutralize infected cells; relevant antibodies include anti-CD45, anti-CD4 and anti-CD163 Abs, as well as anti-CD52g, CD52g being a seminal plasma antigen found on the surface of all cells in semen (Isojima, 1989).

CCR5 inhibition agents, including CCR5 analogs, and Abs targeting CCR5, may influence the migration profile of inoculated infected cells, and thereby limiting the risk of their dissemination through mucosal tissue (Anderson *et al.*, 2010a) (Figure 26).

However, agents with anti-inflammatory properties, for example glycerol monolaurate that decreases the production of chemotactic molecules, may reduce the risk of migration of inoculated infected leukocytes through the epithelial surface.

b. Inhibition of the formation of the virological synapse

The virological synapse (VS) is a zone of close contact between an infected cell and an uninfected target cell, both being $CD4^+$ T cells, macrophages or DCs. As in immunological synapses, cells interact with each other through integrins and their receptors (such as LFA-1 with ICAM, Mac-1 with JAM-C), and CD4 membrane proteins in the target cells bind with the viral *env* on the surface of the infected cell (Sattentau, 2008) (Figure 26). This close interaction initiates the budding of newly produced virions and constitutes the most efficient route for HIV transmission. Interestingly, it has been reported that cell-to-cell HIV transmission via the VS is resistant to the action of neutralizing antibodies targeting HIV (Chen *et al.*, 2007).

Thus, to prevent HIV transmission via cell-to-cell contacts, the initial formation of the VS must be prevented. Specific Abs targeting major components of the VS, for example, Abs targeting LFA-1, Mac-1, ICAM and JAM-C, may inhibit its formation (Anderson *et al.*, 2010a). Abs specifically recognizing CD4 may also help prevent the formation of VS, in addition to initiating leukocyte neutralization. Effective neutralizing Abs, with broad specificity and targeting HIV *env*, may both neutralize cell-free particles and also inhibit VS formation by binding to the proteins expressed on the surface of infected cells (Figure 26).

Also, the attachment of infected leukocytes to epithelial cells, provoking *in vitro* and *ex vivo* transcytosis of virions through the epithelium, must be prevented by inhibitor agents. Aptimers and dendrimers are currently being developed for this purpose (Mumper *et al.*, 2009; Witvrouw *et al.*, 2000).

Finally, CCR5 analogs, such as PSC-RANTES, Abs targeting CCR5 and fusion inhibitors may also inhibit cell-to-cell transmission (Figure 25).

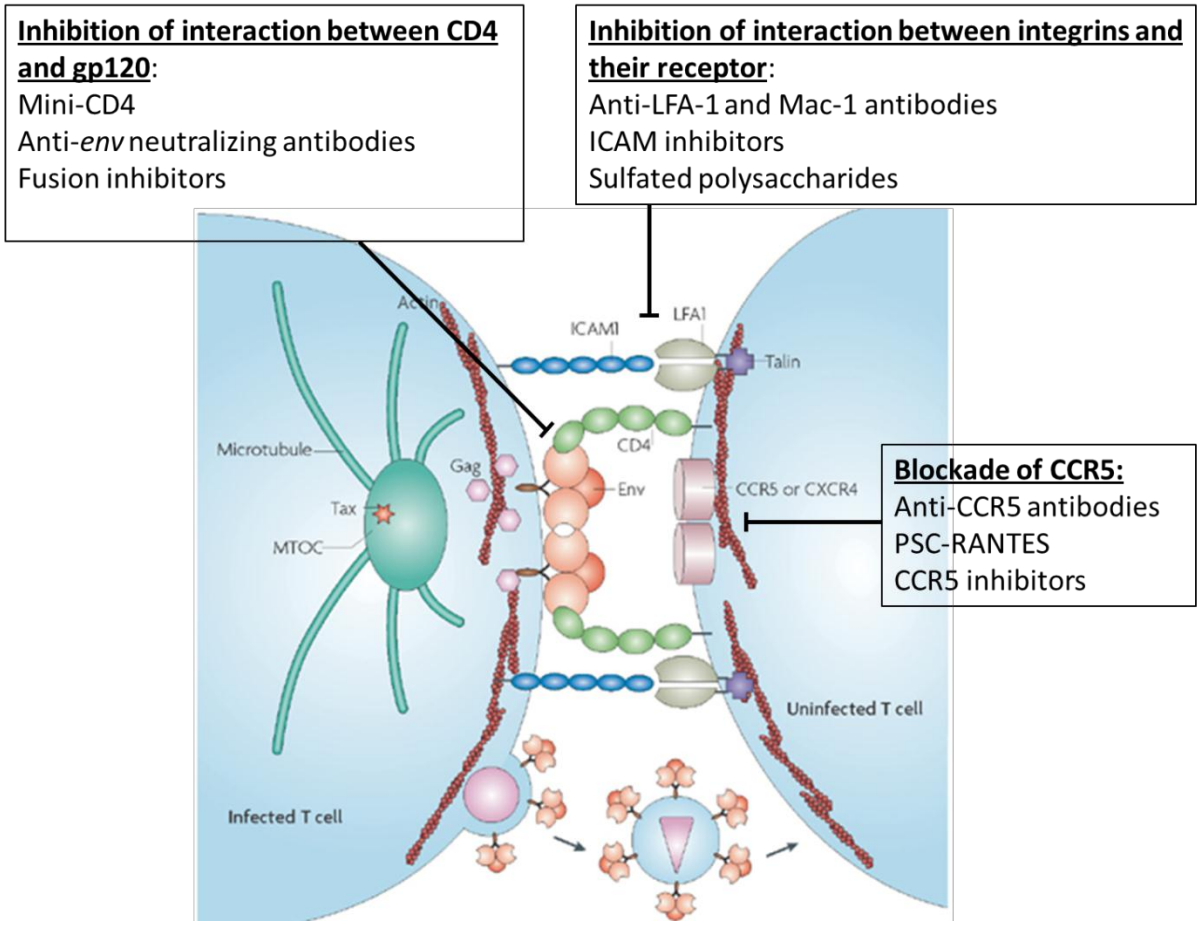

Figure 26 : Potential mechanisms for targeting and preventing cell-to-cell transmission of HIV for putative microbicide strategies. Adapted from (Sattentau, 2008).

To conclude, there is still much work to do to identify and develop potential inhibitors of HIV transmission, especially molecules targeting cell-to-cell transmission; there are still many candidate that remain to be tested. However, for the vast majority of the new prevention strategies currently being developed and evaluated, the issue of the their efficacy against cell-associated virus has not been adequately considered or tested. Therefore, there is an urgent need for novel, physiologically relevant models for studying cell-associated HIV transmission, and in particular for evaluating, during the pre-clinical phase, the efficacy of novel strategies for prevention.

Finally, the scale and geographic distribution of the HIV pandemic is such that products and agents used for its control have to be cheap, and compatible with large-scale production. This may be a limitation for the development and implementation of some these possible strategies (notably the use of Abs as microbicide components).

Objectives

I. Objectives of the study

Over the past decade, numerous studies have been conducted to understand the mechanisms of HIV mucosal transmission. Many potential mechanisms have been proposed, and pioneer works on NHP models helped to detailed description of the earliest steps of infection (Haase, 2011; Li *et al.*, 2009; Miller *et al.*, 2005; Miller and Shattock, 2003; Veazey *et al.*, 1998; Veazey *et al.*, 2001; Veazey *et al.*, 2000b), impossible to investigate in human for obvious ethical reasons. As emphasized in the Introduction Chapter, here is growing evidence that infected cells may play an important role in HIV mucosal transmission.

Cell-associated virus is present in 50 to 75% of the ejaculates of infected men, even under ART (Bagasra *et al.*, 1994; Ball *et al.*, 1999; Dulioust *et al.*, 1998; Mermin *et al.*, 1991; Quayle *et al.*, 1997; Tachet *et al.*, 1999; Xu *et al.*, 1997). However little is known about the nature of these infected cells and their dynamics during HIV infection. On the other hand, many studies have demonstrated that infected leukocytes are able to transmit infection, *in vitro*, *ex vivo* and, most of all, *in vivo* (Anderson *et al.*, 2010a; Salle *et al.*, 2010; Zacharopoulos *et al.*, 1997).

However, all the experiments conducted so far in NHP models of HIV/AIDS have been using blood cells (PBMCs), spleen cells or even cell lines. These cells may not reflect the nature and biology of infected leukocytes present in semen. In addition, viral quasispecies seeding the seminal plasma or harbor by semen leukocytes may significant differ from PBMC isolated SIV strains or laboratory adapted viruses commonly used in challenge studies.

To date, little is known about the nature and the phenotype of the immune cells that are present in the semen of HIV-infected men, and the direct role of semen infected leukocytes has not been directly investigated *in vivo* yet.

We therefore defined different objectives for this work:

- To monitor viral shedding in semen in parallel to blood in SIV-infected macaques to compare our experimental model with the virological parameters of HIV infection;
- To describe the different immune cells that are present in the semen of cynomolgus macaques, to phenotype those that are potential targets for HIV/SIV ($CD4^+$ T cells and macrophages mainly) and investigate whether they are affected by SIV infection;

- To identify the nature of the infected leukocytes present in the semen of SIV-infected macaques at every stages of infection and quantify the number of viral DNA copies present in these cells;
- To investigate their infectivity *in vitro*, by co-culturing semen leukocytes with cells that are permissive to SIV infection;
- Finally, to test their capacity to transmit infection *in vivo*, by inoculating, rectally or vaginally, sorted semen leukocytes of SIV-infected macaques to male and female macaques.

Another objective of our work was to investigate the presence of SIV-specific adaptive responses (antibodies and $CD8^+$ T cells) in the semen of infected macaques and their potential role in mucosal transmission of the virus. In parallel to the SIV-specific expression of certain molecules by T cells, we measured seminal plasma levels of 28 cytokines, chemokines and growth factors, in order to study the inflammatory state which is associated with SIV infection in the semen.

II. Experimental approach

A. Infection of cynomolgus macaques with SIVmac251

1) Use of cynomolgus macaque in HIV/AIDS research

We used here the experimental model of the infection of cynomolgus macaques with SIVmac251 to study the seminal virological and immunological parameters associated with HIV infection. Animals were at different stages of infection and displayed a large diversity of profiles in viral shedding. We also performed a longitudinal follow-up of SIV-infected macaques, from the first week after infection to the chronic stage.

Asian macaques, among them rhesus macaques (*Macaca mulatta*), pigtail macaques (*Macaca Nemestrina*) and cynomolgus macaques (*Macaca* fascicularis), have become the most commonly used and widely accepted models for studying the transmission and the pathogenesis of HIV/SIV infection, as well as for the pre-clinical safety and efficacy testing of candidate antiretrovirals, vaccines and microbicides (Anderson, 2010; Brenchley *et al.*, 2010; Haigwood, 2004; Hatziioannou and Evans, 2012; Karlsson *et al.*, 2007; Mannioui *et al.*, 2009; Miller, 1994; Miller *et al.*, 1989; Veazey *et al.*, 2012).

Differences exist between these different macaque species, regarding the pathogenicity of SIVmac251 (or SIVmac239, close to SIVmac251) and the progression to AIDS.

- **Rhesus macaques of Indian origin and pigtail macaques** are the most utilized NHP model for AIDS, especially in the USA. Initially, the SIVmac251 strain has been isolated from rhesus macaques of Indian descent and is thus particularly well adapted to these animals. SIV infection results in high viral loads and a rate of disease progression particularly rapid, with minimal animal-to-animal variation (Hatziioannou and Evans, 2012; Ling *et al.*, 2002; Marthas *et al.*, 2001).

- **Rhesus macaques of Chinese origin and cynomolgus macaques** are more commonly utilized in Europe. SIV infection results in both species in a different pathogenic profie, with lower setpoint viral loads (Figure 27) and slower progression of CD4+ T cells decrease (Hatziioannou and Evans, 2012; Karlsson *et al.*, 2007; Ling *et al.*, 2002; Mannioui *et al.*, 2009; Marthas *et al.*,

2001). Although rapid progressing models may be more appropriated to give results in NHP compatible with lab constrains, we may prefer slower progressing models which certainly better mimics HIV infection and AIDS in humans.

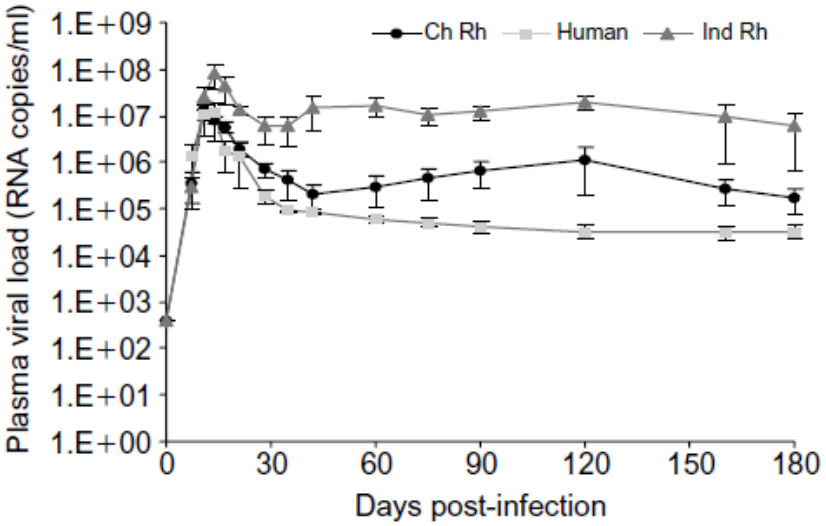

Figure 27 : Blood plasma viral RNA loads in rhesus macaques of Chinese and Indian origin infected with SIVmac239 and in HIV$^+$ individuals. Adapted from (Ling *et al.*, 2002).

Cynomolgus macaques of Mauritius origin that we used for this study display the immune-genetic particularity to descend from a small founder population, resulting in a limited MHC diversity (only 7 MHC haplotypes identified) and thereby in a certain homogeneity in infection profiles, particularly during primary stages (Burwitz *et al.*, 2009; Pendley *et al.*, 2008; Wiseman *et al.*, 2007). However, heterogenic profiles in viral replication control is observed in chronically infected Mauritius cynomolgus macaques, similar to what is reported in HIV$^+$ men (Aarnink *et al.*, 2011a; Aarnink *et al.*, 2011b; Karlsson *et al.*, 2007; Mee *et al.*, 2009).

2) Use of the pathogenic strain SIVmac251

The experimental infection of macaques with the pathogenic strain SIVmac251 has been extensively described as the best model available to date to study HIV pathogenesis and mucosal transmission, as well as to test prevention strategies against HIV-1 infection.

SIVmac strains were first isolated at the New England Primate Research Center, (Massachusetts, USA) from rhesus macaques which had been inoculated in the 1970s with

tissues from SIV-infected sooty mangabeys, which are natural hosts for SIV (Apetrei *et al.*, 2006; Daniel *et al.*, 1985). Many strains have been developed from the original strain, in particular chimeric viruses engineered to have some HIV-1 gene (mostly Env) (Figure 28).

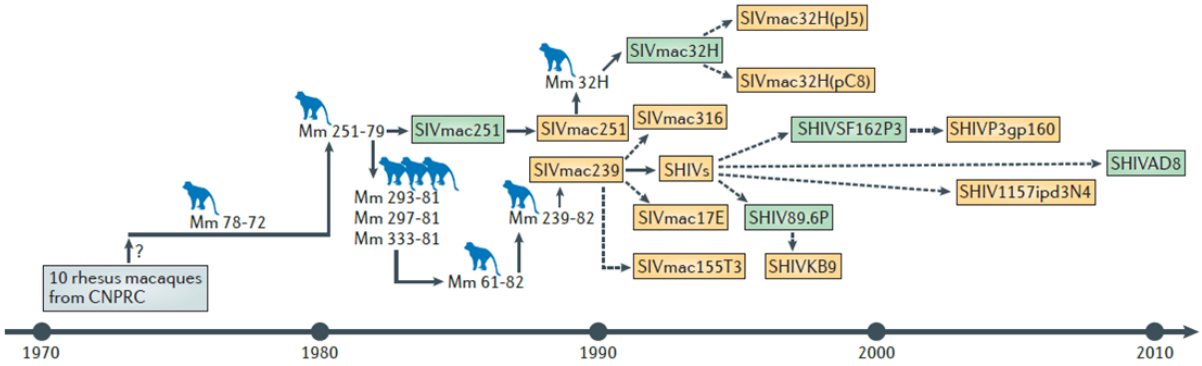

Figure 28 : Origin of the different SIV strains utilized in NHP models for HIV/AIDS. Adapted from (Hatziioannou and Evans, 2012).

SIVmac251, an uncloned strain displaying high genetic diversity like HIV-1, is widely used for mucosal challenges, with rectal, vaginal or exposure, and studying of the infection pathogenesis, whereas SHIV strains are more frequently used for testing prophylactic strategies (Hatziioannou and Evans, 2012; Le Grand *et al.*, 1995; Le Grand, 1993; Miller *et al.*, 1989; Miller *et al.*, 1992a; Veazey *et al.*, 2012).

SIVmac251 is more genetically related to HIV-2 than to HIV-1. In particular, some differences exist concerning the accessory genes (Figure 29) (Hatziioannou and Evans, 2012). Nevertheless, like most naturally transmitted HIV-1 isolates, SIVmac251 uses CCR5 as a co-receptor, replicates predominantly in memory $CD4^+$ T cells and express Env glycoproteins that are resistant to neutralizing antibodies (Hatziioannou and Evans, 2012). Moreover, ongoing replication results in the turnover and progressive loss of $CD4^+$ T cells, mainly in mucosal tissues like gut and genital tract (Karlsson *et al.*, 2007; Le Grand *et al.*, 1995; Le Grand, 1993; Mannioui *et al.*, 2009; Veazey *et al.*, 1998; Veazey *et al.*, 2000a; Veazey *et al.*, 2001).

Commonly used in our laboratory, the virus stocks have been titrated *in vivo* and used many times for mucosal challenges (Karlsson *et al.*, 2007; Le Grand *et al.*, 1995; Le Grand, 1993; Mannioui *et al.*, 2009). These stocks have been obtained in 1989 from a homogenate of splenocytes and PBMCs of a SIV-infected rhesus macaque from the New England Primate Research Center (Le Grand *et al.*, 1995).

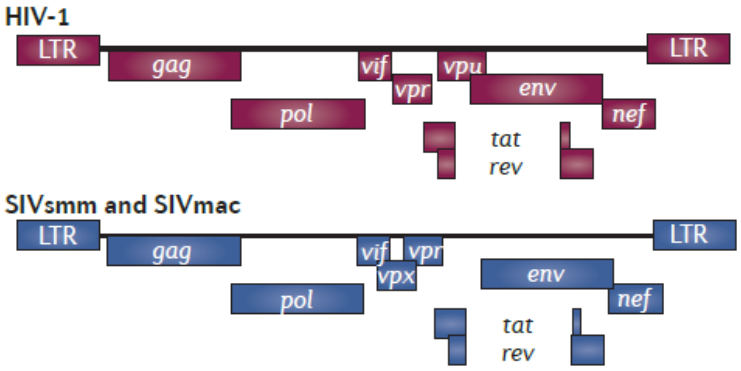

Figure 29 : Genetic discrepancies between HIV-1 and SIVmac strains. Adapted from (Hatziioannou and Evans, 2012).

B. Semen sampling by electroejaculation

Electroejaculation was performed here to collect semen samples. It is a non-invasive method to collect semen samples by endorectal sequential electric stimulations (VandeVoort, 2004). This method is commonly used in the field of animal reproduction, in particular in dog and cat (Okano *et al.*, 2009; Zambelli and Cunto, 2006), but is also used in humans suffering from spinal cord injuries desiring to conceive a child (Soler and Previnaire, 2011).

Some authors reported that the obtained sample volume and the concentrations in spermatozoa may be reduced in comparison to the technique of electro-stimulation (consisting in stimulations using electrodes placed on the penis, performed without anesthesia) (VandeVoort, 2004). However, this technique is largely considered as reliable, reproducible and safe.

Results

I. First article: Semen $CD4^+$ T cells and macrophages are productively infected at all stages of SIV infection in macaques

A. Summary

In this first article, our objectives were 1) to characterize the leukocytes that are present in the semen of macaques, and investigate whether they are impacted by SIV infection; 2) to identify the nature of the infected leukocytes and their dynamics during infection; 3) to assess their infectivity *in vitro*.

First, we validated the relevance of our experimental model in the study of the impact of SIV infection on viral shedding and seminal immune cells dynamics, by measuring by quantitative RT-PCR the viral RNA loads in paired blood and seminal plasmas samples in a large panel of SIV-infected macaques.

We demonstrated, using multi-parameters flow cytometry, that all the HIV/SIV target cells are present in the semen of infected macaques: central memory, activated $CD4^+$ T cells, $CD4^+$ macrophages and dendritic cells, including pDCs. These cells express migration and adhesion markers (CCR5, CXCR4, LFA-1 and Mac-1 integrins), which may facilitate their role in HIV mucosal transmission.

We observed that six pro-inflammatory cytokines and chemokines (IP-10, IL-6, IL-8, RANTES, MCP-1) were increased in leukocytospermic animals, as well as increased during primary infection and/or correlated to blood and semen viral loads, creating an inflammatory state in the seminal plasma which may enhance virus transmission by infected cells.

Finally, we isolated semen $CD4^+$ T cells from HLA-DR^+ antigen-presenting cells (among them, a great majority are macrophages) by flow cytometry sorting. Using quantitative PCR targeting SIV DNA and a co-culture system, we demonstrated that both semen CD4+ T cells and macrophages can be productively infected at all stages of infection, and are infectious *in vitro*.

B. Manuscript

Semen CD4$^+$ T cells and macrophages are productively infected at all stages of SIV infection in macaques

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Running head:

SIV-infected T cells and macrophages in semen

Summary

The mucosal events of HIV transmission have been extensively studied, but the role of infected cells present in the genital and rectal secretions, and in the semen, in particular, remains a matter of debate. As a prerequisite to a thorough in vivo investigation of the early transmission events through infected cells, we characterized in detail the changes in macaque seminal leukocytes during SIVmac251 infection, focusing on T cells, macrophages and dendritic cells. Lymphocytes had a mucosal phenotype and expressed activation (CD69 & HLA-DR) and migration (CCR5, CXCR4, LFA-1) markers. Macrophages predominated at all stages and expressed CD4, CCR5, MAC-1 and LFA-1. Both $CD4^+$ T cells and macrophages were productively infected at all stages of infection. We found that primary infection induced strong local inflammation, which was associated with an increase in the number of leukocytes in semen, both factors having the potential to favor cell-associated virus transmission.

Highlights

- Semen $CD4^+$ T cells and macrophages are productively infected at all stages of infection
- Semen $CD4^+$ T cells are activated and have a mucosal phenotype
- Macrophages express virus receptors, coreceptors and migration markers
- Primary infection induces strong local inflammation associated with leukocytospermia

Introduction

More than 33 million people are currently living with HIV/AIDS worldwide. Almost 80% of new infections occur through sexual intercourse. Semen is thus one of the major factors in HIV transmission. Most studies on HIV sexual transmission have focused on the role of cell-free particles, and the underlying mechanisms of transmission have been extensively described. Moreover, most attempts to develop HIV vaccines and microbicides have focused on blocking cell-free virus transmission. The rectal and vaginal exposure of macaques to free SIV particles has been widely used in studies of the sexual transmission of HIV and evaluations of the efficacy of prophylactic strategies (Haigwood, 2004; Veazey et al., 2012). Most challenge studies use viruses produced *in vitro* in the culture supernatants of human and nonhuman primate (NHP) cells.

However, genital secretions, including semen, contain HIV, in both cell-free and cell-associated forms. The prevalence of proviral DNA in semen ranges from

21% to 65% in HIV-infected patients, and high levels of viral DNA have been associated with high leukocyte counts in semen (Anderson et al., 2010). Moreover, leukocytospermia, the incidence of which is higher in seropositive than in seronegative individuals (Anderson et al., 2010), has been associated with a high degree of semen infectiousness (Politch et al., 2009). This suggests that semen leukocytes may also be an important factor to be taken into account when considering the mucosal transmission of HIV. In the first few years of the HIV epidemic, the hypothesis that HIV could be efficiently transmitted by infected cells, through direct cell-to-cell contact, was proposed, as most retroviruses spread in this way (Anderson and Yunis, 1983).

It is now clearly established that HIV does indeed spread through cell contacts (Alfsen et al., 2005; Groot et al., 2008). Moreover, in reconstituted mucosal models, HIV-infected cells efficiently transmit the virus across epithelial barriers. If the epithelial surface is intact, viral translocation involves transcytosis, which is favored by viral synapses between productive cells and epithelial cells (Bomsel, 1997; Ganor et al., 2010), or direct uptake by local target cells, such as Langerhans cells, macrophages and intraepithelial T cells (Ganor et al., 2012; Hladik et al., 2007). We and others have demonstrated that the vaginal inoculation of humanized mice and macaques with infected leukocytes induces systemic infection. Inoculated CFSE-labeled cells were found in the vaginal tissue and the draining lymph nodes within 21 h, suggesting that these cells were able to migrate through the mucosal epithelium and to disseminate rapidly (Di Fabio et al., 2001; Salle et al., 2010).

We have reported that macrophages and $CD4^+$ T cells present in the various secretory glands of the male NHP genital tract may efficiently seed the semen with free viral particles and infected cells (Le Tortorec et al., 2008). However, semen from

NHP is difficult to collect and process, so previous studies made use of spleen cells from infected animals (our reported work) or *ex vivo* infected PBMCs, as semen cell surrogates (Di Fabio et al., 2001; Kaizu et al., 2006; Salle et al., 2010). These cell-associated virus stocks may not be representative of leukocyte-infected populations in semen. Indeed, semen leukocytes, like mucosal cells, may have a distribution and differentiation and activation phenotypes different from those of blood and spleen cells. Therefore, it is of prime importance to thoroughly investigate the nature and characteristics of the potential cell transmitters in the semen of NHP, since this model is unique to decipher *in vivo* the early events of HIV sexual transmission.

Very few studies have focused on the nature and phenotype of the infected cells present in semen. There is still no formal proof that these cells can transmit HIV across mucosal barriers, and the mechanisms potentially involved have yet to be identified. An understanding of the contribution of semen leukocytes to the sexual transmission of HIV may have significant implications for prevention strategies.

We aimed to carry out a more detailed characterization of the infected cells present in semen, elucidating their role in mucosal transmission, in the experimental infection of macaques with pathogenic SIVmac251 model. We focused on characterization of the semen leukocyte populations, the nature of the infected cells and their capacity to transmit infection *in vitro*. We demonstrated that all major viral target cells, including $CD4^+$ T cells and macrophages are productively infected at every stage of infection.

Results

Virus shedding in the semen of SIVmac251-infected macaques

We assumed that, similarly to men, macaque semen contains various types of infectious material, including free viral particles and infected leukocytes, and that current challenge studies in NHP models of HIV and AIDS do not fully reproduce the conditions of natural transmission. We analyzed the dynamics of free virus shedding in macaque semen, in longitudinal and transverse studies in cynomolgus macaques infected with pathogenic SIVmac251. As in HIV-infected patients, we found a strong positive correlation between blood plasma (PVL) and seminal plasma (SVL) viral RNA (vRNA) loads (Spearman r=0.6381, p=0.0001, n=30) (Baeten et al., 2011; Coombs et al., 1998; Tachet et al., 1999; Vernazza et al., 1997). After the first month of infection, (Fig. 1 A-B), SVL is variable and correlated with PVL. Mean SVL remained systematically equal to, or lower than PVL (Fig. 1 B). However, as observed in humans, discordant profiles are also observed.

Changes in semen leukocytes in SIVmac251-infected macaques

We investigated whether semen leukocytes could be infected and play a role in the mucosal transmission of HIV/SIV, by carrying out a detailed characterization by multiple approaches. We first compared semen cells from uninfected and SIVmac251-infected cynomolgus macaques at various stages of infection.

In uninfected animals, the semen contained various amounts of leukocytes, the numbers of which were strongly correlated with markers of inflammation (Figure 2A). Indeed, leukocytospermic individuals had significantly higher concentrations of inflammatory cytokines, including IP-10 (p=0.0157), MIP1β (p=0.0002), IL-6

(*p*=0.0004), RANTES (*p*=0.0007), IL-8 (*p*=0.0007) and MCP-1 (*p*=0.0089), in seminal plasma. The levels of all these molecules, except MIP-1β, were affected by SIV infection (Suppl. Table 1). IP10, Il-8 and RANTES concentrations were significantly higher in the seminal plasma of macaques during primary infection than in uninfected macaques. The concentrations of IL-8, RANTES, IL-6 and MCP-1 were correlated with PVL and/or SVL.

Macaque semen contains mostly polymorphonuclear cells (PMN, $CD45^+CD11b^+HLA\text{-}DR^-$, 26.02% ± 6.70% of total $CD45^+$ cells), macrophages ($CD45^+CD3^-CD8^-CD11b^+HLA\text{-}DR^+$, 22.22% ± 5.06% of total $CD45^+$ cells) and T cells (8.15% ± 1.94% $CD4^+$ T cells and 8.11% ± 2.36% $CD8^+$ T cells; Figure 2B, Suppl. Fig.1). A small proportion of dendritic cells was also identified, corresponding to a mean of 1.87% ± 1.20% of total $CD45^+$ cells (Suppl. Fig. 1). Inflammation affected the proportions of macrophages and PMN, with a significant increase of the proportion of macrophages among total $CD45^+$ cells (Mann-Whitney test, *p*=0.0022) and a relative decrease in the proportion of PMN (Mann-Whitney test, *p*=0.0769; Fig. 2B).

In infected macaques, leukocytospermia was associated with a higher SVL than in macaques with small numbers of semen leukocytes (Mann-Whitney test, *p*=0.0372; Fig. 2C). In these animals, the number of $CD45^+$ events was strongly correlated with SVL (Spearman correlation, *p*=0.0008, r=0.6858; Fig. 2D). Interestingly, 85.7% (6/7) of infected macaques in the primary phase of infection (10-14 dpi) displayed leukocytospermia (Mann-Whitney test, *p*=0.0062), whereas no significant difference in the number of $CD45^+$ events acquired was found between uninfected and chronically infected macaques (Fig. 2E). The percentages of

macrophages and PMN were not significantly affected by infection, but the T–cell population was significantly modified (Fig. 2F). Semen $CD4^+$ T cells were strongly and persistently depleted (2.50% ± 0.78%), from primary infection onwards (Mann-Whitney test, p=0.0076 and 0.0077, respectively; Fig. 3 C-D). In contrast, the proportion of CD8+ T cells tended to be increased by the infection.

The percentage of $CD4^+$ T cells among total semen T cells was negatively correlated with blood plasma viral load (Spearman correlation, p=0.013, r=-0.5323; Fig. 3A). Conversely, as expected, the proportions of $CD4^+$ T cells among total $CD3^+$ T cells in semen and blood were positively correlated (Spearman correlation, p<0.0001, r=0.679; Fig. 3 B).

Macrophages remained detectable in the semen at all stages of infection, with no significant change to the mean proportion among total $CD45^+$ cells (Fig. 3D-E). However, their frequency in semen was highly variable among individuals (Fig. 3E).

Finally, $CD141^+$ dendritic cells were also present in low quantity in the semen of SIV-infected macaques (Fig. 3F). This population exhibited a tendency to decrease during primary infection (14 dpi), although non significantly, as this analysis could be performed only in 3 animals due to small proportion of these cells in semen (Wilcoxon test, p=0.25, n=3)

We can conclude from this first analysis that all cell types targeted by the virus are present in the semen and that significant changes in the numbers occur in macaques infected with SIV.

Infection of semen $CD4^+$ T cells and macrophages

SIVmac251 DNA, despite being detected at low frequency in total semen DNA extracted from spermatozoa and macaque semen leukocytes, could be amplified by nested PCR with primer pairs binding to *gag* (Supp. Fig. 2). Similar results have been reported for men infected with HIV (Ghosn et al., 2004; Hamed et al., 1993; Krieger et al., 1998; Mayer et al., 1999; Mermin et al., 1991; Quayle et al., 1997; Tachet et al., 1999; Van Voorhis et al., 1991; Vernazza et al., 2000; Xu et al., 1997).

In macaques, sorted $CD4^+$ T cells and macrophages contained SIV-DNA (Suppl. Fig. 3), as demonstrated by quantitative real-time PCR (Table 1). SIV-DNA^+ $CD4^+$ T cells and macrophages were detected as early as 7 dpi, and in 100% of the sorted fractions at 10 dpi. During chronic infection (>90 dpi), SIV-DNA remained detectable but was below the quantification limit (<30 copies). In general, infected cells were more frequently found in the $CD4^+$ T cells fraction and the proportion of infected cells among the total sorted cells was higher than the macrophages fraction.

We also demonstrated, by immunocytofluorescence, that $CD45^+$-enriched fractions of semen leukocytes contained T cells ($CD3^+$) and macrophages ($CD163^+$) harboring SIV proteins (Fig. 4). These results are consistent with real-time PCR results for sorted cells, confirming that both $CD4^+$ T cells and macrophages in semen can be infected with SIVmac251.

Finally, semen leukocytes were found to be productively infected with SIVmac251 during both primary and chronic infection. Transmission could be achieved, albeit with various degrees of efficacy, with both $CD4^+$ T cells and macrophages cocultured with CEMx174 susceptible cells (Suppl. Fig. 4 A-D).

Semen $CD4^+$ T-cell phenotype

As semen $CD4^+$ T cells were found to be productively infected, we investigated their differentiation, activation and migratory profiles, with a view to determining their potential contribution to the mucosal transmission of HIV.

At steady state, most semen $CD4^+$ T cells (94.57%±4.57%) co-expressed $CD95^+$ and $CD28^+$ and could therefore be considered to have a central memory phenotype (Tcm cells, Fig. 5A), whereas such co-expression was less frequent in peripheral blood central memory $CD4^+$ T cells (58.02% ± 8.10, Mann-Whitney test, $p<0.0001$, Fig. 5B). Interestingly, memory $CD4^+$ T cells are the preferential targets of HIV, and these cells are also the principal producers of viral particles (Brenchley et al., 2004; Mattapallil et al., 2005; Veazey et al., 2000a).

CD69 expression on Tcm $CD4^+$ cells is an early marker of activation and proliferation and HLA-DR is a late activation marker. In uninfected macaques, the percentage of $CD69^+$ cells among semen $CD4^+$ T lymphocytes was 26.8 times higher than that among blood cm$CD4^+$ T cells, and the percentage of HLA-DR^+ cells among semen $CD4^+$ T cells was four times that for the corresponding lymphocytes in blood (Mann-Whitney test $p<0.0001$ and $p=0.0002$ respectively; Fig. 5D). Infection with SIV resulted in a significant increase in the proportion of CD69-expressing cells, beginning in primary infection (Mann-Whitney test, $p=0.042$; Fig. 5F). We observed a significant, transient decrease in the proportion of HLA-DR^+ cells among semen $CD4^+$ T lymphocytes at 14 dpi (Wilcoxon matched paired rank test, $p=0.031$), followed by a rapid return to baseline levels.

We also studied the expression of CCR5 and CXCR4, two major coreceptors for HIV/SIV which are also markers of cell migration (Fig. 5G-H). In uninfected macaques, semen T cells expressed both CCR5 and CXCR4 on their surface, with

236 $CCR5^+$ cells more numerous than $CXCR4^+$ cells. A small proportion of double-
237 positive $CCR5^+CXCR4^+$ T cells was also detected (5.512% ± 3.301). In infected
238 macaques, the proportion of $CCR5^+$ cells was significantly higher among $CD4^+$ T
239 cells (1.98 times higher, Mann-Whitney test, p= 0.0022; Fig. 5H). This difference
240 probably reflects the activation of T cells. Intracellular staining showed that all cells
241 were positive for CCR5 and CXCR4, providing evidence of highly regulated surface
242 expression (Suppl. Fig. 5A).

243 Finally, we studied LFA-1 expression in semen T cells. This integrin plays a
244 crucial role in T-cell adhesion to epithelial cells and migration through mucosal
245 tissues. Moreover, LFA-1 has been shown to be an important component of the
246 virological synapse, which mediates the efficient cell-to-cell transmission of HIV-1
247 (Jolly et al., 2004; Rudnicka et al., 2009). LFA-1 is formed by the α-chain CD11a and
248 the β-chain CD18. All $CD4^+$ T cells express CD11a, but only a fraction of these cells
249 express the complete form of LFA-1 (CD11a/CD18; Fig. 5I-J). In our study, most of
250 the $CD4^+$ T cells in semen were $CD11a^+CD18^+$ (60.60% ± 6.18 at steady state), and
251 the proportion of these cells was not affected by SIV infection (Fig. 5J).
252
253 Thus, semen $CD4^+$ T cells were highly activated. They expressed CCR5 and had a
254 memory phenotype. This profile is typical of mucosal cells (Veazey et al., 2000b),
255 and makes semen T cells a potential target for virus infection and replication. These
256 lymphocytes also express migratory and adhesion factors, which might strengthen
257 virological synapse formation and increase the capacity of the cells to migrate
258 towards chemokine-producing tissues and, therefore, the capacity to transmit
259 infection.
260

Semen antigen-presenting cells phenotype

Macaque semen contains a population of antigen-presenting cells (APCs) that is heterogeneous in terms of morphology (using CD45 *versus* SSC-A) and HLA-DR expression (Fig. 3 and Suppl. Fig. 1). We identified two major populations of APCs on the basis of the intensity of CD11b expression: a) a population of HLA-DR^{bright}/CD11b$^{mid\text{-}to\text{-}bright}$ cells, consisting mostly of macrophages, and b) a population of HLA-DR$^{mid\text{-}to\text{-}bright}$/CD11b$^{-\ to\ mid}$ cells, including dendritic cells.

Macrophages were the most abundant HIV/SIV target cell in semen. Variable intensities of CD163, CD14 and CD11b expression were observed (Fig. 6 A), resulting in the definition of three different subsets. The most frequent of these subsets was CD163^{bright}CD14^{bright} cells (31.59% ± 3.51 of CD11b^{+} HLA-DR^{+} cells), 74.27% ± 5.01 of which were CD11b^{bright} (Fig. 6B-C). This profile is typical of activated macrophages. The second subset was CD163^{mid}CD14^{low} cells (27.24% ± 2.39), and the third was CD163^{low}CD14^{-} cells (mean 29.26% ± 3.84). This last subset, which may also include dendritic cells, contained equal proportions of CD11b^{bright} and CD11b^{mid} cells (57.44% ± 6.80 and 53.11% ± 7.06, respectively). The proportion of CD11b^{bright} cells in subset 1 was significantly different from those in subsets 2 and 3 (Mann-Whitney test, p=0.011 and p=0.003, respectively). All semen macrophages expressed CD4 (Fig. 6D-E). We also found no significant difference in macrophages CD4 expression between uninfected and SIV-infected macaques (Fig. 6E).

Interestingly, HLA-DR expression was found to be downregulated at 14 dpi, both on semen macrophages and on CD4^{+} T cells (Suppl. Fig. 6 B, Fig. 3D). By contrast, CD11b had increased (83.70% ± 2.03% of CD11b^{bright}; Wilcoxon test, p=0.0313, Suppl. Fig. 6B). The concentrations of the pro-inflammatory chemokines

MCP-1, MIP-1β and IL-8, which are produced principally by macrophages, also decreased transiently at this time point (Suppl. Fig. 6C). These observations indicate that the acute peak of SVL is accompanied by major changes to the activation and maturation profiles of semen macrophages.

Semen macrophages strongly expressed CCR5 at the membrane, and this expression was not affected by SIV infection (Fig. 6F-G). By contrast, the proportion of $CXCR4^+$ cells was significantly higher in infected than in uninfected macaques (Mann-Whitney test, p=0.011), although this proportion remained low (2.96% ± 0.31). As for $CD4^+$ T cells, almost 100% of semen macrophages contained intracellular CCR5 and CXCR4, indicating high levels of turnover for these molecules (Suppl. Fig. 5B).

Most semen macrophages expressed LFA-1 (76.93% ± 2.79 at steady state). The expression of this molecule was significantly decreased by SIV infection (Mann-Whitney test, p=0.049; Fig. 6H,J). $CD11b^+$ macrophages also expressed Mac-1 (49.08% ± 2.678, Fig. 6I). This integrin is formed by the α-chain CD11b and the β-chain CD18. The mean proportion of double-positive cells was increased significantly (p=0.028) by SIV infection (Fig. 6J).

Discussion

We report here in a macaque model of transmission the first comprehensive characterization of semen leukocytes with the potential to transmit HIV/SIV infection across mucosal barriers. This study was carried out with cynomolgus macaques infected with pathogenic SIVmac251, which is recognized as the most relevant experimental model for studies of HIV infection pathogenesis and mucosal transmission (Lackner and Veazey, 2007; Mannioui et al., 2009; Miller, 1994; Salle et

al., 2010). Macaque semen was found to be very similar to human semen, with similar distributions and phenotypes of semen leukocytes in these two species. Moreover, the dynamics of viral RNA shedding in the semen of SIVmac251-infected macaques was similar to that reported for men. Seminal plasma viral load was correlated with blood plasma vRNA, although some discrepancies were observed in untreated.

Leukocytospermia is frequent in humans and macaques and has been reported to be associated with higher levels of HIV DNA in humans, suggesting that semen leukocytes may contribute to virus transmission across mucosal surfaces (Anderson et al., 2010). We show here that the presence of large numbers of leukocytes in semen is associated with high levels of inflammation markers (IL-6, IL-8, MIP-1β, MCP-1, RANTES and IP-10). Concentrations of these cytokines were correlated with blood and/or seminal plasma viral load. Interestingly, in SIV-infected macaques, leukocytospermic animals had higher seminal vRNA. Moreover, during primary SIV infection, the semen is highly inflammatory and contains large numbers of macrophages, a major target of HIV.

As in other compartments, $CD4^+$ T cells were rapidly and profoundly depleted in the semen of SIV-infected macaques. These cells have a central memory phenotype ($CD95^+CD28^+$) and express CCR5, a profile typical of resident mucosal T cells (Hladik et al., 1999; Prakash et al., 2001; Veazey et al., 2000a). A large proportion of cells expressed CD69, an activation marker observed at all stages of infection. If infected, semen $CD4^+$ T cells should be able to produce replicative viral particles.

Despite the high degree of $CD4^+$ T-cell depletion in semen, SIV DNA was detected in these cells at all stages of infection. Only a few $CD4^+$ T cells could be

sorted from semen, but these cells transmitted the infection when cocultured *in vitro* with a permissive cell line, demonstrating their considerable capacity to produce infectious SIV. However, in the context of chronic infection, $CD4^+$ T cells are present in very small numbers and therefore probably have a very small impact on virus transmission. Further studies are required to confirm this hypothesis.

In our study, semen macrophages formed a heterogeneous population, with different levels of CD163, CD14 and CD11b expression. This population also expressed markers of activation and migration. Interestingly, semen HLA-DR^+ APCs contain SIV DNA at all stages of infection, including the first weeks after infection. Moreover, like T cells, these cells were able to transmit infection to CEMx174 cells when collected at the primary and chronic stages of infection. Thus, semen APCs are also productively infected and produce replication-competent viral particles. Macrophages from chronically infected and leukocytospermic individuals are therefore major candidates for involvement in cell-associated virus transmission.

We also identified other types of APC in semen. This is the first study to report the presence of dendritic cells (DCs), including pDCs, in the semen of infected macaques. More studies are required to characterize semen DCs, particularly for the $CD141^+$ $CD123^-$ subpopulation. As Langerhans cells have been found in the penile mucosa (glans, meatus, foreskin) (Ganor et al., 2010; McCoombe and Short, 2006), they would be expected to be present in semen and their role in the dissemination of viral particles requires investigation.

We found that a large proportion of semen $CD4^+$ T cells and macrophages expressing CCR5, one of the co-receptors of HIV/SIV. The expression of this coreceptor was significantly stronger in infected than in uninfected macaques. This predominance of $CCR5^+$ viral target cells might also account for most of the

transmitted viral founders, after mucosal exposure, being R5 strains. It also provides support for the role of cell-associated virus transmission, because semen leukocytes have a migratory and mucosal profile, and are therefore able to migrate to tissues producing RANTES, MIP-1α and MIP-1β, such as the cervico-vaginal mucosa (Lehner et al., 2000). Furthermore, semen T cells and macrophages express high levels of LFA-1 and/or Mac-1 integrins. Both play an important role in leukocyte adhesion to epithelial cells and transmigration. LFA-1 has also been described as a key player in virological synapse formation and virus transmission (Jolly et al., 2004; Rudnicka et al., 2009).

Taken together, these data suggest that infected semen leukocytes may play a major role in mucosal transmission, even if present in very small numbers. This hypothesis is supported by the observation that infection may be initiated by a very small number of transmission events (Keele et al., 2008; Li et al., 2010; Miller et al., 2005; Salazar-Gonzalez et al., 2009). Mucus, low pH and natural microbicidal molecules secreted by the genital and rectal mucosa may be less effective against infected cells than against free viral particles. In this scenario, semen leukocytes may act as Trojan horses, protecting cell-associated virus from host mucosal defenses (Anderson et al., 2010).

Experimental Procedures

Animals, infection and sample collection

Cynomolgus macaques (*Macaca fascicularis*), weighing 5 to 11 kg were imported from Mauritius and kept according to European guidelines for animal care (*Journal Officiel des Communautés Européennes*, L 358, December 18, 1986). All work on animals was carried out in accordance with institutional guidelines and protocols

approved by the local ethics committee. Adult males were infected by intravenous or intrarectal inoculation with a single dose of 50-5,000 50% animal infectious doses (AID_{50}) of SIVmac251 (Karlsson et al., 2007). Semen and blood were collected from animals sedated by a 5 mg/kg intramuscular injection of Zoletil®100 (Virbac, Carros, France).

Ejaculation was performed by intrarectal electrostimulation of the prostate, with a probe (12.7 mm diameter) lubrified with conductor gel, and an AC-1 electroejaculator (Beltron Instrument, Longmont, USA). Sequential stimulations were performed, with a pattern of 6 cycles, each cycle consisting of nine two-second stimulations followed by a tenth stimulation lasting 10 seconds. The voltage was increased every two cycles (1-3 V for the first two cycles, 2-4 V volts for the third and fourth cycles and 6-8 V for the last two cycles). If a complete ejaculate had not been obtained after six cycles of stimulation, a 7^{th} cycle of stimulation at 7- 10 V was performed. The complete ejaculate was immediately diluted in 1.2 ml of phosphate-buffered saline (PBS) and centrifuged.

Blood sample were collected into BD Vacutainer® Plus Plastic K_3EDTA tubes (BD Biosciences, Le Pont de Claix, France).

Seminal plasma and cell preparation

Seminal plasma was isolated from total semen immediately after collection, by centrifugation for 15 minutes at 775 x *g*. The seminal cell pellet was resuspended in 14 ml of complete medium, consisting in RPMI-1640 Glutamax medium (Invitrogen, Carlsbad, USA) supplemented with a mixture of penicillin, streptomycin and neomycin (Invitrogen) and 10% FCS (Lonza, Allendale, USA), and kept at room temperature for no more than one hour. Cells were then centrifuged for 10 min at

411 1,500 x *g*, filtered through a sieve with 70 µM pores and washed with 5 ml of PBS
412 supplemented with 10% FCS.
413
414 **Blood and semen RNA viral load quantification**
415 Blood plasma was isolated from EDTA-treated blood samples by centrifugation for 10
416 min at 1,500 x *g*, and stored frozen at -80°C. Seminal plasma samples were
417 maintained on ice for no more than one hour and were frozen at -80°C. Semen vRNA
418 was prepared from 500 µl of seminal plasma with the QIAamp UltraSens Virus kit
419 (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. RT-
420 PCR on blood and seminal plasma RNA was performed as previously described
421 (Karlsson, 2007, J Virol). The quantification limit (QL) was estimated at 111 copies/ml
422 and the detection limit (DL) was estimated at 37 copies/ml. Samples from chronically
423 infected macaques on antiretroviral treatment (ART) were treated in the same way,
424 increasing the amount of plasma to increase sensitivity (QL and DL of 37 and 12.3
425 copies of vRNA/ml, respectively).
426
427 **Phenotypic characterization of semen leukocytes**
428 Staining was performed on either whole blood or PBMC. Except for whole-blood
429 assays, staining was performed after the saturation of Fc receptors by incubation
430 with healthy macaque serum (produced in-house) for 1 h at 4°C. Amine-reactive
431 Live/dead® Fixable blue dye (Life Technologies) was used to assess cell viability and
432 to exclude dead cell from the analysis. Cells were stained with monoclonal antibodies
433 by incubation for 30 min at 4°C, washed in PBS/10% FCS and fixed in CellFIX^{TM} (BD
434 Biosciences). Five different antibody panels were used (see Supplementary Table 2).
435 Corresponding isotype controls for CD163, CD14, CCR5, CXCR4, CD11a and CD18

were used at the same concentrations as the reference antibody. Semen sample were considered leukocytospermic if it was possible to acquire a minimum of 50,000 $CD45^+$ events per ml on flow cytometry. Acquisition was performed on a BD LSRII machine equipped with four lasers (355, 405, 488 and 633 nm), with Flowjo v9 (Tree Star, Ashland, OR) used for analysis.

Sorting of semen $CD4^+$ T cells and antigen-presenting cells

Total semen cells were filtered and washed (see above), and then incubated for 15 min at 4°C with 20 µl of anti-CD45 magnetic beads (Miltenyi Biotec) and washed once with 2 ml of cold PBS supplemented with 0.5% BSA and 2 mM EDTA (sorting buffer). The $CD45^+$ cell fraction was then enriched by magnetic bead sorting, with LS columns (Miltenyi Biotec), used according to the manufacturer's instructions. Cells were eluted in 4 ml of sorting buffer. Cells were stained as described above, using Live/Dead® dye to identify the dead cells, and the same antibodies as for leukocyte phenotyping: CD45, CD3, CD8, CD4 and HLA-DR. Cells were washed twice and stored at 4°C in PBS/10% FCS. Following the magnetic bead-based enrichment process, $CD4^+$ T cells and HLA-DR^+ APCs were sorted by simultaneous four-way sorting on a FACSAria flow cytometer (BD Biosciences). The enrichment of cell fractions was estimated by flow cytometry with BDDiva (BD Biosciences) and FlowJo software (TreeStar).

Detection of SIV DNA in the cellular components

After semen collection and the separation of the cellular components from the seminal plasma, the cells were centrifuged and the cell pellet was kept at -80°C until further tests. Nested PCR targeting SIVmac251 *gag* was performed as previously

described (Benlhassan-Chahour et al., 2003). Twenty tests were carried out for each semen DNA sample. A second amplification was then carried out, as we previously described (Le Torterec et al).

After the sorting of $CD4^+$ T cells and macrophages, the cells were washed once in PBS and centrifuged for 10 min at 1,500 x *g*. The supernatant was discarded and the dry cell pellets were frozen at -80°C. PCR was derictly performed on cell lysates as we previously describe (Mannioui et al). The number of cells was determined by amplifying the CCR5 gene (see Supplementary Table 3) with serial 10-fold dilutions of SIV-negative PBMCs (starting with 1 million cells) as a standard and a lysate of SIV-negative PBMCs and nuclease-free water as the negative control. SIV DNA was quantified with serial dilutions over five orders of magnitude of a pCR4-TOPO plasmid (Invitrogen) containing the SIVmac251 *gag* cDNA sequence and diluted in SIV-negative PBMCs as the standard. The quantification threshold was 30 SIV-DNA copies and the detection threshold varied between 1 and 10 copies of SIV-DNA.

Coculture of sorted semen leukocytes with CEMx174

After sorting, semen $CD4^+$ T cells and macrophages were washed once with 10% FCS in PBS and transferred to a U-bottomed 96-well plate. CEMx174 cells were added to each well, the number of cells added being three times the number of sorted cells. If this number was below 10,000 cells, we added a minimum of 50,000 CEMx174 cells per well. Cells were cultured in a final volume of 250 µl of complete medium (see above), at 37°C, under an atmosphere containing 5% CO_2. The positive control was CEMx174 cells cultured with 50 µl of highly concentrated SIVmac251 particles, and the negative control was CEMx174 cells alone. Cells were passaged

three or four days after the initiation of the coculture and then every two or three days thereafter. Coculture was stopped after 8 days. At each passage, half the cell suspension was replaced with fresh medium. Supernatants and cell pellets were cryopreserved at -80°C until further analysis. SIV-DNA and SIV-RNA were quantified as described above.

Immunofluorescence staining of cytospun semen cells

Cells were washed once in PBS, then diluted in a maximal volume of 450 µl and cytospun on Superfrost slides at 500T for 10 min, in a Cytospin 4 Cytocentrifuge (Thermo Shandon, Thermo Fisher Scientific, Waltham, USA), at a concentration of 200,000 cells per spot. Cytospun cells were allowed to dry at room temperature for two hours and were then fixed by incubation in a mixture of 50% ice-cold methanol and 50% ice-cold acetone for 15 min. Slides were then allowed to dry at room temperature for 20 min and frozen at -20°C for later use.

Before staining, the slides were thawed and allowed to dry at room temperature for 20 min. They were then washed three times, for 5 min each, in PBS. Cells were permeabilized by incubation with 0.025% Triton in PBS for 5 min and the slides were then washed three times, for five minutes each, in PBS. Non specific binding sites were saturated by incubation for 1 hour at room temperature with 5% BSA and 10% macaque serum in PBS. Immunofluorescence staining was performed with the following antibodies: anti-CD3 Alexa Fluor 700 (BD Biosciences, clone SP34-2), anti-CD163 Alexa Fluor 488 (BD Biosciences, clone GHI/61) and anti-SIV nef (NIBSC, Center for AIDS Reagents, clone KK75) and anti-SIV gp41 (NIBSC, Center for AIDS Reagents, clone KK7a) both coupled with Alexa Fluor 594, in 10% BSA/0.05% Tween 20 in PBS, for 30 min, at room temperature, in a dark chamber.

511 Slides were washed three times with 0.05% Tween 20 in PBS and three times in
512 PBS alone. Finally, slides were mounted in ProLong® anti-fade mounting medium
513 (Invitrogen) and stored at 4°C until further analysis.
514 Images were acquired with a Leica confocal microscope (equipped with 4
515 lasers) and analyzed with ImageJ® software. The final images were generated with
516 Adobe Photoshop®.
517
518 **Cytokine quantification in semen**
519 Concentrations of IL-6, IL-8, MIP-1β and MCP-1 in seminal plasma were determined
520 with the Milliplex® Map Non-Human Primate Cytokine Magnetic Bead Panel -
521 Premixed 23-plex (Merck Millipore, Darmstadt, Germany). The concentrations of
522 RANTES and IP-10 were determined with a 2-plex Milliplex kit. Assays were
523 performed in duplicate, with 25 µl of seminal fluid. Samples were thawed at room
524 temperature and centrifuged for 10 min at 1,500 x *g* to harvest any cellular
525 components. Immunoassays were performed according to the manufacturer's
526 instructions. Data were acquired with a Bio-Plex Instrument 200 and analyzed with
527 Bio-Plex Manager Software version 6.1 (Bio-Rad, Hercules, USA).
528
529 **Data visualization and statistical analysis**
530 All data visualization and statistical analyses were carried out with GraphPad
531 Prism 5.03 software (GraphPad software, La Jolla, USA). Nonparametric Spearman's
532 rank correlation tests were used to investigate the relationships between parameters.
533 The nonparametric Mann-Whitney test was used to compare groups of macaques,
534 and the nonparametric Wilcoxon rank sum test was used to compare data from same
535 macaques at different time points, before and after SIV infection. *P* values of 0.05 or

536 less in two-tailed tests were considered significant, *:$p<0.05$, **: $p<0.01$, ***: $p<0.001$,
537 ****: $p<0.0001$.

538

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551

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744

Figure legends

Figure 1: Viral shedding in blood and semen in SIVmac251-infected macaques

(A-B) Longitudinal follow-up of RNA viral loads in blood (B) and seminal plasma (C) in 8 macaques infected intravenously with high doses of SIVmac251 (5,000 AID_{50}); each animal is represented by different dot. The solid black line indicates the mean PVL. The dotted horizontal line represents the limit of quantification (111 and 37 copies/ml in blood and semen plasma respectively).

Figure 2: Semen leukocytes in SIVmac251-infected macaques

(A) Seminal concentrations of six pro-inflammatory molecules in normal and leukocytospermic animals. (B) Proportion of each studied leukocyte subset among total $CD45^+$ cells. (C) Semen vRNA load in SIV^+ macaques with normal (*n*=11,●) or leukocytospermic (*n*=8,◓) semen. (D) Spearman's correlation between semen RNA viral load and the number of $CD45^+$ acquisition events (*n*=20). (E) Number of $CD45^+$ events acquired by flow cytometry, for control uninfected (*n*=20), and SIV-infected macaques, at 10 and 14 dpi (*n*=7) and during chronic infection. The dotted line represents the leukocytospermia threshold (10,000 $CD45^+$ events acquired) (*n*=13). (F) Proportion of each subset among total $CD45^+$ events in SIV^- macaques (*n*=12,●) and SIV^+ macaques during primary infection (*n*=7,▲) and chronic infection (*n*=9,◆).

Figure 3: Changes in semen T cells, macrophages and DCs during SIV infection

(A) Spearman's correlation between the proportion of $CD4^+$ T cells among total T cells in the semen and PVL (*n*=6 macaques at 14 dpi – ▲, n=15 at chronic stage – ◆). (B) Spearman's correlation between the proportions of $CD4^+$ T cells among total

770 T cells for semen and blood (*n*=6 macaques at 14 dpi – ▲, n=15 at chronic stage –
771 ◆, and n=6 SIV^- macaques). (C) RNA viral loads in blood (dotted line,▲) and semen
772 (dotted line,▼) and changes in $CD4^+$ (solid line,●) and $CD8^+$ T-cell proportions (solid
773 line,■) during SIV infection; mean and SEM are represented (D) Longitudinal follow-
774 up of $CD4^+$ and $CD8^+$ T cells, $CD11b^+$ HLA-DR^{bright} macrophages, $CD11b^{bright}$ HLA-
775 DR^- polymorphonuclear cells and $CD141^+$ dendritic cells, including $CD123^+$ pDCs.
776 Dot plots from a representative animal infected with 5,000 AID_{50} IV (E). Longitudinal
777 follow-up of $CD11b^+$ HLA-DR^{bright} macrophages in 6 macaques infected with 5,000
778 AID_{50} IV; each line represents an animal (H). Longitudinal follow-up of $CD123^+$
779 $CD141^+$ pDCs in 4 SIV^+ macaques.
780
781 **Figure 4: SIV antigens in semen $CD4^+$ T cells and macrophages**
782 Immunocytofluorescence staining targeting CD3 (red), CD163 (green) and SIV nef
783 (pink) on cytospun $CD45^+$-enriched semen cells from SIV-infected and uninfected
784 macaques.
785
786 **Figure 5 : Semen $CD4^+$ T-cell phenotype**
787 (A) D Gating of central memory $CD4^+$ T cells ($CD95^+CD28^{+)}$ (B) Comparison of $CD4^+$
788 T-cell differentiation markers between blood (◓) and semen (●) (*n*=13 uninfected
789 macaques). (C) Gating strategy for CD69 and HLA-DR expression. (D) CD69 (●) and
790 HLA-DR (■) expression in $CD4^+$ T cells in blood (half plain dots) and semen (plain
791 dots) from SIV^- macaques (*n*=13). (E) CD69 (●) and HLA-DR (■) expression in
792 semen $CD4^+$ T cells from SIV^- (*n*=6, empty dots) and SIV^+ macaques, at different
793 stages of infection (*n*=10, plain dots). (F) Longitudinal follow-up of CD69 (solid line,●)
794 and HLA-DR (solid line,■) expression in semen $CD4^+$ T cells during infection (*n*=6).

795 Dotted lines represent PVL (▲) and SVL (▼) (G). CCR5 and CXCR4 expression in T
796 cells from a representative uninfected macaque. From left to right: contour plots
797 representing outliers, overlay of CCR5 and CXCR4 staining (black line) and isotype
798 control (solid gray curve). (H) Comparison of $CCR5^+$, $CXCR4^+$ and $CCR5^+$ $CXCR4^+$
799 $CD4^+$ T cells between SIV^- (*n*=6, ○) and chronically SIV-infected macaques (*n*= 6,●).
800 (I) Gating strategy for LFA-1 integrin expression. From left to right: contour plots
801 representing the outliers, overlay of CD11a and CD18 staining (black line) and
802 isotype control (solid gray curve) (J) Comparison of $CD11a^+$ $CD18^+$ (LFA-1) $CD4^+$ T
803 cells between SIV^- (*n*=6,○) and chronically SIV-infected macaques (*n*=7,●). Mean
804 and SEM are represented.
805
806 **Figure 6: Semen macrophage phenotype**
807 (A) Gating strategy for CD163 and CD14 expression in $CD11b^+$ HLA-DR^{bright} cells.
808 (B) Intensity of CD11b expression by subset, in a representative animal. (C)
809 Proportion of $CD11b^{bright}$ cells in each subset in 5 SIV^- (○) and 4 SIV^+ animals (●).
810 (D) CD4 expression in each subset from a representative uninfected animal. (E)
811 Comparison of CD4 expression in each subset between 4 SIV^- (○) and 6 SIV SIV^+
812 macaques (●). (F) CCR5 and CXCR4 expression in a representative SIV^- macaque.
813 From left to right: contour plots representing the outliers, overlay of CCR5 and
814 CXCR4 staining (black line) and isotype control (solid gray curve). (G) Comparison of
815 $CCR5^+$, $CXCR4^+$ and $CCR5^+$ $CXCR4^+$ macrophages between SIV^- (*n*=6,○) and SIV^+
816 macaques (*n*= 6,●). (H) Gating strategy for LFA-1 integrin expression. From left to
817 right: contour plots representing outliers, overlay of CCR5 and CXCR4 staining (black
818 line) and isotype control (plain gray curve). (I) Gating strategy for Mac-1 integrin
819 expression. (J) Comparison of LFA-1 and Mac-1 expression by macrophages

820 between 6 SIV^- (○) and 8 SIV^+ (●) macaques. SIV^+ macaques were all at chronic
821 stage of infection. Mean and SEM are represented.

822

Animal ID	Days post-infection	Cell type	Number of sorted cells	Number of SIV DNA copies	Frequency of positive test
BB259	7	Macrophages	16,763	<LD	0/1
		$CD4^+$ T cells	4,892	1-30	1/1
BB461		Macrophages	25,288	<LD	0/1
		$CD4^+$ T cells	6,305	<LD	0/1
BB343		Macrophages	2,065	1-30	1/1
		$CD4^+$ T cells	190	1-30	1/1
31047	10	Macrophages	6,250	45.95	1/1
		$CD4^+$ T cells	1,250	680.43	1/1
10999		Macrophages	13,100	1-30	1/1
		$CD4^+$ T cells	2,286	1-30	1/1
29860		Macrophages	27,369	1-30	1/1
		$CD4^+$ T cells	6,494	43.05	1/1
31052		Macrophages	3,759	1-30	1/1
		$CD4^+$ T cells	906	1-30	1/1
30602	28	Macrophages	12,115	<LD	0/1
		$CD4^+$ T cells	1,002	1-30	1/1
30690		Macrophages	30,836	<LD	0/1
		$CD4^+$ T cells	8,430	1-30	1/1
31044		Macrophages	209,059	1-30	1/1
		$CD4^+$ T cells	12,222	66.74	1/1
30717	135	Macrophages	4,359	<LD	1/3
	140		3,862	1-30	
	162		2,853	<LD	
	135	$CD4^+$ T cells	689	1-30	3/3
	140		298	1-30	
	162		759	1-30	
21362R	135	Macrophages	623	<LD	0/3
	140		286	<LD	
	162		1,037	<LD	
	135	$CD4^+$ T cells	73	1-30	2/3
	140		27	<LD	
	162		102	1-30	
30838	244	Macrophages	4,004	<LD	1/3
	249		4,987	<LD	
	271		5,853	1-30	
	244	$CD4^+$ T cells	163	1-30	3/3
	249		130	1-30	
	271		241	1-30	

Table 1: Quantification of SIV DNA copies in sorted semen $CD4^+$ T cells and macrophages at various stages of infection;

LD: limit of detection. The limit of quantification was estimated at 30 copies.

Supplementary figure legends

S1: Gating strategy for semen leukocyte characterization

(A) Exclusion of all events other than those for leukocytes; from left to right, doublets, cell debris and dead cells are excluded and leukocytes are identified with the pan-leukocyte marker CD45. (B) The SSC-A *versus* CD45 gate distinguishes lymphocytes from macrophages and polymorphonuclear cells on the basis of morphology. (C) CD11b and HLA-DR distinguish HLA-DR^{bright} CD11b$^{mid\text{-}to\text{-}bright}$ antigen-presenting cells from CD11b^{bright} HLA-DR$^{negative\text{-}to\text{-}low}$ polymorphonuclear cells. (D) CD3$^+$ T cells are gated against HLA-DR, and CD4$^+$ T cells are separated from CD8$^+$ T cells. (E) Gating strategy for dendritic cell identification: after gating on CD45 and SSC-A, all cells positive for theCD3, CD8, CD20 lineage are excluded and HLA-DR$^{mid–to\text{-}bright}$ CD11b$^{low\text{-}to\text{-}negative}$ cells are gated. Cells negative for CD14 and CD163 are selected, most being CD141$^+$ (BDCA3) dendritic cells, although some are CD123$^+$ pDCs.

S2: Detection of SIV-positive cells in the cellular compartment of whole semen at all stages of infection

(A) Nested PCR, by pre-amplification of the whole SIV genome by PCR, followed by a second step to amplify the SIV *gag* region by real-time PCR (B) Percentage of positive tests in macaques with primary infection (*n*=8) and chronic infection (*n*=14).

S3: Purity of the sorted CD4$^+$ T-cell and macrophage fractions

(A) Control of the purity of the sorted CD4$^+$ T-cell fraction. (B) Control of the purity of the sorted HLA-DR$^+$ macrophage fraction.

S4: Macroscopic evidence for virus transmission and quantification of SIV DNA copies in cocultures of sorted semen $CD4^+$ T cells and macrophages

(A) Negative (culture medium only) and positive (SIVmac251) controls at 8 days of coculture. (B) Coculture with semen macrophages from 1 macaque at 10 dpi (#31047) and 2 macaques with chronic infection (#21362R and 30717). (C) Coculture with semen $CD4^+$ T cells from 1 macaque at 10 dpi (#31047) and 1 macaque at 35 dpi (#31044). (D) Number of SIV DNA copies in cells from cocultures of sorted semen $CD4^+$ T cells and macrophages with CEMx174 cells (log copy number per one million cells). Each dot and line represents one set of conditions.

S5: Intracellular CCR5 and CXCR4 in semen $CD4^+$ T cells and macrophages

(A) Gating strategy for $CD4^+$ T cells. (B) Gating strategy for macrophages.

S6: Changes to semen macrophages during SIV infection

(A) Longitudinal follow-up of the mean fluorescence intensity (MFI) for HLA-DR in infected macaques (at 14 and 35 dpi, data were available for only 4 macaques). (B) Longitudinal follow-up of the proportion of $CD11b^{bright\ and\ mid}$ among total $CD11b^+$ $HLA\text{-}DR^{bright}$ cells (C) Dynamics of seminal plasma MCP-1, Il-8 and MIP-1b concentrations, as determined with Luminex technology. (A-C) Dotted lines represent PVL (▲) and SVL (▼).

Supplementary table 1: Influence of SIV infection on molecules more abundant in leukocytospermic semen

Molecule	Uninfected macaques (pg/ml)		Mann-Whitney test *p* control *versus* primary infection	Correlation with PVL		Correlation with SVL	
	Normal semen	Leukocyto-spermia		Spearman correlation *p*	Spearman correlation *r*	Spearman correlation *p*	Spearman correlation *r*
MCP-1	3,646 ± 1,020	26,775 ± 4,762	0.2475*	0.0099	0.5376	0.5820	0.1211
IL-8	548.00 ± 144.30	7,838 ± 1,997	0.0027	<0.0001	0.7445	<0.0001	0.7494
RANTES	94.29 ± 25.34	355.00 ± 49.12	0.0007	0.0004	0.6857	0.0023	0.6031
IL-6	14.69 ± 4.13	60.20 ± 11.80	0.1765	0.0157	0.5082	0.0033	0.5854
IP-10	0.36 ± 036	15.58 ± 5.412	0.0108	0.144	-0.5046	0.0347	-0.6809

***:** Mann-Whitney test *p* control *versus* chronic infection. Mean and SEM are specified. Normal semen group: n=12, leukocytospermic semen group: n=13.

Supplementary material 1: Antibodies panel for semen T cells and antigen-presenting cells phenotyping

Antibody	Fluorochrome	Clone	Supplier
Backbone (shared by panels 1-4)			
CD45	PerCp	B058-1283	BD Biosciences
CD3	V500	SP34-2	BD Biosciences
CD4	PE-Cy7	L200	BD Biosciences
CD8	V450	BW138/80	Miltenyi Biotec
CD11b	Alexa Fluor 700	ICRF44	BD Biosciences
HLA-DR	APC-H7	G46-6	BD Biosciences
Panel 1: T cell activation and differentiation			
CD69	FITC	FN50	BD Biosciences
CD95	APC	DX2	BD Biosciences
CD28	ECD	25-0289-73	Clinisciences
Panel 2: Macrophages characterization			
CD14	V450	M5E2	BD Biosciences
CD66	FITC	TET2	Miltenyi Biotec
CD163	APC	215927	R&D Systems
Panel 3: CCR5 and CXCR4 expression on T cells and macrophages			
CCR5	APC	3A9	BD Biosciences
CXCR4	PE	12G5	BD Biosciences
Panel 4: LFA-1 and Mac-1 expression on T cells and macrophages			
CD11a	PE	HI111	BD Biosciences
CD18	APC	6.7	BD Biosciences
Panel 5: Macrophages and dendritic cells characterization			
CD45	PerCp	B058-1283	BD Biosciences
CD11b	Alexa Fluor 700	ICRF44	BD Biosciences
HLA-DR	APC-H7	G46-6	BD Biosciences
CD3	V450	SP34-2	BD Biosciences
CD8	V450	BW138/80	BD Biosciences
CD20	V450	L27	BD Biosciences
CD141 (BDCA3)	PE	1A4	BD Biosciences
CD14	FITC	M5E2	BD Biosciences
CD123	PE-Cy7	7G3	BD Biosciences
CD163	APC	215927	R&D Systems

Supplementary material 2: Primers and Taqman probe specific to cynomolgus macaque CCR5 gene

CCR5 gene amplification	
Forward primer	CTG CAG CTC TCA TTT TCC A
Reverse primer	CCC GAG TAG CAG ATG ACC
Probe	ACA AGC AGC GGC AGG ACC AGC C

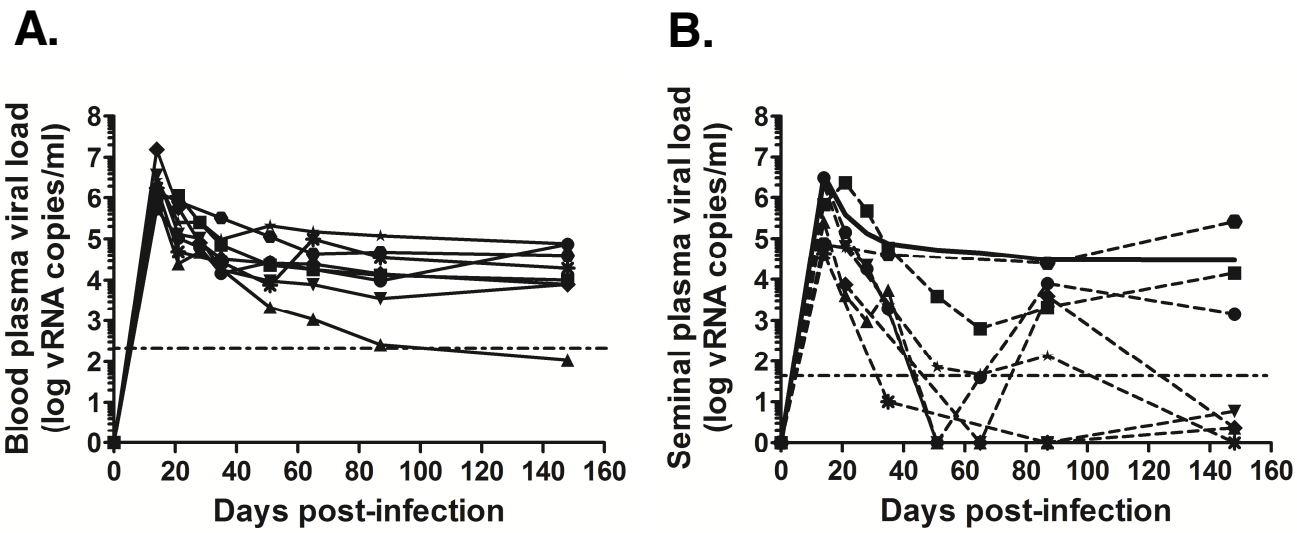


Figure 1: Viral shedding in blood and semen in SIVmac251-infected macaques

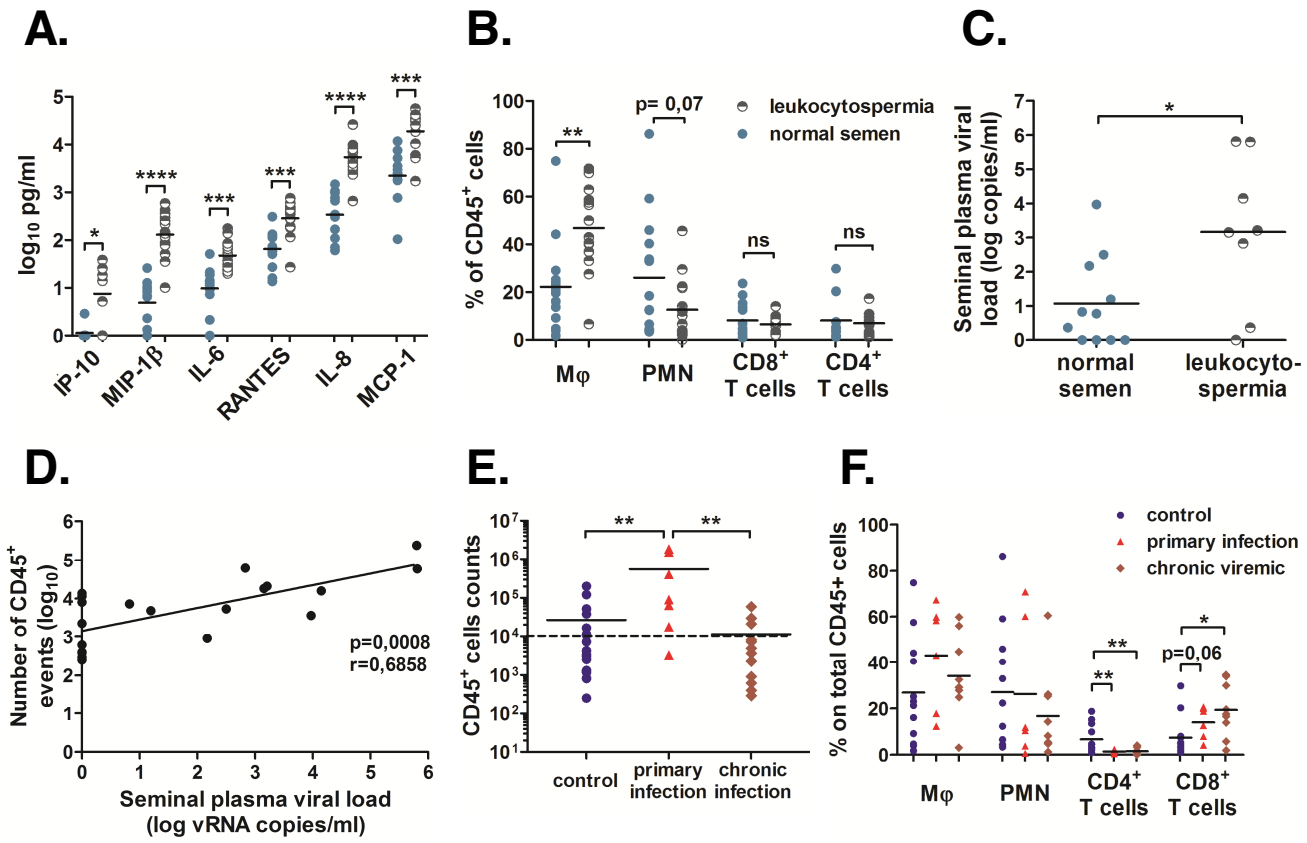


Figure 2: Semen leukocytes in SIVmac251-infected macaques

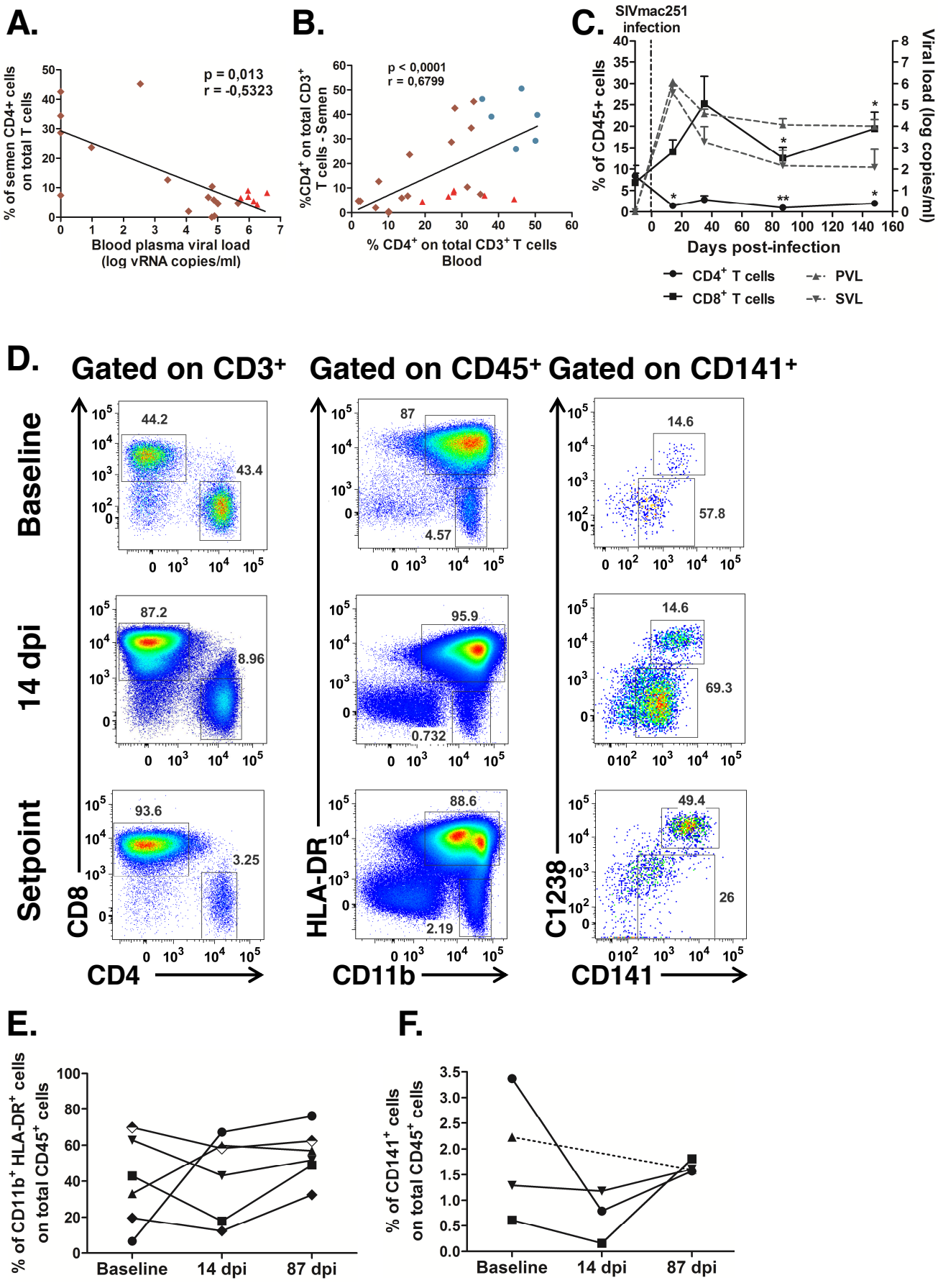


Figure 3: Changes in semen T cells, macrophages and DCs during SIV infection

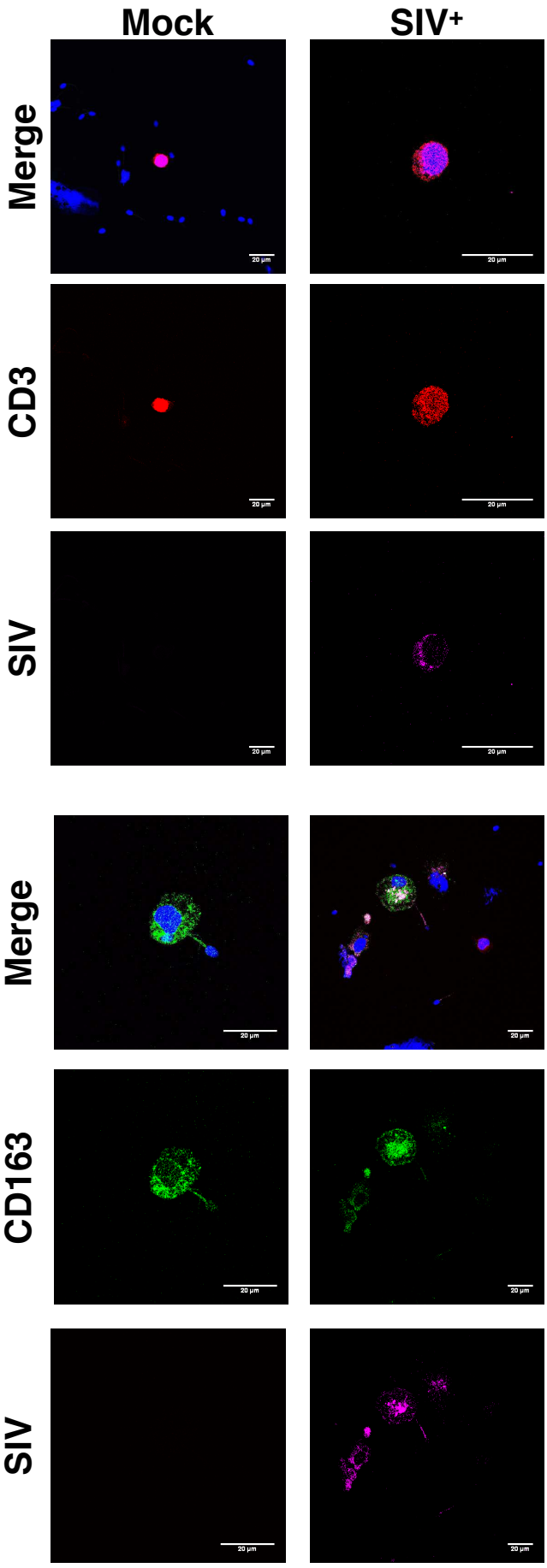


Figure 4: SIV antigens in semen CD4+ T cells and macrophages

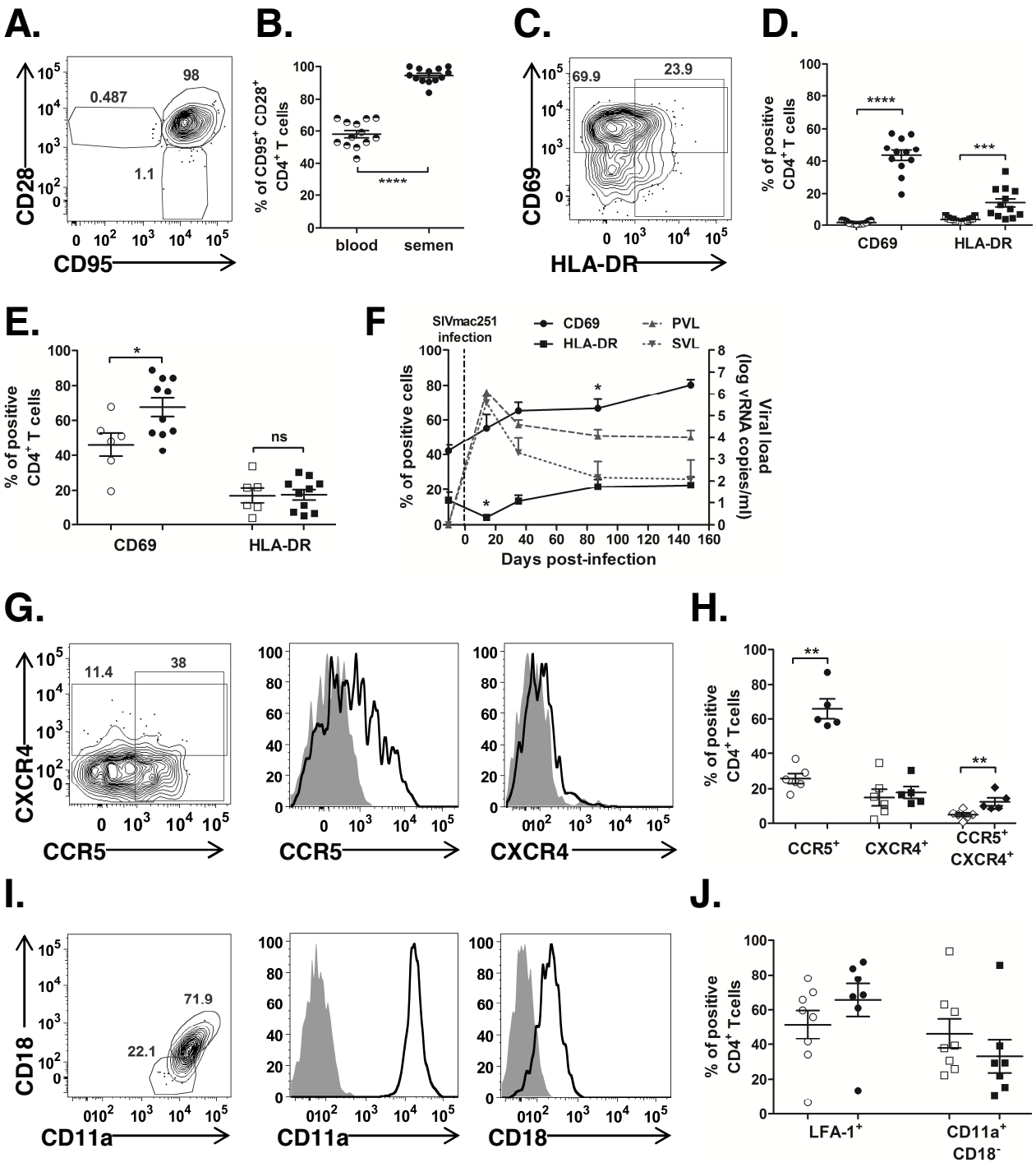


Figure 5: Semen CD4⁺ T-cell phenotype

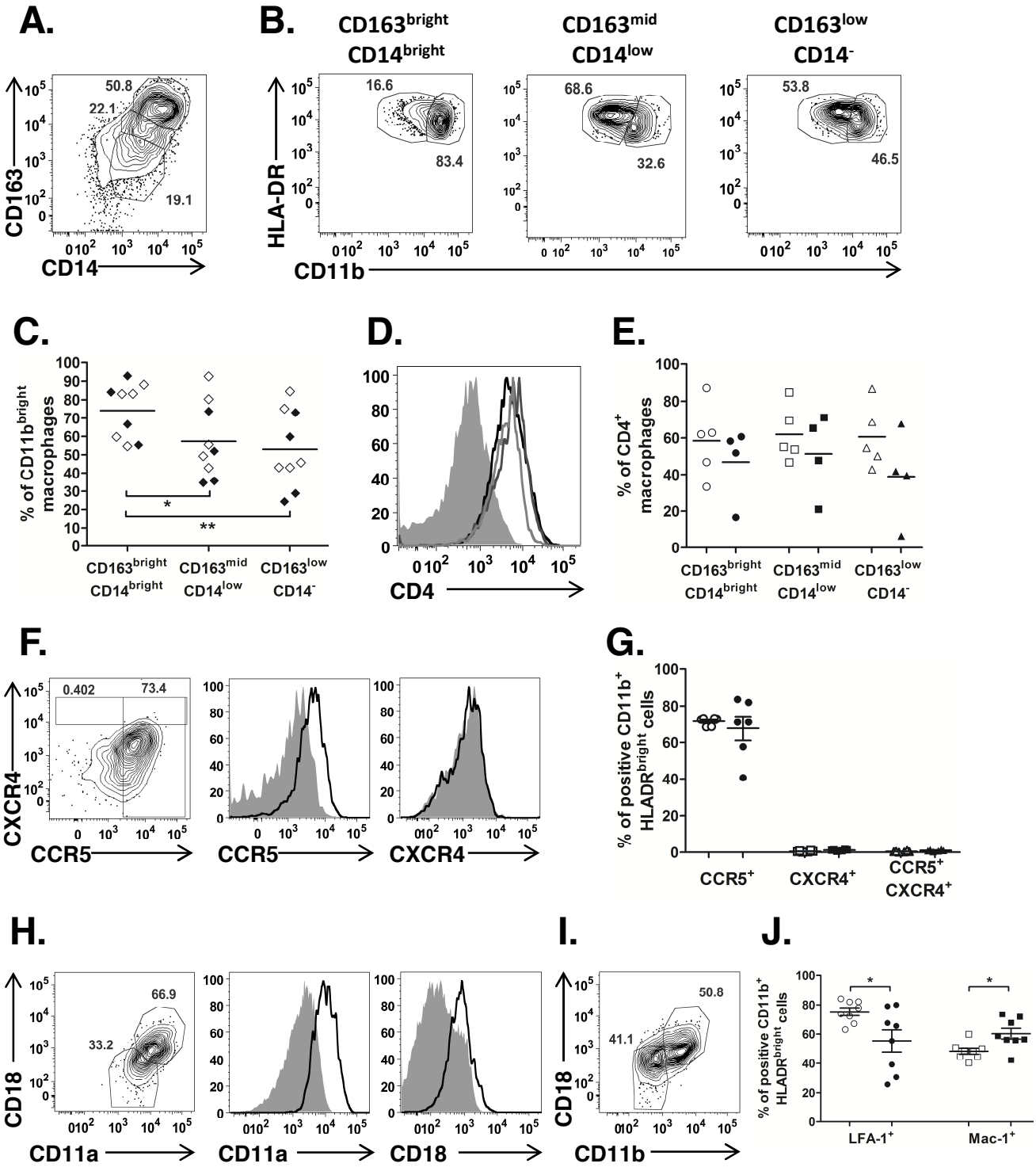
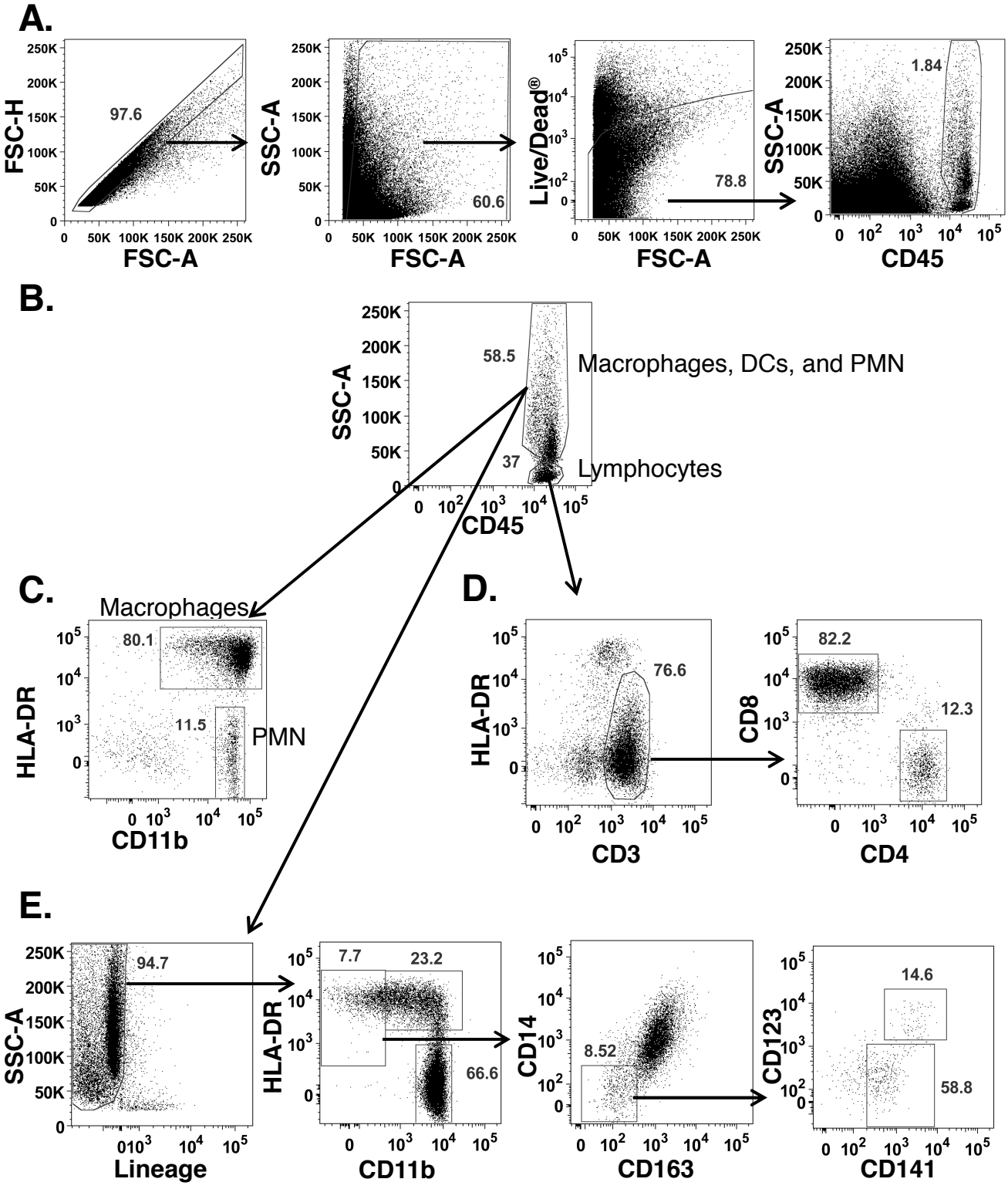
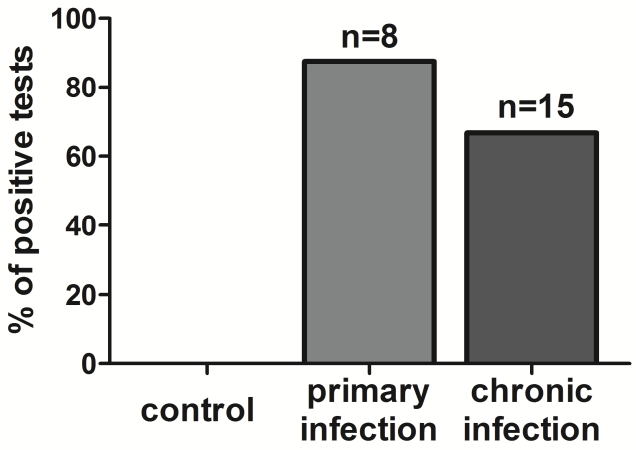


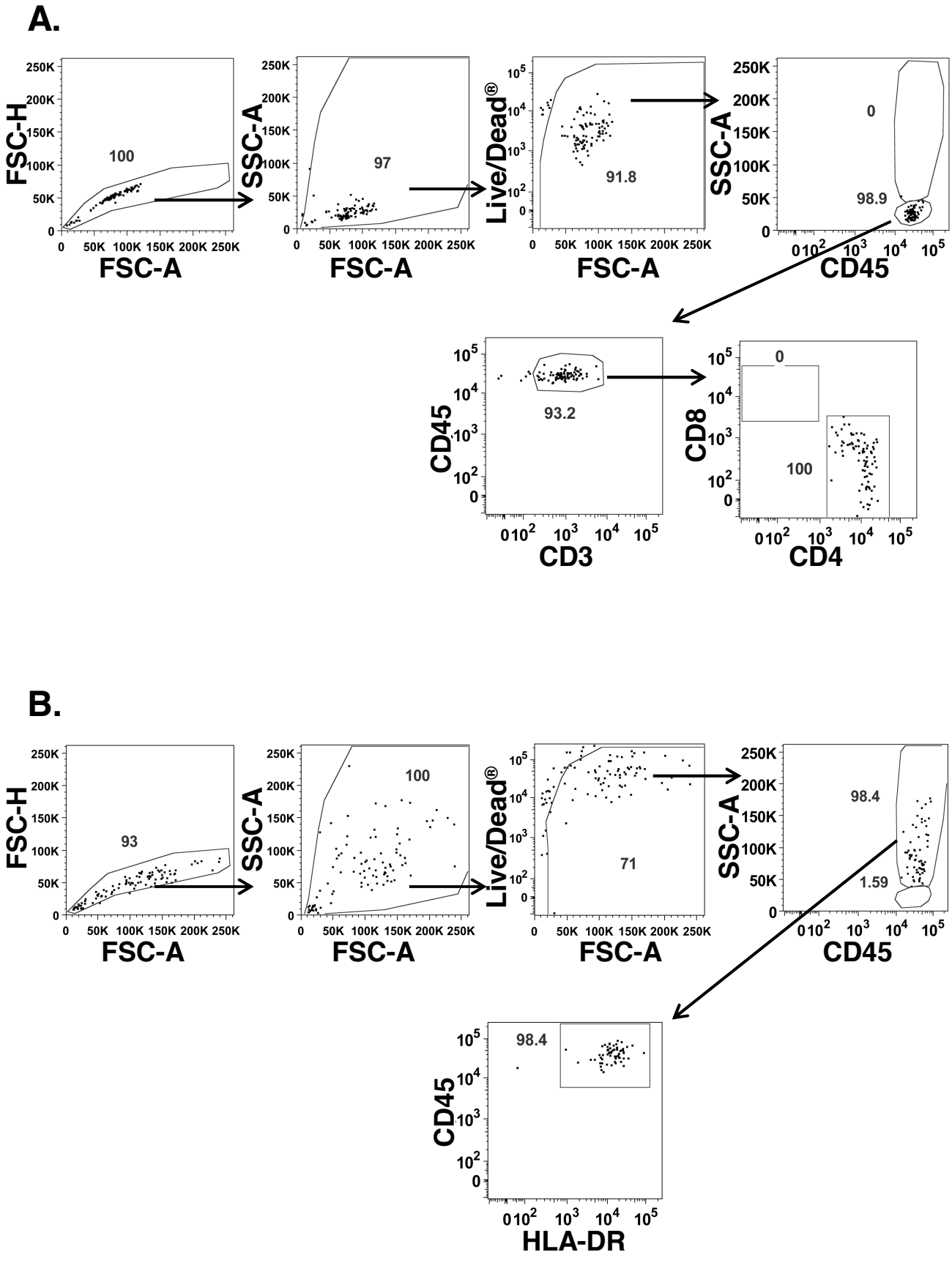
Figure 6: Semen macrophage phenotype



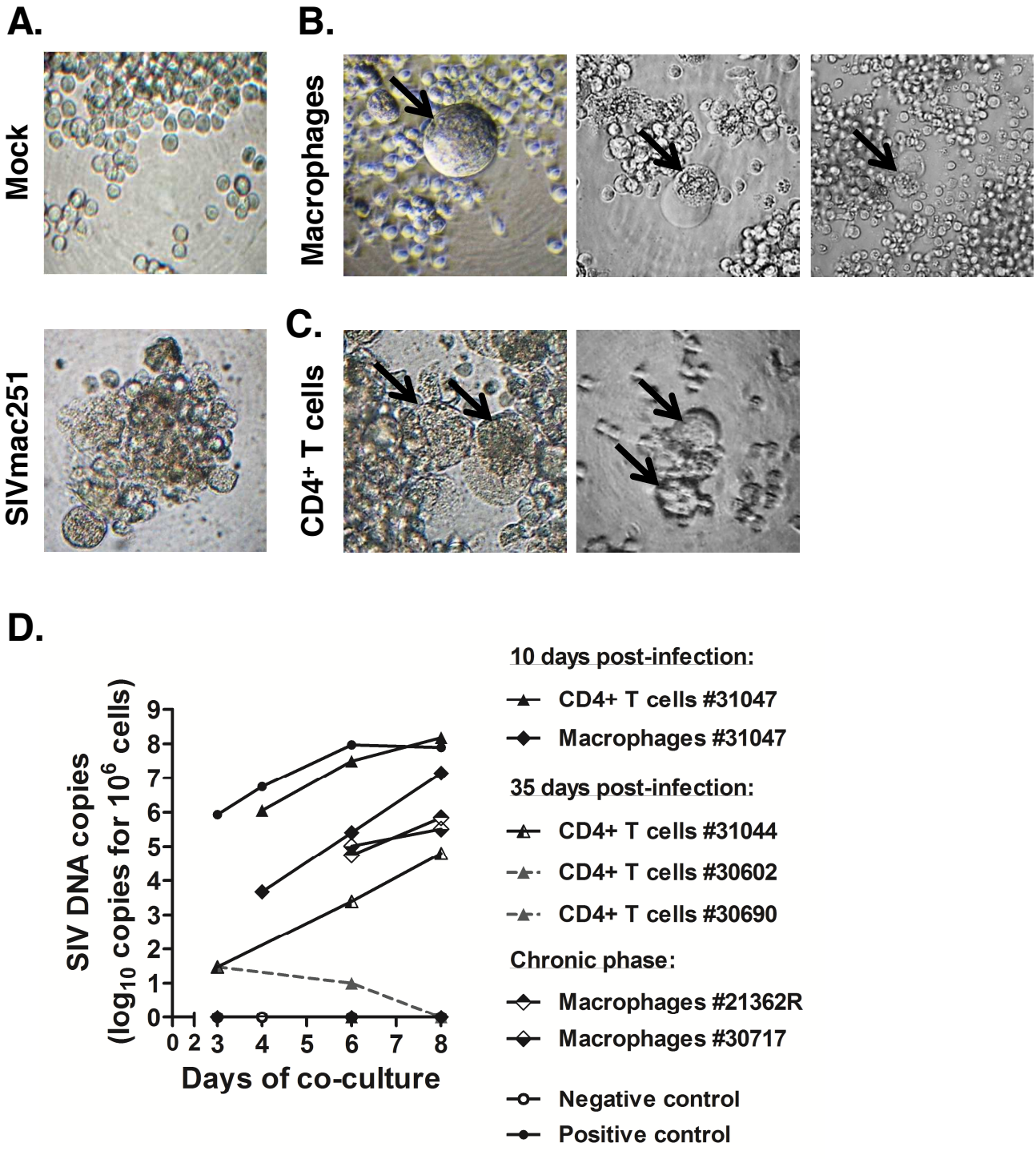
S1: Gating strategy for semen leukocytes characterization



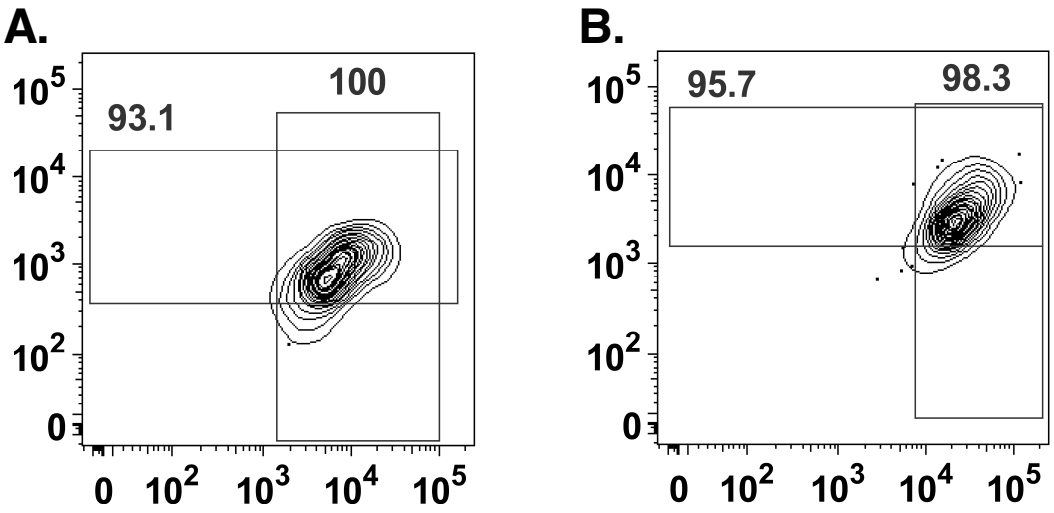
S2: Detection of SIV-infected cells by nested PCR in cellular components of whole semen, at all stages of infection



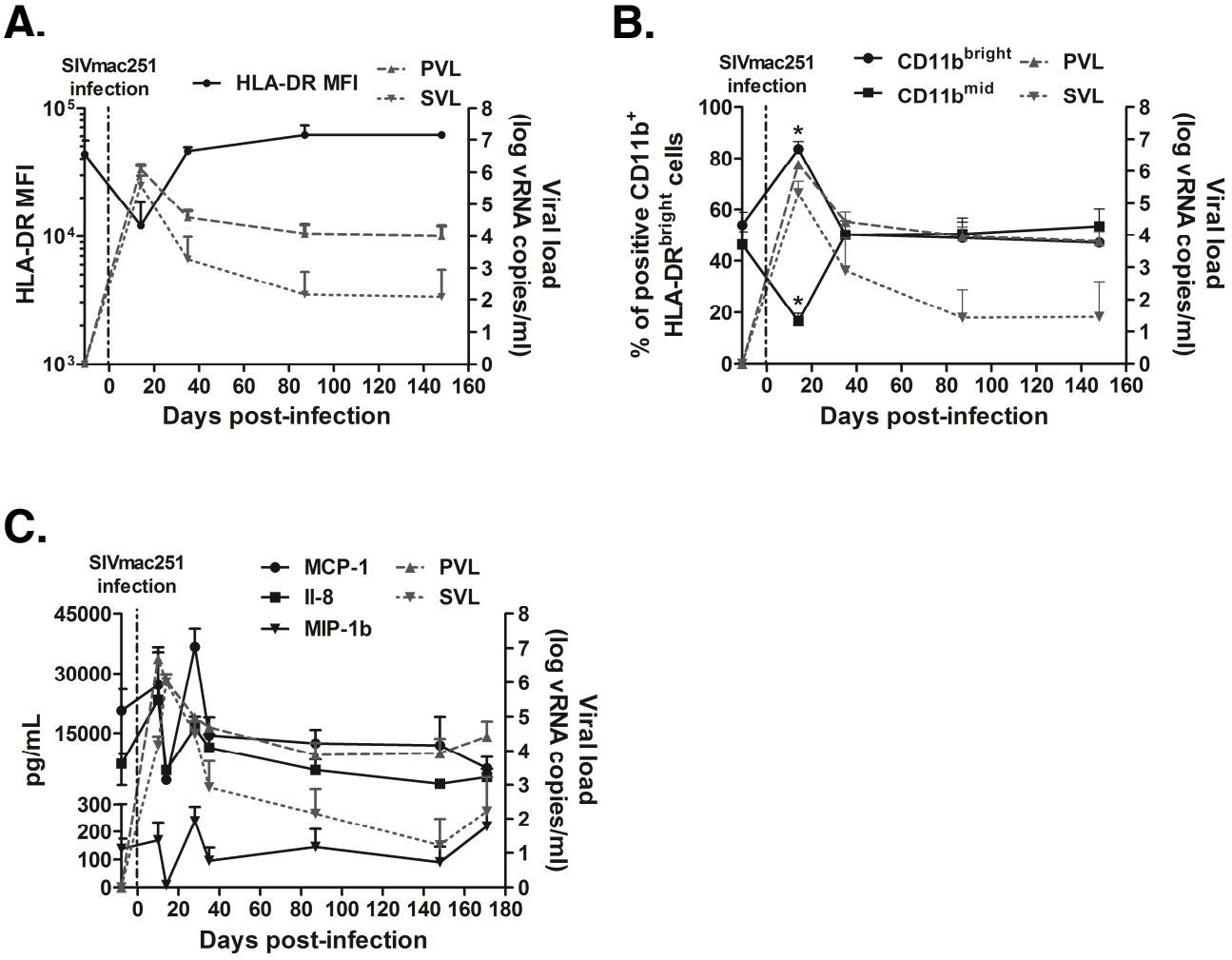
S3: Purity of the sorted CD4⁺ T-cell and macrophage fractions



S4: Microscopic evidence for viral transmission and quantification of SIV DNA copies in cocultures of sorted semen CD4+ T cells and macrophages



S5: Intracellular CCR5 and CXCR4 in semen $CD4^+$ T cells and macrophages



S6: Changes to semen macrophages during SIV infection

II. Second article: SIV-specific innate and adaptive immune response in the semen of infected cynomolgus macaques

A. Summary

In this second article, our objectives were to study the anti-SIV innate and adaptive immune response in the semen. First, we decided to investigate the antibody response, with quantification of SIV-specific IgG and IgA in the semen of macaques at different stages of infection. Secondly, the cellular response was studied using *ex vivo* stimulation of semen T cells with or without SIV gag and the intracellular staining with antibodies targeting CD45RA, IL-2, IFN-γ, TNF-α and MIP-1β.

Furthermore, we reported in the first article the impact of SIV infection and leukocytospermia on several seminal cytokines and chemokines (IL-6, IL-8, MIP-1β, MCP-1 and RANTES). Indeed, these molecules have been described as playing a role in inflammation and leukocytes chemotaxis, and therefore a potential role in cell-associated HIV mucosal transmission. However, we studied also the seminal levels of 23 other molecules in uninfected and SIV^+ macaques, which are here fully described.

We detected SIV-specific IgG in the semen of macaques at different stages of infection, with no correlation between IgG titers and viral RNA loads in semen. Quantification of IgA will be performed soon, as well as neutralization tests to evaluate this functionality in seminal SIV-specific antibodies, and to investigate whether the viral RNA shedding may be affected by the presence of them. We also observed that the proportion of semen $CD8^+$ T cells on total $CD45^+$ leukocytes was significantly increased in SIV^+ macaques.

Fifteen cytokines/chemokines were affected by SIV infection: 8 were significantly increased in SIV^+ macaques (TNF-α, IFN-γ, RANTES, IL-8, IL-15, IL-13, IL-15 and IL-18), and 12 were correlated to blood and/or semen viral loads: the pro-inflammatory molecules Il-1β, IL-6, IL-8, IL-15, IL-18 and RANTES, the Th2 cytokines IL-4, IL-5 and IL-13, and the immune-modulatory cytokines TFG-β1-3.

Finally, semen $CD8^+$ T cells displayed a memory and activated phenotype. Moreover, the proportion of $CCR5^+CXCR3^+$ cells, exhibiting a Th1 profile, was significantly increased by

SIV infection. A minority of macaques displayed SIV-specific $CD8^+$ T cells in semen, which did not correlated to seminal viral shedding.

B. Manuscript

Anti-SIV innate and adaptive response in semen of cynomolgus macaques

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Abstract

HIV-1 infection is mostly transmitted by sexual exposure, with genital and rectal secretions being the principal contaminating fluids. The HIV-specific immune response that takes place in the male genital tract and the semen remains poorly studied. We investigated the innate and adaptive immune response in the semen of cynomolgus macaques infected by SIVmac251.

We show that SIV infection induces an inflammatory state in the semen, with an increase of several pro-inflammatory molecules: IFN-γ, TNF-α, IP-10, IL-8, IL-13, IL-15, IL-18 and RANTES. Six pro-inflammatory molecules (IL-1β, IL-6, IL-8, IL-15, IL-18, RANTES), three Th2 cytokines (IL-4, IL-5 and IL-13) and TGF-β1-3 were correlated to semen and/or blood plasma RNA viral loads.

SIV-specific IgG were detected in most semen samples. Seminal antibodies titers were generally lower than the blood plasma levels, with a larger variability. We provide here an extensive characterization of semen $CD8^+$ T cells. They displayed a memory ($CD95^+$) and activated phenotype (CD69 and HLA-DR), with an important proportion of effector cells ($CD95^+CD28^-$). SIV infection significantly increased the proportion of semen T cells exhibiting a Th1 profile ($CCR5^+CXCR3^+$) A minority of infected macaques displayed a specific and polyfunctional ($IFN\text{-}\gamma^+MIP\text{-}1\beta^+TNF\text{-}\alpha^{+/-}$) CD8 T cell response in semen. Neither SIV-specific antibodies titers nor cellular response did correlate with local viral shedding.

Introduction

More than 80% of the new worldwide HIV infections occur during sexual intercourse, with a mucosal transmission of the virus[1-3]. Mucosal and genital tract tissues represent the major source of HIV-1 contaminating fluids. In particular, the semen which contains cell-free particles and infected cells, mostly leukocytes[4-6], accounts for a large proportion of HIV sexual transmission. However, because technical limitations, mechanism of transmission associated to semen remains largely unknown. Seminal viral load and stage of infection are correlated with transmission. Different factors are also known to affect semen infectivity like concomitant genital infections and inflammation, natural antimicrobial molecules (β-defensins, secretory leukocytes peptidase inhibitor –SLPI-, lysozyme, lactoferrin), cytokines (IL-7)[7] and male circumcision[8-12]. Semen-derived amyloid fibrils have been described as powerful enhancers of HIV transmission, by capturing virions and promoting their attachment to target cells[13,14]. However, very few is known about the actors of innate and HIV-specific adaptive immune response present in semen and which may limit or enhance viral transmission.

HIV-specific antibodies may prevent or partially control mucosal HIV-1/SIV entry[15,16]. In macaques, systemic and mucosal application of neutralizing IgG succeeded to prevent infection after intravenous or vaginal inoculation of SHIV[17-20]. Furthermore, neutralizing HIV-specific IgA have been reported in the semen and vaginal washes from HIV-1 exposed and seronegative sex-workers, although this remains subject of controversy[21,22]. HIV-specific non-neutralizing antibodies may also play a protective role through antibody-dependent cell-mediated cytotoxicity (ADCC), induction of viral aggregation, or prevention of viral uptake[23]. HIV/SIV-specific antibodies have been described as present in abundant quantities and high frequencies (81-100%) in the semen of HIV^+ men and SIV^+ macaques, which are mostly IgG[16,21,24]. However, their ability to neutralize the virus and induce ADCC, remains poorly investigated[25].

Whereas the role for cell-mediated immunity in reducing HIV replication and killing the infected cells has been fully established[26], very few studies have assessed the presence of HIV-specific $CD8^+$ T cells and their functionality in semen[27-30]. Sheth *et al.* reported that HIV-specific $CD8^+$ T cells in semen do not correlate with reduction of local virus shedding[31]. Nevertheless, it remains unclear whether the presence of HIV-specific polyfunctional cytotoxic cells in the semen may have an impact on the local shedding of viral particles or infected cells and on HIV transmission.

Here we focused on the innate and adaptive immune response in the semen of SIV infected macaques we used as a model of HIV infection and AIDS. We studied dynamics of seminal viral load, cytokine levels, SIV-specific antibodies and $CD8^+$ T cells. Inflammation was associated with SIV acute infection. Pro-inflammatory cytokines/chemokines and Th2 cytokines concentrations correlated with blood and semen plasma viral loads. This was not the case for levels of SIV-specific antibodies. A minority of macaques displayed strong, polyfunctional response, which was not associated with reduced viral shedding in semen.

Results

Virus shedding and cytokine profiles in semen of SIVmac251 infected macaques

Viral shedding, as HIV cell-free particles and infected cells, has a major impact in the infectivity of semen. We and others hypothesized that seminal levels of inflammatory ctokines cells may impact on HIV mucosal transmission.

We measured these parameters in paired blood and semen samples of macaques infected intravenously with 5,000 animal infectious dose 50% (AID50) (Fig. 1A-C). Blood and semen viral RNA (vRNA) levels are strongly correlated (Spearman test, $p<0.0001$, $r=0.7643$, $n=30$). Viral load peaked at 10 days post infection (dpi), followed by a rapid decrease and a stabilization in both compartments at 3 months post-infection (Fig. 1A).

The seminal plasma levels of 28 cytokines and chemokines were measured by Luminex assay (Table 1, Fig. 2A). VEGF, active form of TGF-β1-3, MCP-1 and IL-8 were present at high levels (mean superior to 1,000 pg/ml). A significant increase ($p<0.05$) of several pro-inflammatory and immunomodulatory molecules was observed in SIV infected macaques (Fig. 2B). IP-10 was increased during both primary and chronically stages of infection (Mann-Whitney test, $p=0.0108$ and 0.0072, respectively). Interestingly, two peaks of TNF-α were observed, at 14 and 87 dpi, with different profiles among the 6 tested macaques (Fig. 2F). IL-13, IFN-γ, IL-18, RANTES, IL-15 and IL-8 were increased only at 10 and 14 dpi (Mann-Withney test, $p=0.0378$, 0.0229, 0.0003, 0.0007, 0.0014 and 0.0027, respectively). Moreover, 6 pro-inflammatory cytokines (Il-1β, Il-6, IL-15 and IL-18) and chemokines (IL-8 and RANTES) correlated with both PVL and SVL (Fig. 2C and Suppl. Fig. 2A-F). The same correlation profile was observed with three main Th2 cytokines: IL-4, IL-5 and IL-13 (Suppl. Fig. 3A-C) and TGF-β1, 2 and 3 (Suppl. Fig. 4A-C), whereas none of the main Th1 cytokines (IFN-γ, TNF-α and IL-2) were associated with viral loads (Fig. 2 E).

SIV-specific IgG and T cells dynamics in the semen of SIVmac251 infected macaques

SIV-specific IgG were detectable at low levels after 35 dpi in blood and semen (mean titer of 57.15 ± 17.22 and 41.08 ± 28.17, respectively). At chronic stage, IgG titers were significantly higher (Mann-Whitney test, p=0.0317) in blood (mean titer of 1880 ± 770.4) than in seminal plasma (200.9 ± 138.0, ;Fig. 1B).

A large diversity of profiles in SIV-specific IgG titers was observed in blood and semen (Fig. 1D-E). The sensitivity of the ELISA we used to measure antibody titers was higher in blood serum than in seminal plasma which induces a higher background. A few macaques displayed close titers in both compartments, whereas blood titers were much higher than SP's for the majority of animals. Interestingly, at chronic stage, the most elevated titers found in the two compartments were observed in one macaque (#30838) displaying constant high viral shedding in blood and semen ("high shedder") and another macaque which strongly controlled blood and semen viral loads ("SIV controller or SIC"; Fig. 1D). Besides, we didn't found any correlation between SIV-specific IgG titers and viral loads in the two compartments, at any stage of infection.

Proportions of semen T cell were strongly affected by SIV infection, with a rapid, significant and durable depletion of $CD4^+$ T cells (Wilcoxon matched-pairs signed rank test, p=0.0313 at 14) (Fig. 1C). These data confirms that macaques infected with SIVmac251 recapitulates what has been described in human HIV patients[21]. A depletion of CD4+ T cells was found, as expected, in peripheral blood, but no significant increase of the percentage of CD8+ T cells on total blood CD45+ cells was observed (Fig. 1G). Interestingly, the percentage of semen $CD8^+$ T cells in total semen $CD45^+$ cells correlated with blood viral RNA load (Spearman correlation, p=0.0364, r=0.4825) at chronic stage (Suppl. Fig. 1A), whereas

a same correlation wasn't found with blood $CD8^+$ T cells (Suppl. Fig. 1B). This suggests that blood and semen T cells undergo different dynamics in the two compartments.

Thus, SIV infection induces SIV-specific IgG in semen, with a large diversity of profiles among individuals, as well as important changes in T cells dynamics. Moreover, infection is associated with inflammation in SP, especially during the first weeks of infection, with pro-inflammatory molecules levels correlating to seminal viral shedding.

Phenotype of semen CD8+ T cells

As most of the cytokines which are increased in semen of SIV+ macaques are known to induce T cells activation, we pursued by phenotyping semen T cells and studying the specific responses to SIV. Since $CD4^+$ T cells are severely depleted in infected macaques, and thereby not in sufficient quantities to perform significant analysis, we focused on $CD8^+$ T cells only and the SIV-specific Th1 response (Fig. 3).

In macaques, CD95 and CD28 are commonly used in flow cytometry analysis to discriminate naïve (Tn, $CD95^-CD28^+$) from central memory (Tcm, $CD95^+CD28^+$) and effector memory (Tem, $CD95^+CD28^-$) T cells (Fig. 2A). In contrast with peripheral blood, semen $CD8^+$ T cells were all of memory phenotype ($CD95^+$), with a mean of 59.42% ± 3.71 of Tcm cells and 38.17% ± 3.23 of Tem cells, with no difference between non infected and SIV^+ macaques (Fig. 3A, Suppl. Fig. 5). The expression of CD45RA by memory cells is considered as a marker of highly, end stage, differentiation, as reviewed in [32]. We observed a low proportion of $CD45RA^+$ $CD8^+$ T in uninfected macaques' semen (mean of 6.20% ± 3.04), in contrast with peripheral blood (mean of 82.00% ± 3.70; Suppl. Fig. 5B). In SIV+ macaques, CD45RA+ cells were increased, however without reaching a significant difference (Mann-Whitney test, p=0.16).

We studied the expression of CD69 and HLA-DR, two T cells activation markers commonly described to be increased by HIV/SIV infection. The proportion of CD69 and HLA-DR positive $CD8^+$ T cells was much higher in semen than in blood (with an increase of 12.22- and of 5.85-fold for CD69 and of 3.56 and 4.52-fold for HLA-DR in both subsets, respectively; Suppl. Fig. 5C). The proportion of $CD69^+$ cells was increased in semen of SIV^+ animals in both, Tcm (Mann-Whitney test, p=0.06) and Tem (p=0.0046) subsets, (Fig. 3C), whereas in blood, increase was only observed for Tem cells (Mann-Whitney test, p=0.0292, Suppl. Fig. 5E). A durable increase in the proportion of $CD69^+$ cells was observed in 8 infected macaques followed longitudinally (Fig. 3D). Moreover, the proportion of $CD69^+$ cells in both Tcm and Tem $CD8^+$ cells was correlated between blood and semen (Fig. 3E). In contrast, the percentage of HLA-DR^+ cells in semen was not affected by SIV infection, although it was the case in blood Tcm cells (Mann-Whitney test, p=0.0091, Fig. 3C, Suppl. Fig. 5E).

CCR5 and CXCR3 are receptors for the chemokines RANTES, MIP-1α and -1β (CCR5) and IP-10 and MIG (CXCR3). Their expression on the cell surface of T cells will trigger their migration to tissues expressing a gradient of these chemokines, as cervico-vaginal and gut mucosa. Moreover, $CCR5^+CXCR3^+$ T cells have been associated with a Th1 profile[33,34]. At steady state, semen CD8+ T cells are of 21.97% ± 4.77 $CCR5^+$, of 73.00% ± 4.59 $CXCR3^+$ and of 17.75% ± 4.04 $CCR5^+$ $CXCR3^+$. Interestingly, SIV infection induces a significant increase in the proportion of $CCR5^+$ and $CCR5^+$ $CXCR3^+$ cells (Mann-Whitney test, p=0.0022 for both subsets, mean of 67.17% ± 4.94 $CCR5^+$ and of 53.68% 4.65 $CCR5^+$ $CXCR3^+$ cells, respectively). This suggests that, in SIV^+ macaques, a majority of semen $CD8^+$ T cells display Th1 profile. This is consistent with capacity of Th1 T cells to home into inflammatory tissues[35]. Finally, LFA-1 integrin, formed by the α-chain CD11a and the β-chain CD18, is a major marker of adhesion and transmigration of T cells (Fig. 3G) [36]. Semen $CD8^+$ T cells displayed a high expression of this integrin, with 100% of cells positive for CD11a and a mean of 40.33% ± 8.68 LFA-1^+ cells. SIV infection slightly increased the proportion of LFA-1^+ cells(mean of 54.50 ± 8.70, Mann-Whitney test, p=0.3823). Thus, at steady state, semen

184 $CD8^+$ T cells display a memory, activated profile. These cells are all $CD95^+$, expressing
185 CD69, HLA-DR, CXCR3 and LFA-1 in large proportions and CCR5 in to a lower extent. This
186 profile is typical of mucosal T cells [37,38]. SIV infection induces considerable changes in their
187 phenotype, as well as in their dynamics: the proportion of CD69 and CCR5 positive cells is
188 significantly increased, which is consistent with a boost of their activation. Interestingly, the
189 expression of CD69, an early activation marker increased with HIV/SIV infection, in $CD8^+$ T
190 cells is correlated between blood and semen cells, with a higher proportion of positive cells in
191 the seminal compartment. Furthermore, a majority of cells are of $CCR5^+CXCR3^+$ Th1 profile.
192 Therefore we followed our work by a characterization of the SIV-specific $CD8^+$ T cells
193 response.

194

195 **Semen SIV-specific $CD8^+$ T cells response**

196 We performed a 5 hours *ex vivo* stimulation with a pool of SIV *Gag* peptides on semen
197 and peripheral blood mononuclear cells to assess and quantify the presence of SIV-specific
198 $CD8^+$ T cells. We assayed on seven chronically infected macaques the intracellular
199 expression of 4 cytokines expressed by activated, HIV-specific CD8+ T cells in human [39-41]:
200 IFN-γ, TNF-α, IL-2 and MIP-1β (Fig. 4A). No significant expression of IL-2 was detected,
201 neither in blood nor in semen. The majority of animals (4 of 7) displayed no SIV-specific
202 response in both compartments (Fig. 4A and Suppl. Fig. 7A-B). In two macaques (#31044
203 and #30690), the proportion of SIV-specific cells was much higher in semen than in blood

204 The animal #31044, displayed a high proportion of SIV-specific $CD8^+$ T cells in blood and
205 semen, and express all 3 cytokines, IFN-γ (0.352% of IFN-γ^+ cells in blood, and 4.28% in
206 semen), TNF-α (0.169% of TNF-α^+ cells in blood and 1.57% in semen) and MIP-1β (0.681%
207 of MIP-$1\beta^+$ cells in blood, and of 5.87% in semen). Four types of SIV-specific cytokines
208 producing cells were observed: a majority of IFN-γ^+MIP-$1\beta^+$TNF-α^- and IFN-γ^-MIP-$1\beta^+$TNF-α^-

cells, followed by IFN-γ^+MIP-1β^+TNF-α^+ (highly polyfunctional) and finally of IFN-γ^+MIP-1β^- TNF-α^- cells, with the same distribution in CD45RA positive and negative cells.

The animal #30690 also showed a significant response for the 3 cytokines upon Gag peptides stimulation in both compartments. Seminal cells were in a large majority Ag-experienced polyfunctional cells expressing CD45RA$^+$IFN-γ^+MIP-1β^+TNF-α^+ (11.07% of positive Gag-stimulated cells (Fig. 4A-B). Interestingly, a basal, production of these cytokines is also observed in non SIV-specific semen CD8$^+$ T cells (4.92% of positive non-stimulated cells). This suggests that local environment, and high levels of cytokines we observed, may account for chronic activation of CD8+ T cells seeded in semen. For both of these macaques, the SIV-specific response displayed the same dominant profiles in blood (Suppl. Fig 7A): a majority of CD45RA$^-$IFN-γ^+MIP-1β^+TNF-α^- cells (0.22% and 0.11% of positive cells for #30690 and #31044 respectively), and of CD45RA$^-$IFN-γ^-MIP-1β^+TNF-α^- cells (0.18% and 0.09% of positive cells for #30690 and #31044, respectively). The proportion of these SIV-specificities was much lower in blood than in semen.

The animals #OBCS5 and #30742, excluded from analysis because insufficient CD8$^+$ T cells acquired from semen sample, displayed the highest SIV-specific responses in blood CD8$^+$ T cells (Suppl. Fig 7A). Among these SIV-specific cells, #OBCS5 displayed a majority of CD45RA$^+$IFN-γ^-MIP-1β^-TNF-α^+ (0.14%), whereas #30742 had a majority of CD45RA$^+$IFN-γ^+MIP-1β^+TNF-α^- (0.53%), CD45RA$^+$IFN-γ^+MIP-1β^+TNF-α^+ (highly polyfunctional cells) (0.26%) and CD45RA$^+$IFN-γ^-MIP-1β^+TNF-α^- (0.19%). Both displayed low PVL (#OBCS5: 2.35 log10 copies/ml and #30742: 2.02 log10 copies/ml) and undetectable SVL. Animal #OBCS5 was of MHC-M6 haplotype, which has been associated with a high control of viremia in SIV$^+$ cynomolgus macaques [42], and had controlled viremia for years.

For each compartment (blood and semen), the proportion of IFN-γ^+, TNF-α^+, and MIP-1β^+ cells was not correlated to control of vRNA load (PVL or SVL, respectively). However, in semen, the macaques with the highest SIV-specific responses in CD8$^+$ T cells displayed the

highest SVL (Fig. 4C). The same trend was observed in blood, with the exception of one macaque (#30742), which had the highest proportion of cells positive for the three cytokines and the lowest BVL (Fig. 4C).

Thus, the diversity of SIV-specific Th1 response we observed in semen $CD8^+$ T cells is consistent with what we observed on the TNF-α level in SP: whereas some macaques responded strongly, others didn't display any response. In both compartment, the majority of SIV-specific cells display high polyfunctionality, with concomitant expression of at least two of the three cytokines IFN-γ, MIP-1β and TNF-α. The presence of SIV-specific $CD8^+$ T cells in semen seems to be positively related to SVL, although results from more animals are needed to confirm this observation. In blood, the two macaques displaying the best control of their viremia also had the highest proportion of SIV-specific IFN-γ and MIP-1β positive $CD8^+$ T cells.

Discussion

We report here a comprehensive characterization of semen SIV-specific adaptive immune response in macaques we used as a model of HIV/AIDS[42-45,46 43-46,47] . Dynamics of viral RNA loads and SIV-specific IgG in the blood and semen of SIVmac251-infected macaques were similar to what reported for men[6,48,49]. Moreover, semen T cells were strongly affected by SIV infection, with a rapid and profound depletion of $CD4^+$ T cells and a parallel significant increase of the proportion of $CD8^+$ T cells on the total semen $CD45^+$ leukocytes population. Such observations have been also described in human patients[50,51]. Interestingly, whereas $CD4^+$ T cells depletion was observed in blood and in semen, the strong increase in the proportion of semen $CD8^+$ T cells n total leukocytes in infected macaques, which correlated with BVL and not SVL, was not observed in peripheral blood.

We studied the SIV-specific antibodies levels in paired blood and semen samples in macaques at different stages of infection. SIV-specific IgG were detected in almost all semen samples, generally at lower levels that in serum. Interestingly, viral RNA loads and SIV-specific IgG titers did not correlate in both compartments, suggesting that the local humoral response fail to control viral shedding. This is consistent with the observation made in human patients whose systemic antibody response to HIV following infection is mostly ineffective, with an early B-cell response being largely directed against non-neutralizing epitopes of HIV envelope. Response during late stages is impaired by B-cell aberrant hyperactivation and abnormalities[52-54]. Further functional assays needs to be performed to investigate the impact of semen SIV-specific IgG on SIV transmission. Indeed, although a few studies reported the presence of neutralizing antibodies[21,55], mostly IgA, seminal HIV-specific antibodies activity remains poorly described. Interestingly, studies on HIV controllers reported a higher level of antibodies with ADCC activity than in progressor patients[56]. Therefore it would be also interesting to evaluate ADCC activity in semen SIV-specific antibodies in infected macaques, and investigate whether it might be associated with local viral shedding.

HIV/SIV infection is associated with an increased systemic production of cytokines and chemokines. In particular, acute infection induces a so-called “cytokine storm”, with strongly elevated levels of numerous molecules (Il-1β, IL-6, IL-8, IL-12, IL-15, IL-18, TNF-α, RANTES, MIP-1β, MCP-1, MIP-3α, IP-10, IFN-γ, etc.)[57-62]. Some of them are also elevated in the seminal plasma of chronically infected men[59,60,63-65], although their dynamics in the course of infection have been poorly studied. We provide here one of the first extensive studies of the influence of the different stages of SIV infection on the seminal proteins network. Many molecules correlated to PVL and/or SVL: pro-inflammatory molecules (Il-1β, IL-6, IL-8, IL-15, IL-18, RANTES), Th2 cytokines (IL-4, IL-5, IL-13) and the immunomodulatory cytokine TGF-β. These molecules are known to modulate immune cells activation and migration profiles as well as to initiate T cells effector functions. They may both impact the leukocytes that are present in the donor’s semen and the partner’s mucosal tissue, thereby influencing the efficacy of virus transmission.

Since cell-mediated immunity plays a major role in controlling HIV replication and killing the infected cells[26], we investigated the SIV-specific $CD8^+$ T cells response in semen, and its potential role in HIV/SIV transmission. We found that all semen $CD8^+$ T cells display a memory phenotype ($CD95^+$), with similar proportions of central memory and effector memory cells, whatever the status of infection. However, the expression of CD45RA, a marker of terminal differentiation by memory cells, increases in infected macaques. Semen $CD8^+$ T cells displayed an activation profile, with a higher proportion of $CD69^+$ and $HLA\text{-}DR^+$ cells than in peripheral blood. Besides, the proportion of $CD69^+$ cells was increased in SIV infected macaques in both compartments, particularly in effector cells, as it has been previously described in HIV^+ men[66] and SIV^+ macaques[67]. The percentage of positive cells correlated between blood and semen T cells, in favor with a generalized immune activation in SIV-infected macaques. Furthermore, SIV infected macaques displayed high proportions of $CCR5^+$ and $CCR5^+CXCR3^+$ cells, a phenotype associated with Th1 effector profile[33,34]. Altogether, these observations strongly suggest that semen $CD8^+$ T cells are of mucosal

origin and undergo strong modifications in their activation profile and effector function in infected animals. The role of such effector T cells in the semen and whether their excretion is active or passive remain unclear. Interestingly, activated, cytotoxic (TIA-1$^+$) CD3$^+$ T cells infiltrates have been described along the male genital tract (mostly in the prostate, seminal vesicles and epididymis) of SIV-infected macaques displaying high viremia[68]. Semen CD8$^+$ T cells may originate from these cellular infiltrates of the different semen-producing organs, and therefore reflect the inflammatory status within the male genital tract. If this hypothesize is confirmed, the activation profile of semen T cells may be used as a marker of the peripheral immune activation that is largely described in HIV$^+$ individuals.

CD8$^+$ T cells play an important role in controlling HIV/SIV replication and disease progression. Moreover, the presence highly polyfunctional T cells has been reported in elite controllers and long-term nonprogressor HIV patients[39,40]. Interestingly, the two studied macaques displaying the strongest control viral replication had the highest polyfunctional responses in blood. Unfortunately, we didn't obtained enough numbers of semen T cells to perform the analysis. Furthermore, in semen, the majority of macaques didn't display a detectable SIV-specific T cell response, with the exception of two animals. Both had a high proportion of IFN-γ, TNF-α and MIP-1β producing cells, with a majority of polyfunctional cells. Their responses were about 10-fold higher in semen than in peripheral blood. Importantly, this high SIV-specific response in semen was associated with an elevated local viral shedding. This suggests that the SIV-specific T cells do not significantly impact virus replication. Given the small number of studied macaques, no significant trend in the relation between specific T cell response and local viral shedding was reached. We therefore need to increase the number of animals in this study. Moreover, it would be highly interesting to assess if semen SIV-specific T cells are able to kill semen infected cells, and thereby to impact semen infectivity.

Taken together, our results suggest that the semen SIV-specific adaptive immune response, including antibodies and CD8+ T cells effector function, does not impact on the local viral shedding. Further neutralization and cytotoxicity assays need to be done to evaluate their potential impact on semen infectiousness.

Material and Methods

Animals, infection and samples collection

Cynomolgus macaques (*Macaca fascicularis*), weighing 5 to 11 kg were imported from Mauritius and kept according to European guidelines for animal care (Journal Officiel des Communautés Européennes, L 358, December 18, 1986 and new directive 63/2010). All work related to animals was in compliance with institutional guidelines and protocols approved by local ethical committee. Adult males were infected by intravenous or intrarectal inoculation of 50-5,000 animal infectious doses 50% (AID50) of SIVmac251 isolate (Karlson et al). Semen and blood were collected in sedated animals with 5 mg/kg intra-muscular injection of Zoletil®100 (Virbac, Carros, France).

Electroejaculation was performed by intrarectal electrostimulation of prostate, using a probe (12.7 mm diameter) lubrificated with conductor gel, and an AC-1 electroejaculator (Beltron Instrument, Longmont, USA). Sequential stimulations were performed with a pattern of 6 cycles, each cycle of 9 stimulations of 2 seconds followed by a tenth stimulation lasting 10 seconds. Voltage is increased every two cycles (1-3Vfor the 2 first, 2-4V volts for series 3-4, 6-8V for series 5-6). If no complete ejaculum was obtained after 6 cycles of stimulations, a 7^{th} cycle at 7- 10V was performed. Complete ejaculum was immediately diluted in 1.2 ml of phosphate buffer saline (PBS) and spinned 1.

Blood samples were taken in BD Vacutainer® Plus Plastic K_3EDTA tubes (for plasma viral load quantification and in BD Vacutainer® Plus Plastic SST tubes (for serum preparation) (BD Biosciences, Le Pont de Claix, France).

Seminal plasma and cells preparation

Seminal plasmas were isolated from total semen immediately after collection by spinning 15 minutes at 775 g. After separation from seminal plasma, seminal cells were diluted in 14 ml of complete medium, consisting in RPMI-1640 Glutamax medium (Invitrogen, Carlsbad, USA) supplemented by a mixture of Penicillin, Streptomycin and Neomycin (Invitrogen) and 10% FCS (Lonza, Allendale, USA), and kept at room temperature during 1 hour maximum. Cells were then spinned 10 min at 1,500g, filtered through a 70 µM sieve and washed with 5 ml of PBS supplemented with 10% FCS.

Blood and semen RNA viral load quantification

Blood plasma was isolated from EDTA blood sample by spinning 10 min at 1,500g, and cryopreserved at -80°C. Seminal plasma were maintained on ice for 1 hour maximum and cryopreserved at -80°C. Blood viral RNA was prepared from 250µL of cell-free plasma using the kit Nucleospin 96 RNA kit (Macherey Nagel GmbH&Co KG, Düren, Germany), according to the manufacturer's instructions. Retro-transcription and cDNA amplification and quantification was performed in duplicate by RT-qPCR using Superscript III Platinum one-step quantitative RT-PCR system (Invitrogen, Carlsbad, USA). RT-PCR was performed as we previously described (Karlsson, 2007, J Virol). Quantification limit (QL) was estimated at 111 copies/ml and detection limit (DL) to 37 copies/ml. Samples from chronically infected macaques under ART were treated the same way using increased amounts of plasma in order to increase sensitivity (QL and DL of 37 and 12.3 copies of vRNA/ml, respectively). Semen vRNA was prepared from 500µL of seminal plasma using the QIAamp Ultrasens Virus kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. Quantitative RT-PCR was performed in the same conditions as above, with QL and DL of 37 copies/ml and 12.3 copies/ml, respectively.

Quantification of SIV/HIV-2-specific IgG titer in blood and semen

383 Blood serum was isolated from SST blood sample by spinning 10 min at 1,500g, and
384 cryopreserved at -80°C. Seminal plasmas were isolated as described above. ELISA assay
385 was performed using the kit Genscreen HIV-1/2 Version 2 (Biorad, Marnes-la-Coquette,
386 France), according to the manufacturer's instructions. A reference positive serum from a
387 SIVmac251 infected cynomolgus macaque was used as Standard. The titer was calculated
388 using a cut-off calculated separately for blood and semen samples, using the mean value of
389 samples from uninfected nor immunized monkeys. The maximal titer was fixed at 8,000,
390 extrapolated from the Standard scale.

391

392 **Phenotypic characterization of semen leukocytes**

393 Staining was performed in parallel on whole blood samples.

394 All stainings but whole blood assays, were performed after saturation of Fc receptors in
395 healthy macaque serum (in-house production) for 1h at 4°C. Amine-reactive dye Live/dead®
396 Fixable blue (Life technologies) was used to asses cell viability and exclude dead cell from
397 analysis, cells were stained with monoclonal antibodies for 30 min at 4°C, washed in
398 PBS/10% FCS and fixed in CellFIX™ (BD Biosciences). Three different antibody panels were
399 used. Panel 1, targeting semen leukocytes subpopulations and T cells activation, was
400 constituted with BD Biosciences antibodies (BD, Franklin Lakes, USA) anti-CD45 PerCp
401 (clone B058-1283), anti-CD3 V500 (clone SP34-2), anti-CD4 PE-Cy7 (clone L200), anti-
402 CD11b Alexa Fluor 700 (clone ICRF44), anti-HLA-DR APC-H7 (clone G46-6) and anti-CD69
403 FITC (clone FN50), anti-CD95 APC (clone DX2); with the Miltenyi Biotec antibody anti-CD8
404 V450 (clone BW138/80) (Miltenyi Biotec GmbH, Bergisch Gladbach Germany); with the
405 CliniSciences antibody anti-CD28 (clone 25-0289-73) (CliniSciences, Nanterre, France).
406 Panel 2, targeting CCR5 and CXCR3 expression on T cells, was constituted with the same
407 antibodies against CD45, CD3, CD4, CD8, CD11b and HLA-DR, and with the BD
408 Biosciences antibodies anti-CCR5 APC and anti-CXCR3 FITC (IgG1, clone 1C6).

Corresponding isotype controls of CCR5 and CXCR3 were used at the same concentrations as the reference antibody. Panel 3, targeting LFA-1 and Mac-1 expression on T cells, was constituted with the same antibodies against CD45, CD3, CD4, CD8, CD11b and HLA-DR, and with the BD Biosciences antibodies anti-CD11a PE (IgG1, clone HI111) and anti-CD18 APC (IgG1, clone 6.7). Corresponding isotype controls of CD11a and CD18 were used at the same concentrations as the reference antibody.

Acquisition was performed on BD LSRII equipped with 4 lasers (355, 405, 488 and 633nm) and analyzed by Flowjo v7.6 (Tree Star, Ashland, OR).

Ex vivo stimulation and intracellular CD45RA, IFN-γ, TNF-α, MIP-1β, and IL-2 staining on peripheral blood and semen mononuclear cells

Briefly, $1x10^6$ PBMCs were incubated for 1 h at 37°C in 5% CO2 with medium alone, staphylococcus enterotoxin B (2µg/100 µL), or a commercial pool of SIV gag peptides (89 peptides from p15 and p27, ProteoGenix, Schiltigheim, France) at the concentration of 0.2 µg/100µL, in presence of the co-stimulatory antibodies CD28 (clone L293, IgG1) and CD49d (clone L25, IgG2b). Semen cells were split in two vials and incubated 1 h with medium alone or the SIV gag peptides pool/co-stimulatory Abs. Brefeldin A (BD Biosciences) was then added (1µg/100µL), and samples were incubated for 4 h, permeabilized, and stained with combinations of CD45-PerCp (clone D058-1283, IgG1), CD3-APC-Cy7 (clone SP34-2, IgG1), CD8-V500 (clone RPA-T8, IgG1), CD45RA-PE-Cy7 (clone L48, IgG1), CD154-FITC (clone TRAP1, IgG1), IL-2-APC (MQ1-17H12, IgG2a), MIP-1β-PE (clone D21-1351, IgG1), TNF-α-Alexa Fluor 700 (clone Mab11, IgG1) and IFN-γ-V450 (clone B27, IgG1) (BD Biosciences). All antibodies used in this panel were from BD Bioscience. In PBMCs, a positive response was defined as an SIV-specific response if: 1) the response in Gag-stimulated cells was at least 2-fold higher than the unstimulated control and 2) the $CD8^+$ SIV-specific response frequency was of >0.1%; in semen, a positive response was defined as

specific if: 1) more than 500 CD8+ T cells were acquired, 2) the frequency of positive cells was of >1%.

Cytokines quantification in semen

Concentration of IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23, IL-13, IL-15, IL-17, IL-18, sCD40L, GM-CSF, G-CSF, TGFα, IFNγ, MIP-1α, MIP-1β, MCP-1, TNFα and VEGF were measured in seminal plasma using the Milliplex® Map Non-Human Primate Cytokine Magnetic Bead Panel - Premixed 23-plex (Merck Millipore, Darmstadt, Germany). Levels of RANTES were and TGF-β1, 2 and 3 were mesured by monoplex anda 3-plex Milliplex kits. Assays were performed in duplicates using 25 µL of seminal fluid. Samples were thawed at room temperature and spinned for 10 min at 1,500g to harvest any cellular component. Immunoassays were performed according to the manufacturer's instructions. Data was acquired by a Bio-Plex Instrument 200 and analyzed using the Bio-Plex Manager Software version 6.1 (Bio-Rad, Hercules, USA).

Data visualization and statistical analysis

All data visualization and statistical analysis were carried out with GraphPad Prism 5.03 software (GraphPad software, La Jolla, USA). Nonparametric Spearman rank correlation test was used to investigate the relationship between parameters. The nonparametric Mann-Whitney test was used to compare different groups of macaques, and the nonparametric Wilcoxon rank sum test was used to compare data from same macaques at different time points before and after SIV infection. P values of 0.05 or lower in 2-tailed tests were considered significant, *:$p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$

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636

637 Figure legends

638 **Figure 1. Viral loads, IgG anti-SIV titers and semen T cells during SIVmac251 infection**
639 **in cynomolgus macaques**

640 (A-C) Longitudinal follow-up of viral loads, IgG targeting SIV/HIV-2 and semen $CD4^+$ and
641 $CD8^+$ T cells dynamics during the first months of SIV infection, in 8 macaques infected
642 intravenously with 5,000 Animal Infectious Dose 50 (AID50). Mean and SEM are
643 represented. (A) Blood and semen viral RNA loads (BVL -▲, plain line- and SVL -▼, dotted
644 line- respectively). (B) IgG targeting SIV titer in blood serum (◆, plain line) and seminal
645 plasma (■, dotted line) (n=6 macaques). Cut off was calculated separately for blood and
646 semen, from mean titer values at baseline. (C) Percentage of semen $CD4^+$ (●) and $CD8^+$ (●)
647 T cells on total $CD45^+$ leukocytes. Significant differences with baseline are indicated

(Wilcoxon matched-pairs signed rank test, *p<0.05, **p<0.01. (D) Blood (●) and seminal plasma (□) titers in SIV-specific IgG for each macaque (the 2 titer values are connected by a plain line) at baseline (n=6), at one month post-infection (n=12) and at chronic stage (n=9). Maximal titer was extrapolated at 8,000. (E) SIV-specific IgG titers in blood serum (plain lines) and seminal plasma (dotted lines) of 6 macaques, each symbol representing an animal. (F-G) Percentage of $CD4^+$ and $CD8^+$ T cells on total $CD45^+$ cells in semen (F) and peripheral blood (G), in uninfected (○, n=12), and SIV^+ macaques at 14 dpi (▲, n=6) and at chronic stage (◆, n=12). Lines represent mean and SEM and significant differences between groups are represented (Mann-Whitney test, *p<0.05 and **p<0.01).

Table 1. Seminal levels of 28 cytokines and chemokines.

Levels of these molecules were measured in the seminal plasma of 26 SIV^- macaques.

Figure 2. Cytokines and chemokines affected by SIV infection in macaques

(A) Mean and SEM of 28 cytokines and chemokines levels in seminal plasma of 26 uninfected macaques (n=17 for RANTES, 15 for TGF-β and 12 for IP-10). (B) Mean and SEM of 8 pro-inflammatory molecules in SIV^- macaques (n=12) and SIV^+ macaques at primary stage (10 or 14 dpi, n=10) and at chronic stage (n=16). Lines represent mean and SEM and significant differences between groups are represented (Mann-Whitney test, *p<0.05, **p<0.01 and ***p<0.001). (C-E) Longitudinal follow-up of seminal plasma levels of cytokines and chemokines and BVL (▲) and SVL (▼) (both are represented by dotted lines) in 13 macaques infected with 5,000 AID50 IV (n=13 at baseline, 10 at 10 dpi, 4 at 14 dpi, 7 at 28, 35 and 87 dpi, 6 at 148 dpi and 5 at 171 dpi). Mean and SEM are represented for each time point. (C) Levels of 5 pro-inflammatory molecules correlated to BVL and SVL: IL-8 (dark red), IL-15 (red), IL-18 (light brown), IL-6 (orange), IL-1β (beige). (D) Levels of 3 Th2

673 cytokines correlated to BVL and SVL: IL-4 (light purple), IL-5 (dark purple) and IL-13 (pink).
674 (E) Levels of 3 Th1 cytokines that are not correlated to BVL or SVL: TNF-α (green), IFN-γ
675 (light blue) and IL-2 (dark blue). (F) Individual variations of TNF-α level in seminal plasma of
676 4 SIV^+ macaques, each color representing an animal.

677

678 **Figure 3. Phenotype of semen $CD8^+$ T cells**

679 (A) Differentiation profile of semen $CD8^+$ T cells. Left: gating strategy, dot plots from a
680 representative SIV^+ animal. $CD95^+CD28^+$ cells are central memory (cm) and $CD95^+CD28^-$
681 are effector memory (em). Right: Percentage of each subset (cm -round dots- and em -
682 square dots- $CD8^+$ T cells) in SIV^- (empty dots, n=9) and SIV^+ macaques (plain dots, n=17).
683 (B) CD45RA expression of semen $CD8^+$ T cells. Left: gating strategy, dot plots from a
684 representative SIV^+ animal. Right: Percentage of semen $CD45RA^+CD8^+$ T cells in SIV^-
685 (empty dots, n=4) and SIV^+ (plain dots, n=7) macaques. (C) CD69 and HLA-DR expression
686 in semen $CD8^+$ T cells. Left: gating strategy, dot plots from a representative SIV^+ animal.
687 Middle: % of $CD69^+$ cells of each subset, and right: % of HLA-DR^+ cells, of each subset (cm
688 $CD8^+$ T cells: round dots, em $CD8^+$ T cells: square dots) in SIV^- (empty dots, n=6) and SIV^+
689 (plain dots, n=17) macaques. (D) Longitudinal follow-up of percentage of $CD69^+$ $CD8^+$ T cells
690 (●: cm and ■: em $CD8^+$ T cells) and of BVL (▲) and SVL (▼), in 8 macaques infected with
691 5,000 AID50 IV. Significant differences with baseline are represented (Wilcoxon matched-
692 pairs signed rank test, *$p<0.05$). (E) Spearman correlation between % of $CD69^+$ $CD8^+$ T cells
693 in blood and semen; left: cm cells, right: em cells; n=15 chronically infected macaques. (F)
694 CCR5 and CXCR3 expression on semen $CD8^+$ T cells. Left to right: gating strategy, dot plots
695 from a representative SIV^+ animal with overlays of CCR5 and CXCR3 expression (black
696 lines) on isotype control (plain grey curves); % of $CCR5^+$ (round dots), $CXCR3^+$ (square dots)
697 and $CCR5^+$ $CXCR3^+$ cells (diamond dots) in SIV^- (empty dots, n=6) and SIV^+ (plain dots, n=6)
698 macaques. (G) LFA-1 integrin ($CD11a^+CD18^+$) expression on semen $CD8^+$ T cells. Left to

699 right: gating strategy, dot plots from a representative SIV^+ animal with overlays of CD11a and
700 CD18 expression (black lines) on isotype control (plain grey curves); % of $LFA\text{-}1^+$ (round dots
701 and $CD11a^+CD18^-$ cells (square dots) in SIV^- (empty dots, n=8) and SIV^+ (plain dots, n=8)
702 macaques. Mean and SEM are represented.

703

704 **Figure 4. Th1 profile of semen $CD8^+$ T cells**

705 (A-C) Intracellular staining for Th1 cytokines in blood and semen $CD8^+$ T cells, in response of
706 a 5h-stimulation with SIV *gag* peptide, or without stimulation (mock). (A) IFN-γ and TNF-α
707 expression in semen CD8+ T cells of all studied animals (n=6), with (down) or without (up)
708 stimulation with *gag*. (B) Polyfunctionality of semen $CD8^+$ T cells, with (black bars) or without
709 stimulation (mock, empty bars), in 2 chronically infected macaques displaying SIV-specific
710 CD8 T cells (#30690 and #31044). IL-2 was not included, because not expressed in relevant
711 amounts. (C) Association between viral loads in semen (up, half-plain dots) and in blood
712 (down, plain dots) and percentage of, left to right: IFN-γ, TNF-α and MIP-1β.

713

Supplementary figure legends

Supplementary figure 1: Correlation between percentage of $CD8^+$ T cells on total $CD45^+$ cells in semen and peripheral blood

(A) Spearman correlation between blood vRNA load (BVL) and percentage of $CD8^+$ T cells on total semen $CD45^+$ cells. (B) BVL and percentage of $CD8^+$ T cells on total blood $CD45^+$ cells are not correlated.

Supplementary figure 2: Seminal inflammatory molecules correlated to BVL and SVL

(A-F) Spearman correlation between seminal plasma levels of 6 pro-inflammatory cytokines and chemokines and BVL (left column) and SVL (right column). (A) IL-1β. (B) IL-6. (C) IL-8. (D) IL-15. (E) IL-18. (F) RANTES.

Supplementary figure 3: Seminal Th2 cytokines correlated to BVL and SVL

(A-C) Spearman correlation between seminal plasma levels of 3 Th2 cytokines and BVL (left column) and SVL (right column). (A) IL-4. (B) IL-5. (C) IL-13.

Supplementary figure 4: Correlation between seminal TGF-β and BVL and/or SVL

(A-C) Spearman correlation between seminal plasma levels of TGF-β1-3 and BVL (left column) and/or SVL (right column). (A) TGF-β1. (B) TGF-β2. (C) TGF-β3.

Supplementary figure 5: Phenotype of blood $CD8^+$ T cells and comparison with semen

(A) Differentiation profile of peripheral whole blood $CD8^+$ T cells. Left: gating strategy, dot plots from a representative SIV^+ animal. $CD95^-CD28^+$ cells are naïve, $CD95^+CD28^+$ cells are

central memory and $CD95^+CD28^-$ are effector memory. Right: Percentage of cm (round dots) and em (square dots) $CD8^+$ T cells, in blood (half-plain dots) and in semen (plain dots) of 12 uninfected macaques. (B) CD45RA expression of peripheral blood $CD8^+$ T cells. Left: gating strategy, dot plots from a representative SIV^+ animal. Right: Percentage of semen $CD45RA^+CD8^+$ T cells in SIV^- (empty dots, n=4) and SIV^+ (plain dots) macaques in blood (empty plots, n=10 SIV^+ animals) and semen (plain dots, n=7 SIV^+ animals). (C-E) Comparison of CD69 and HLA-DR expression on $CD8^+$ T cells in semen and in peripheral blood. (C) Gating strategy in blood cells, dot plots from a representative SIV^+ animal. (D) Left: % of $CD69^+$ cells of each subset, and right: % of HLA-DR^+ cells, of each subset (cm $CD8^+$ T cells: round dots, em $CD8^+$ T cells: square dots) in blood (half-plain dots, n=12) and semen (plain dots, n=12) macaques. (E) Left: % of $CD69^+$ cells of each subset, and right: % of HLA-DR^+ cells, of each subset (cm $CD8^+$ T cells: round dots, em $CD8^+$ T cells: square dots) in blood, in uninfected (empty dots, n=6) and SIV+ (plain dots, n=15) macaques.

Supplementary figure 6: CCR5, CXCR3 and LFA-1 expression by semen $CD4^+$ T cells

(A) Percentage of $CCR5^+$ (round dots), $CXCR3^+$ (square dots) and $CCR5^+$ $CXCR3^+$ cells (diamond dots) in SIV^- (empty dots, n=6) and SIV^+ (plain dots, n=4) macaques. (B) Percentage of LFA-1^+ (round dots) and $CD11a^+CD18^-$ cells (square dots) in SIV^- (empty dots, n=8) and SIV^+ (plain dots, n=7) macaques. Mean and SEM are represented and significant differences between groups are specified (Mann-Whitney test, **p<0.01).

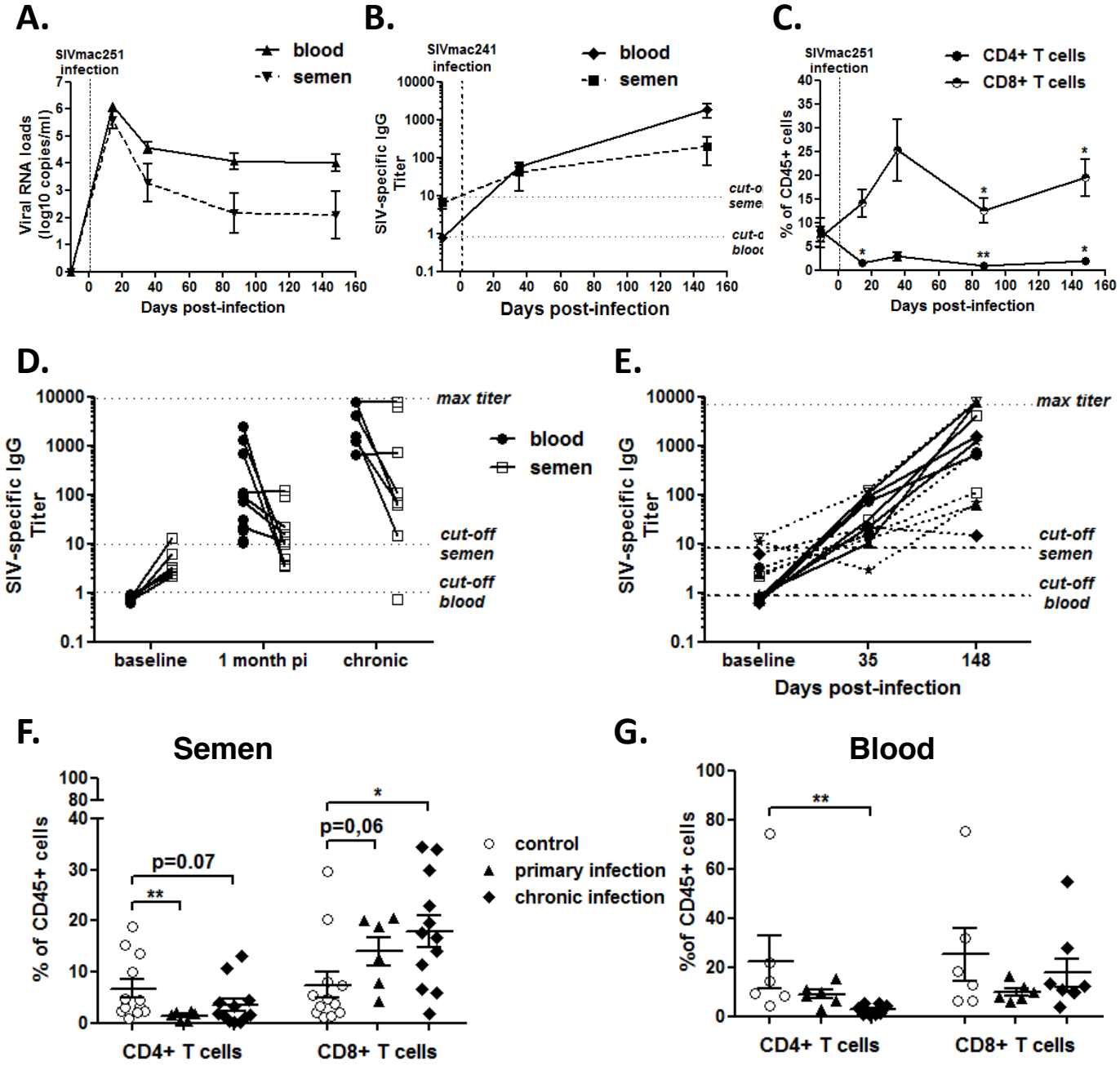


Figure 1. Viral loads , IgG anti-SIV titers and semen T cells during SIVmac251 infection in cynomolgus macaques

Table 1. Seminal levels of 28 cytokines and chemokines

	Mean (pg/ml)	S.E.M	Minimum	Maximum	Lower 95% CI of mean	Upper 95% CI of mean
Molecules affected by SIV infection						
IL-1b	5.732	1.058	0,00	22.64	3.553	7.911
IL-13	10.91	1.484	0,00	33.84	7.853	13.97
IL-5	12.36	1.373	2.61	29.1	9.53	15.19
IFNγ	25.02	5.155	0.22	120.1	14.4	35.64
IL-6	40.95	8.252	0,00	175.1	23.95	57.94
IL-4	51.29	9.282	0,00	215,00	32.17	70.41
TNF-α	77.3	35.19	0,00	879.7	4.828	149.8
IL-18	94.35	20.12	0,00	330.1	52.91	135.8
RANTES	317.2	50.35	16.12	753.4	210.4	423.9
IL-15	368.6	58.91	13.2	1338,00	247.2	489.9
TGF-β3	684.7	150.2	123.4	2130,00	362.5	1007,00
IL-8	4754,00	1346,00	61.27	26128,00	1982,00	7525,00
TGF-β1	6255,00	2485,00	0,00	35063,00	925.5	11584,00
MCP-1	16174,00	3520,00	104.3	56380,00	8893,00	23456,00
TGF-β2	22465,00	6887,00	5256,00	91756,00	7694,00	37236,00
Molecules unchanged by SIV infection						
IL-17	7.324	1.265	0,00	29.58	4.718	9.931
IP-10	8.745	3.702	0,00	38.62	0.5981	16.89
GM-CSF	18.53	2.701	1.71	58.09	12.97	24.1
MIP-1α	28.8	8.039	0,00	176.7	12.24	45.36
IL-2	32.03	4.885	3.05	106.2	21.97	42.09
TGF-α	36.72	7.067	2.35	187,00	22.17	51.28
sCD40L	60.29	13.36	3.72	357.5	32.77	87.81
IL-12/23	64.96	7.068	21.98	164,00	50.4	79.52
MIP-1β	115.5	30.22	0,00	587.7	53.28	177.7
IL-10	248.7	63.95	9.7	1664,00	117,00	380.4
G-CSF	290.4	58.76	24.48	1410,00	169.4	411.4
IL-1RA	551.4	80.77	65.39	1647,00	385,00	717.7
VEGF	46276,00	11649,00	4323,00	299477,00	22285,00	70268,00

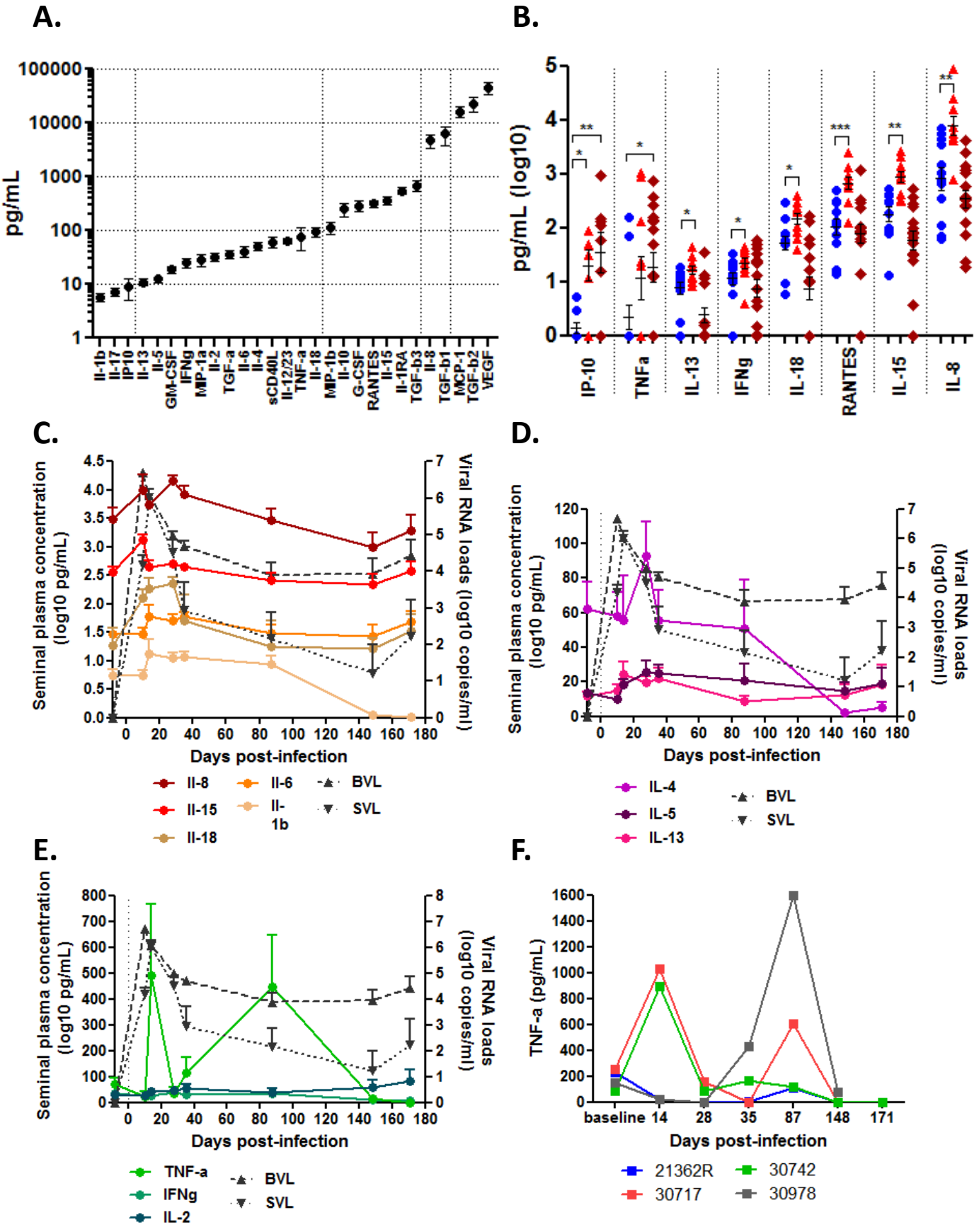


Figure 2. Cytokines and chemokines affected by SIV infection in macaques

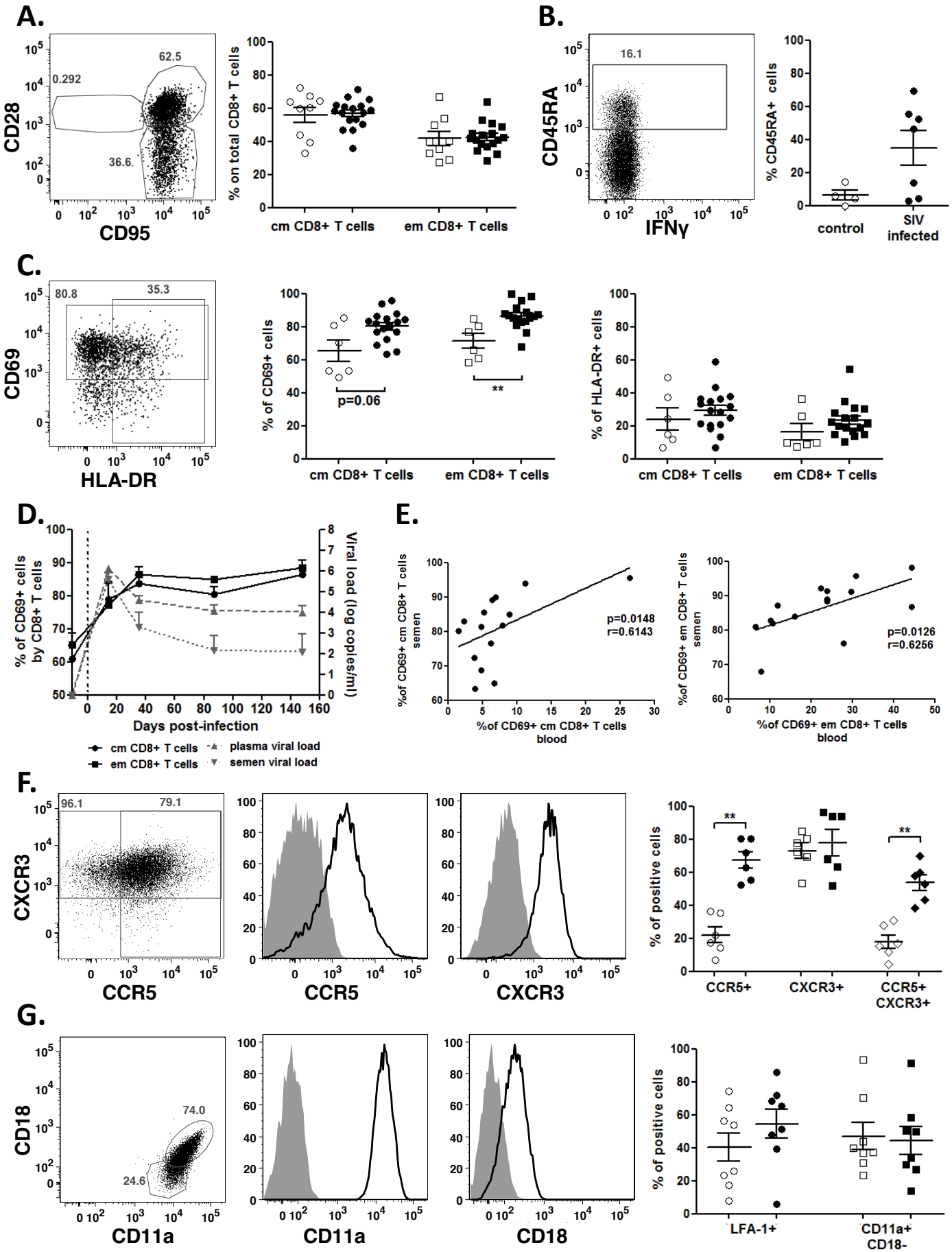


Figure 3. Phenotype of semen CD8+ T cells

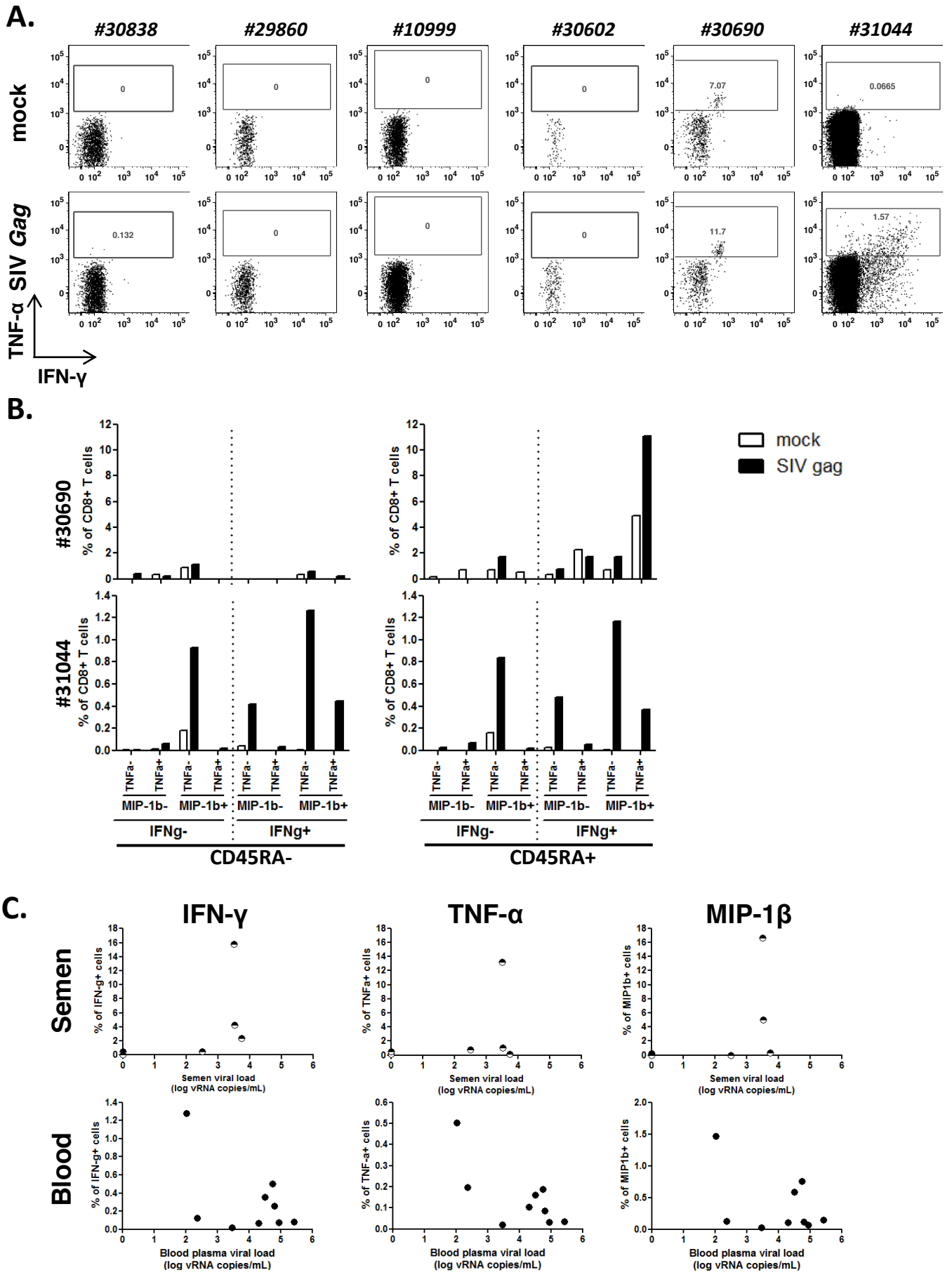
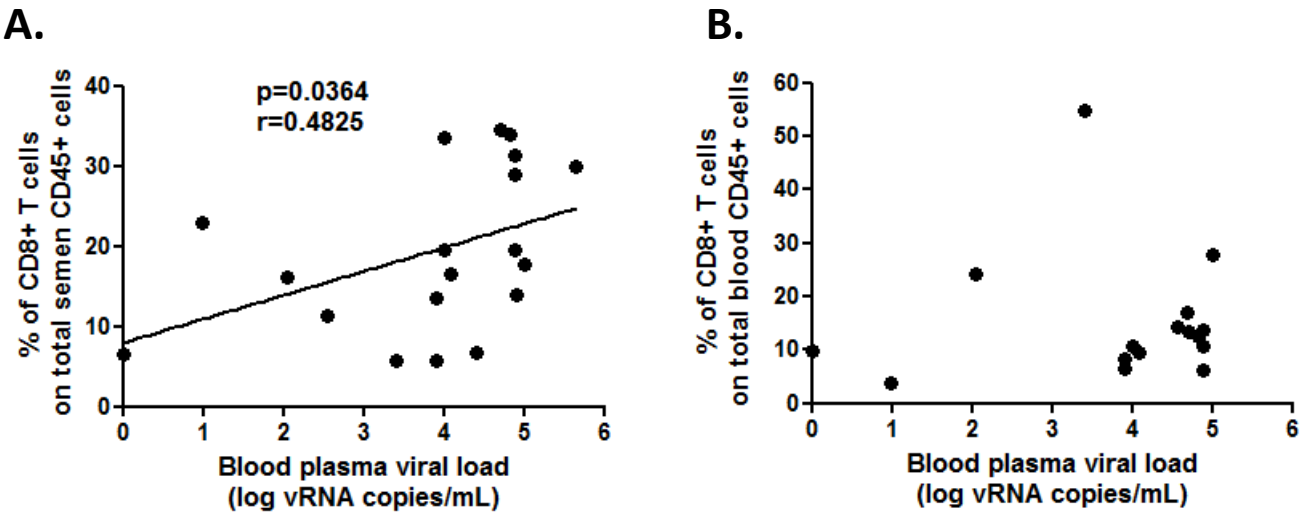
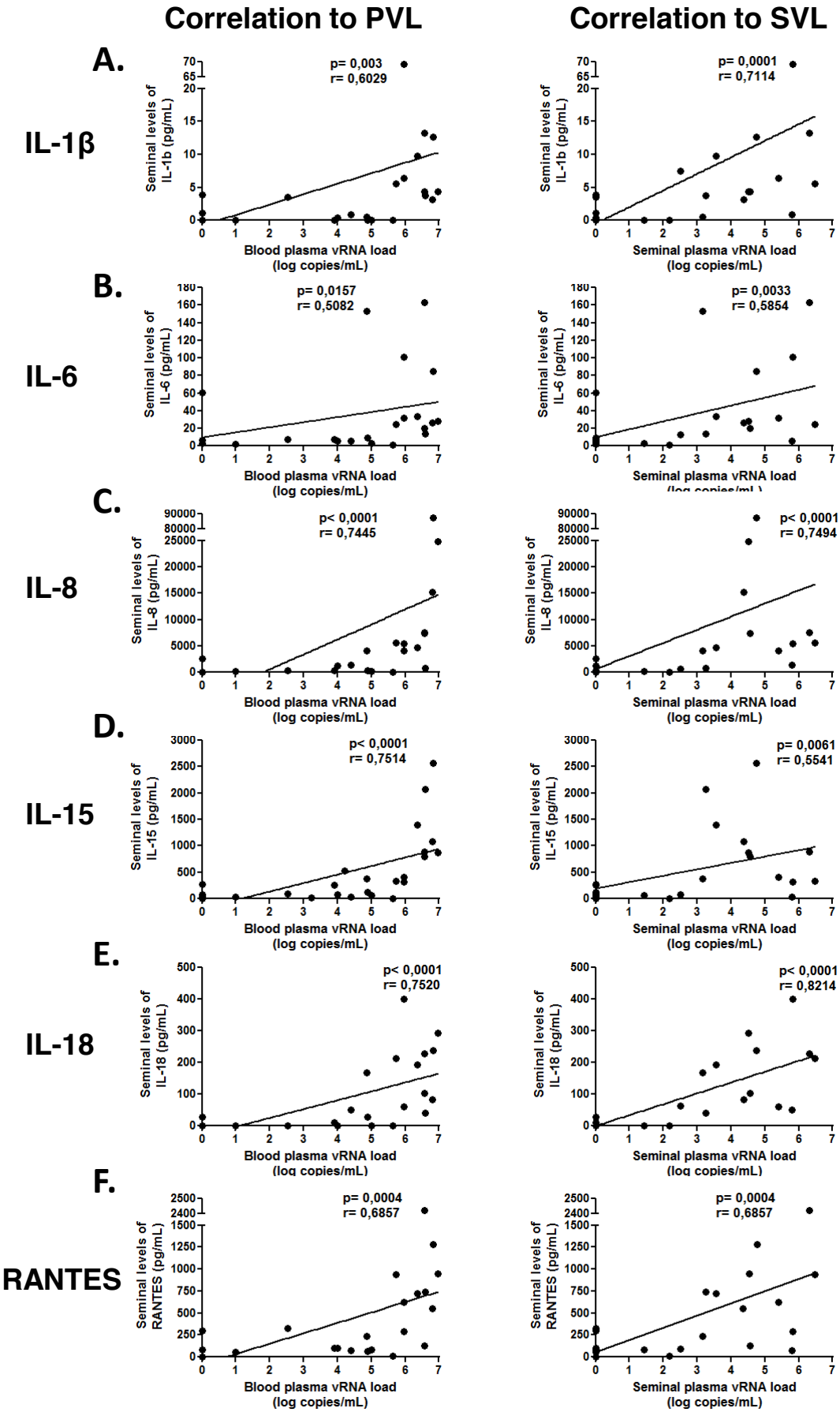


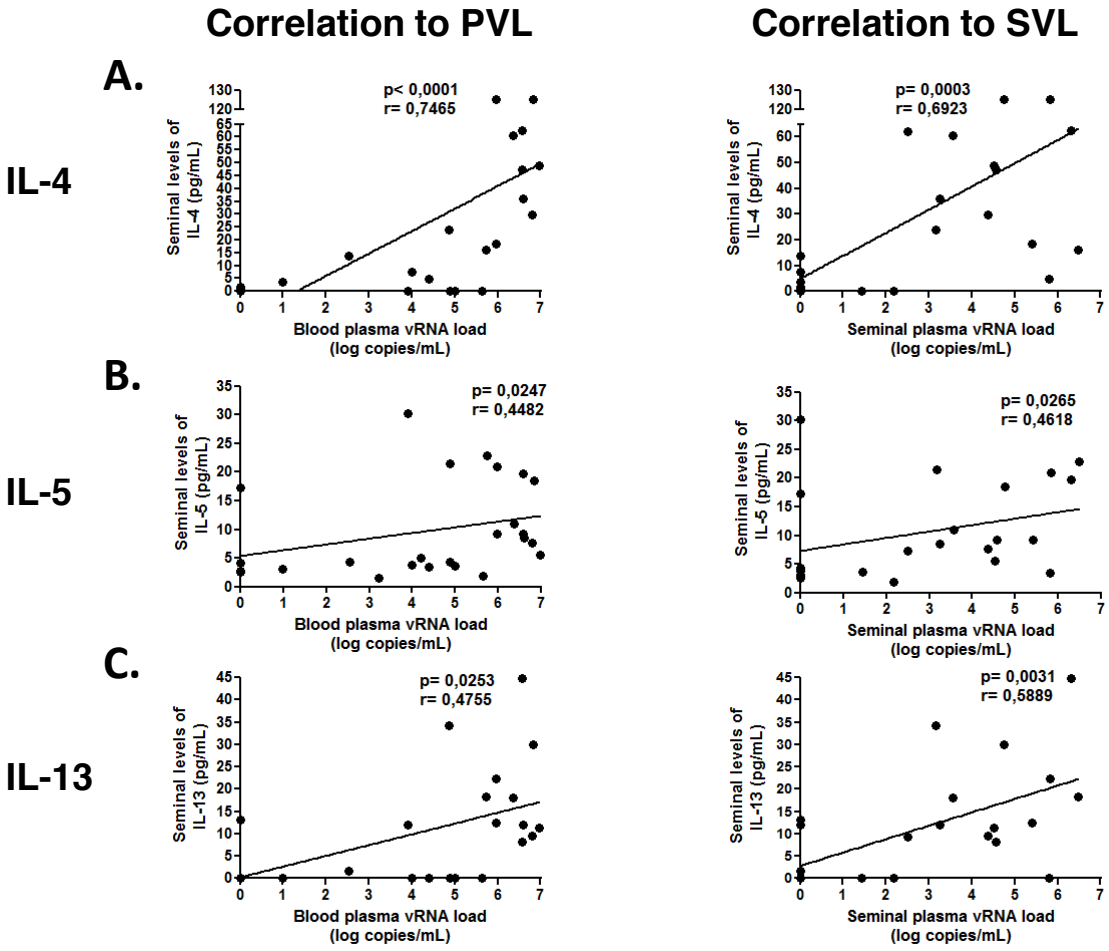
Figure 4. SIV-specific CD8⁺ T cells response in semen



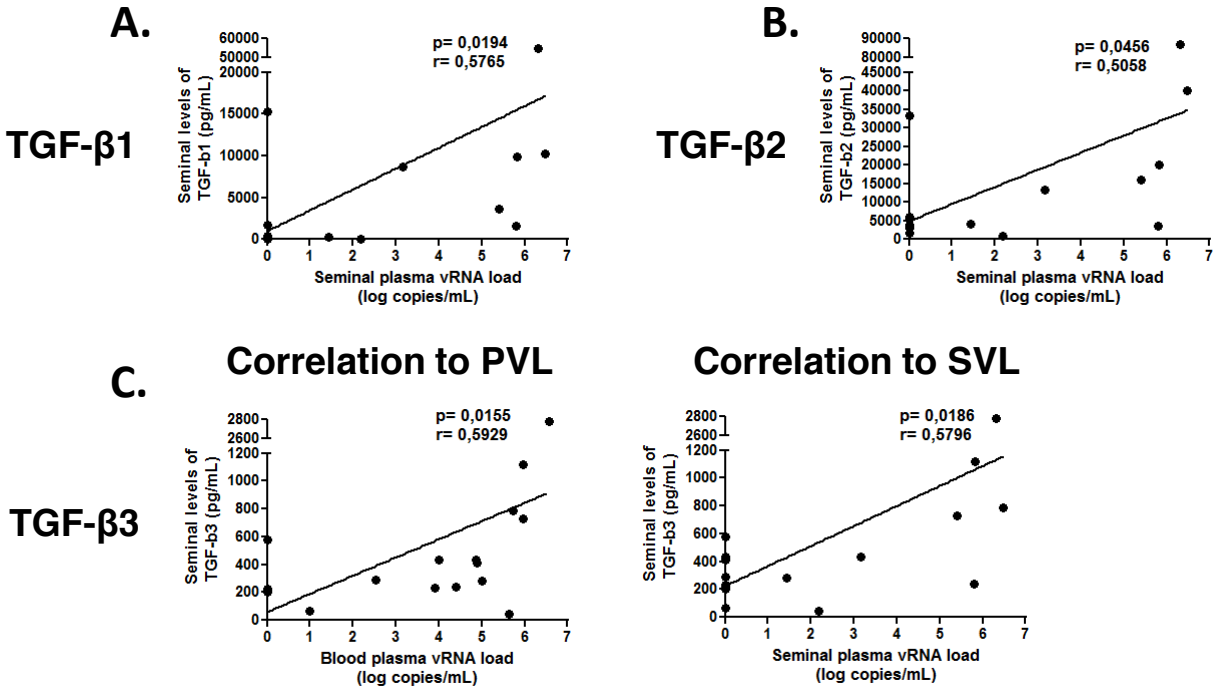
Supplementary figure 1. Correlation between percentage of $CD8^+$ T cells on total $CD45^+$ and BVL in semen and blood.



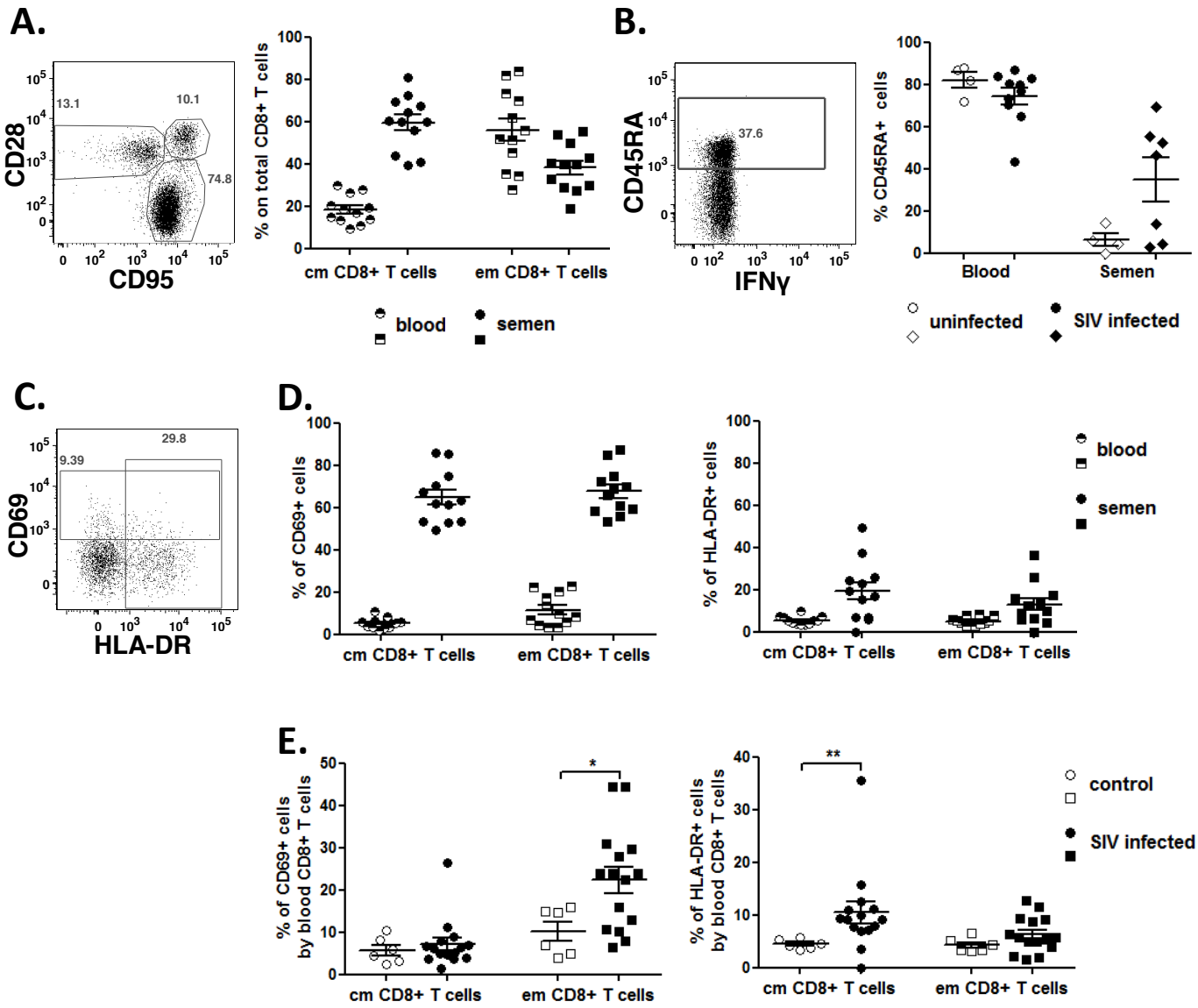
Supplementary figure 2. Seminal inflammatory molecules correlated to PVL and SVL



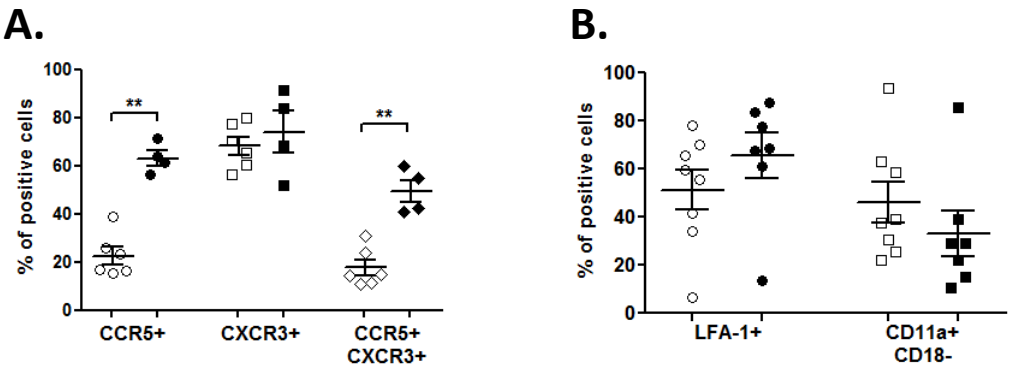
Supplementary figure 3. Seminal Th2 cytokines correlated to PVL and SVL



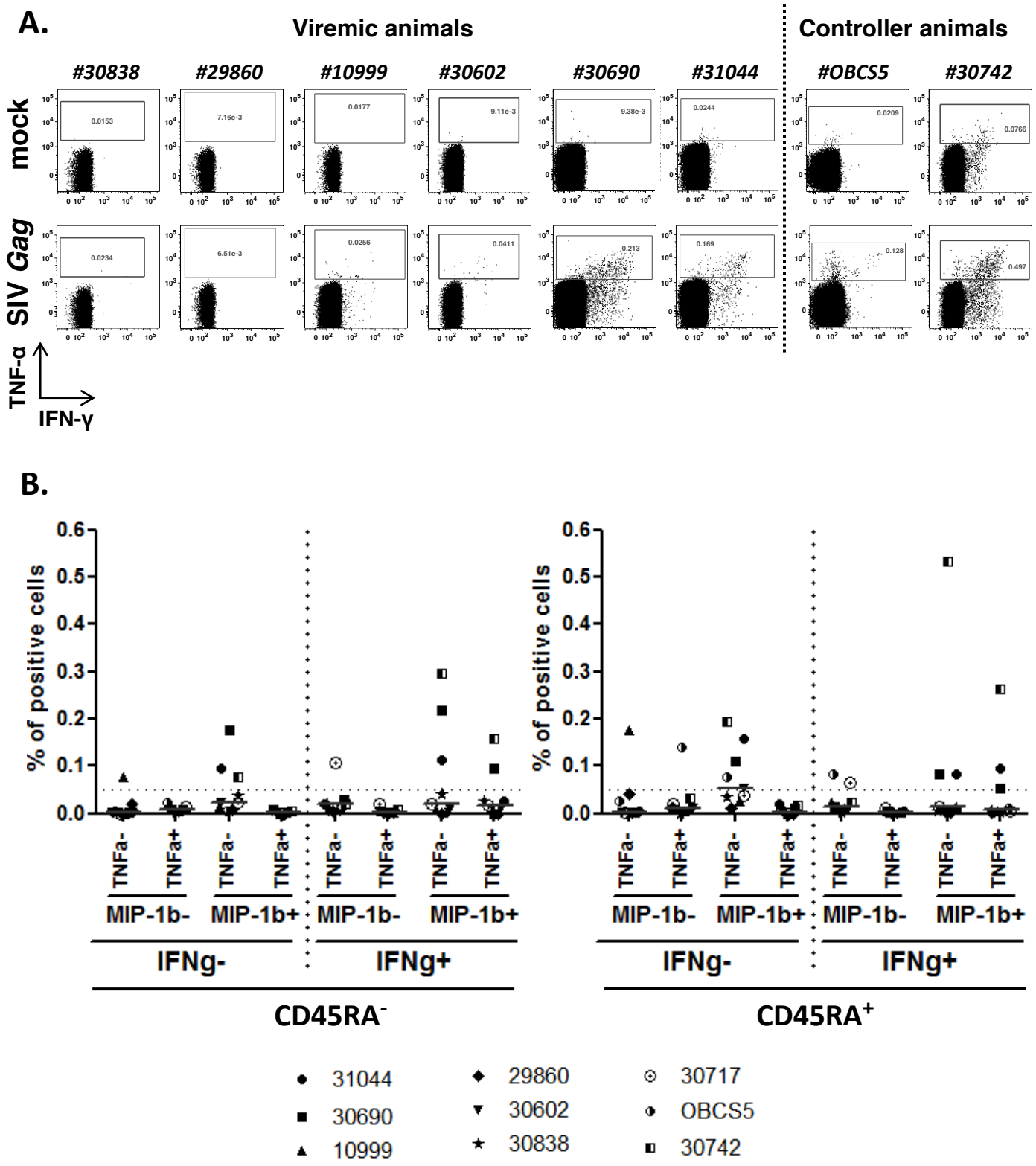
Supplementary figure 4. Correlation between seminal TGF-β and BVL and/or SVL



Supplementary figure 5. Phenotype of blood CD8⁺ T cells and comparison with semen



Supplementary figure 6. CCR5, CXCR3 and LFA-1 expression by semen $CD4^+$ T cells



Supplementary figure 7. SIV-specific CD8⁺ T cells in blood

III. Complementary results: *in vivo* assay of semen leukocytes infectivity

Our study of the capacity of semen leukocytes to transmit infection was pursued by inoculating sorted semen cells by mucosal route to macaques.

Two distinct groups of macaques were included in this study:

- **The "semen donors" group**: leukocytospermic adult male macaques were included in this group, in order to maximize the chances to get a consistent number of leukocytes from their semen samples,
- **The "semen cells receivers" group**: adult macaques, which were inoculated with the sorted semen leukocytes, by rectal or vaginal routes.

The CMH haplotype of each macaque included in this study was known, and animals were dispatched in each group of "semen givers" and "semen cells receivers" in order to avoid:

- Animals of M6-haplotype, which is associated with a control of HIV replication (Aarnink *et al.*, 2011a; Aarnink *et al.*, 2011b; Mee *et al.*, 2009),
- CMH haplotypes matching between the semen giver and the semen cells receivers.

In order to identify the semen leukocytes that are infected and test their capacity to transmit infection *in vivo*, we developed a flow cytometry cell sorting technique.

Since the yield of isolation of target cells is quite low with this technique, only leukocytospermic animals were used. Nevertheless, the number of total isolated cells was limited, especially $CD4^+$ T cells, which are profoundly depleted in infected animals, which constitutes one major limitation of this technique.

A. Modeling HIV mucosal transmission by inoculation of sorted semen leukocytes

1) Inoculation of sorted cells

Our objective was to assess the infectivity *in vivo* of the leukocyte population in semen, and in particular of $CD4^+$ T cells and macrophages. Importantly, the purity of the inoculated cells was one major criterion, in order to avoid contamination with spermatozoa. Indeed, the potential role of spermatozoa as a mobile vector for HIV has been recently proposed, since it is now established that they can carry free particles attached on the cell surface (Ceballos *et al.*, 2009). In these conditions, the best technique to isolate the cellular fraction of interest was flow cytometry sorting.

However, it has been demonstrated that flow cytometry cell sorting represents a traumatic event to the cells, inducing cellular stress, activation and increased mortality (Muller and Nebe-von-Caron, 2010). In the context of this study, we can hypothesize that infected cells (especially $CD4^+$ T cells) may be more fragile and therefore suffer a higher mortality rate than other cells, thereby limiting the number of sorted functional and infectious cells. Nevertheless, sorted CD4+ T cells conserved their infectiousness *in vitro*.

Before the step of cell sorting by flow cytometry, we enriched the total cell fraction in leukocytes using magnetic beads sorting, with a positive enrichment of $CD45^+$ cells. Both steps are known to induce important loss of cells of interest. As much as 50% of the leukocytes were lost during the process. Since the leukocytes counts in semen are already limited, the low yield of this cell sorting technique represents an important limitation.

2) Repeated, low-dose mucosal challenges

Even if the number of inoculated cells was much lower than the leukocytes counts in an ejaculate, our system of repeated, low-dose mucosal challenges was close to the usual conditions of a HIV sexual transmission.

Nevertheless, the repeated inoculations of cells raise the problem of alloimmunization of the receiver against the MHC of the different donors. Indeed, in human, the study of monogamous couples practicing unprotected sex demonstrated the existence of a reciprocal alloimmunization against the partner cells. This phenomenon conferred *in vitro* an increased protection of the $CD4^+$ T cells against the infection with HIV (Peters *et al.*, 2004). The same phenomenon has been described in rhesus macaques inoculated repeatedly by vaginal or

rectal route with PBMCs from different donors. This alloimmunization is dose-dependent and confers a protection of the receiver's CD4+ T cells against SIV infection *in vitro* (Bergmeier *et al.*, 2005).

Therefore, the repeated inoculation of a very small number of infected cells may actually initiate an immune response in the receiver animal that may confer a protection against infection by the cell-associated virus.

3) Depo-Provera treatment of the inoculated females

As mentioned earlier (see Introduction, Chapter II), the treatment of women and female macaques with Medroxyprogesterone acetate (DPMA, Depo-Provera®) induces a thinning of the vaginal epithelium, mimicking the luteal phase of the menstrual cycle (Bahamondes *et al.*, 2000; Hild-Petito *et al.*, 1998; Mauck *et al.*, 1999). Progesterone implants drastically enhance the susceptibility to SIV infection in macaques exposed vaginally (Marx *et al.*, 1996).

Furthermore, DMPA treatment induces changes in mucosal immune system, notably on NK and T cells activation (Genesca *et al.*, 2007). The genetic diversity of the transmitter founder virus population is higher in treated female macaques, suggesting an increase of the number of variants that cross the epithelial barrier.

Nevertheless, we considered that the potential bias introduced by DMPA treatment was not a major barrier to our study, and we decided to treat our receiver females.

4) Absence of seminal fluid within the inoculum

Since our objective was initially to test the capacity of semen leukocytes to transmit infection *in vivo*, we decided to exclude any source of modulation of leukocytes infectivity. In this condition, the adjunction of seminal plasma to the inoculum might constitute a source of undesired complexity.

Notably, seminal plasma contains certain factors programing cellular death, and thereby displays cytotoxicity *in vitro* (Okamoto *et al.*, 2002). However, that has not been demonstrated *in vivo* yet, and we may hypothesize that the dilution of seminal plasma by vaginal or rectal secretions may decrease its cytotoxicity.

B. Single rectal inoculation of purified semen CD4$^+$T cells and macrophages

In a first study, six macaques (#30602, #30690, #31044, #10999, #29860 and #31052) were infected intravenously with 5,000 AID50 of SIVmac251. Blood viral loads remained elevated during the study, whereas different profiles were observed in semen viral loads (Figure 30). Since we observed that at 10 dpi, macaques display frequently leukocytospermia and that semen CD4$^+$ T cells are not yet depleted, unlike in peripheral blood, we purified CD4$^+$ T cells and macrophages from the semen of macaques at this time point.

Each semen leukocytes fraction (CD4$^+$ T cells or macrophages) was purified from three donors (animals #30602, #30690 and #31044) and the cells were pooled together. Each pool (the CD4$^+$ T cells pool and the macrophages pool) was inoculated by rectal route to a male macaque.

A small part (10,000 to 20,000 cells) of each sorted fraction was used to perform quantitative real-time PCR on the cell pellet, in order to quantify the number of SIV DNA copies per cell.

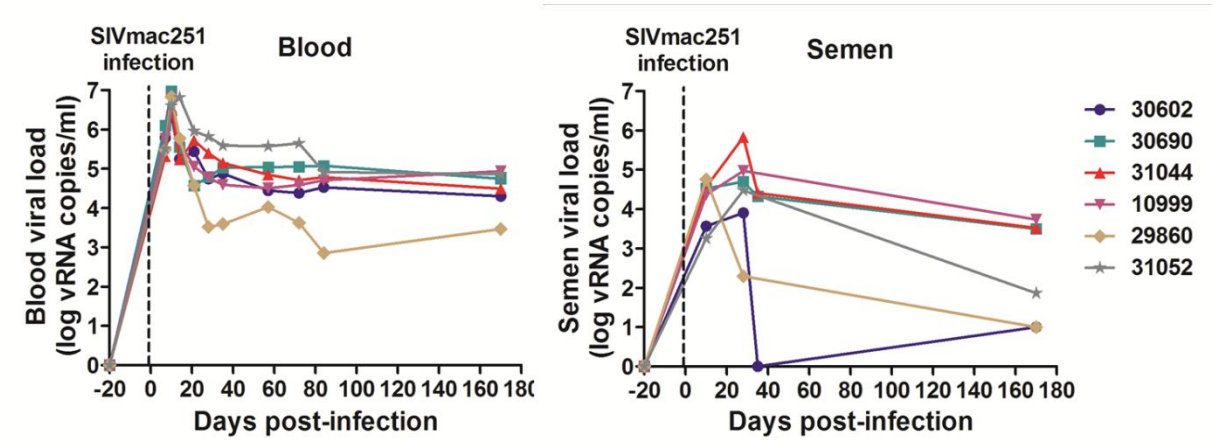


Figure 30 : Blood and semen viral RNA loads in the "semen donor" group.

As a control, the last wash supernatant of each cell fraction was inoculated to an other macaque, in order to invalidate the hypothesis of a transmission by cell-free particles present in the supernatant, and not by the semen cells.

Only a small number of cells were obtained and pooled from the three first macaques (#30602, #30690 and #31044): a total of 33,708 CD4+ T cells and 156,406 macrophages were inoculated to the two receivers animals. Among the two fractions of 10,000 sorted cells that we kept for qRT-PCR, SIV DNA was not detected (Table 4).

Unfortunately, no event of transmission was observed. The blood plasma RNA viral load was measured once weekly during the first month after inoculation, and the presence of vRNA was assayed by qRT-PCR in the peripheral lymph nodes after one month post-inoculation. Finally, we performed an ELISA test on serum samples to measure the SIV-specific IgG titers. All results were negative.

The second sorting experiment from the semen of the three other donor macaques (#10999, #29860 and #31052), the same order of magnitude in number of sorted cells was obtained for both fractions. We then decided to not inoculate them because of these two small pools of cells, and to adapt our protocol of experiments.

C. Repeated rectal inoculations of sorted semen leukocytes

Indeed, we decided to adjust the study protocol, using the same 6 semen-donors macaques (during the first three months of infection):

- the total $CD45^+$ leukocytes fraction was sorted in order to minimize the number of cell loss, pooled from 3 infected macaques, and inoculated to one receiver macaque by rectal route,
- Inoculations were repeated every two weeks, using the same semen cells donors for each receiver macaque.

In total, three inoculations were performed per semen cells-receiver, with a mean of 300,000 $CD45^+$ cells inoculated per challenge (Table 4).

None of the two macaques inoculated with the sorted semen $CD45^+$ cells displayed detectable viremia or seroconversion after three months post-inoculation.

Experiment	Semen cells givers ID	CD4+ T cells fraction (number of sorted cells)	Macrophages fraction (number of sorted cells)	CD45+ cells fraction (number of sorted cells)	SIV DNA detection
1	30602, 30690, 31044	33,708	156,406		negative
	10999, 29860, 31052	21,654	252,010		positive
2	10999, 29860, 31052			110,000	positive
3	30602, 30690, 31044			320,115	positive
	31044, 30717, 30838			345,000	positive
	31044, 30717, 30838			253,841	positive
	31044, 30717, 30838			310,803	négative
	31044, 30717, 30838			300,442	négative

Table 4 : Summary of the two first *in vivo* experiments (rectal exposure).

D. Repeated vaginal inoculations of sorted semen leukocytes

In a third study that is currently in progress, semen $CD45^+$ cells from 4 SIV^+ macaques are sorted, pooled together and inoculated vaginally to female macaques. The females will be previously treated with Depo-Provera in order to induce a thinning of the vaginal epithelial barrier.

Twice weekly repeated inoculations, with a total of 6 consecutives challenges, will be done.

Discussion and perspectives

I. Validation of the experimental approach

A. Relevance of the experimental infection of cynomolgus macaque with SIVmac251 for studying semen virological parameters

Besides the high similarities in the systemic viral replication and immune-pathogenesis profiles of SIVmac251 in cynomolgus macaques, we demonstrated here that semen viral shedding is very close to what is reported in HIV^+ men:

- Blood and semen viral RNA loads are strongly correlated;
- A high diversity in semen viral shedding is observed, with macaques displaying undetectable SVL, others with intermittent and low shedding and finally some "high shedders", with elevated and constant SVL;
- Viral DNA is detected by nested PCR in a high proportion of infected macaques, at all stages of infection;
- Both $vDNA^+$ $CD4^+$ T cells and macrophages were detected.

Therefore we considered that our experimental model is relevant for studying the role of semen cell-associated virus in HIV mucosal transmission.

B. Characteristics of macaque semen and comparison with human

The volume of semen samples that we obtained from electroejaculation ranged from 100 to 500 µl. This is close to what has been described in rhesus macaques (VandeVoort, 2004). In contrast, the mean volume of a human ejaculate is 3.2 ml, with a range comprised between 100 µl and 11 ml.

The pH was slightly basic, ranging from 7 to 9, similarly to human semen of which average pH is 7.7 (Owen and Katz, 2005).

Using multi-parameters flow cytometry we studied the semen leukocytes population. As in human semen (Anderson *et al.*, 2010a; Wolff and Anderson, 1988a), polymorphonuclear cells, macrophages and T cells were the major leukocytes found in macaque semen. Small discrepancies in the relative proportions of each population were observed between our data and what has been described in human (Table 5).

Population	Human semen	Cynomolgus semen
Polymorphonuclear cells	60-70%	26% ± 6.70%
Macrophages	20-30%	22.22% ± 5.06%
Lymphocytes	4-5%	15.90 ± 4.29%*

Table 5 : Comparison between the proportion of each semen leukocyte population between human and cynomolgus macaques (semen sampling by electroejaculation). Adapted from (Anderson *et al.*, 2010a; Wolff and Anderson, 1988a). *: relative proportion of $CD3^+$ T cells.

Notably, the relative proportions in polymorphonuclear cells (PMN) and lymphocytes are quite different, with much less PMN and more T cells in macaque semen samples that in human semen. These discrepancies may be due to different causes. First, whereas the technique usually used to quantify leukocytes populations in human semen is numeration from semen cells smears, we used flow cytometry analysis. PMN may be lost during the process of cell preparation or data acquisition by the flow cytometer, or our gating strategy may somehow exclude a part of semen PMN. On the other hand, variability between human and macaques may really exist.

Finally, we measured the concentration of 28 cytokines, chemokines and growth factors in the semen of macaque. We compared our data with the levels reported in human semen, when the information was available, and found very similar orders of magnitude for most of all tested molecule.

To conclude, we considered that cynomolgus macaque semen shares enough similarities with human semen to constitute a relevant model for studying the role of cell-associated virus in HIV mucosal transmission.

II. Discussion

A. *Characterization of semen leukocytes in uninfected and SIV$^+$ cynomolgus macaques*

1) Characterization of semen leukocytes subpopulations

We demonstrate here that macaque semen is very similar to human semen. Notably, the major HIV/SIV target cells (CD4$^+$ T cells and macrophages) that are found in human semen are also present in macaque semen. They remain present at all stages of infection, despite a profound depletion of CD4$^+$ T cells.

Interestingly, the total leukocytes counts correlate with semen viral shedding, as it has been reported in HIV$^+$ patients (Anderson *et al.*, 1992). A global increase of the proportion of semen CD45$^+$ cells and of macrophages was observed during the acute infection. Concomitantly a "cytokine storm" is observed, with increased levels of pro-inflammatory cytokines and chemokines. This inflammatory state may favor the transmission events by cell-associated virus.

2) Phenotype of semen T cells

We provide here an extensive characterization of semen T cells. Since we stipulated that their phenotype may influence the ability of infected CD4$^+$ T cells to transmit infection, and the capacity of SIV-specific CD8$^+$ T cells to modulate semen infectivity, we studied their expression of differentiation, activation, migration and adhesion markers.

First, semen T cells display a profile that is typically found in mucosal resident cells. They are of memory phenotype (CD95$^+$), express in large proportions the activation markers CD69 and HLA-DR, the migration marker CXCR3 and the integrin LFA-1. In uninfected macaques, the percentage of CCR5$^+$ and CXCR4$^+$ cells is relatively low (mean of 25.77% and 15.12% positive cells, respectively) and the one of CCR5$^+$CXCR4$^+$ is even lower (around 5%). The relative low proportion of CCR5$^+$ cells is quite surprising, since mucosal T cells (especially in genital mucosa) are commonly described as expression in majority high amounts of CCR5 (Bomsel and David, 2002; Hladik *et al.*, 1999; Lederman *et al.*, 2006; Meng *et al.*, 2002; Veazey *et al.*, 2001, 2003). Interestingly, 100% of CD4$^+$ and CD8$^+$ T cells are positive for intracellular CCR5 and CXCR4. The same observation about CCR5

intracellular expression has been reported in human peripheral blood T cells (Shirvani *et al.*, 2011), even if this observation has been recently subject of controversy (Pilch-Cooper *et al.*, 2011). It may suggest that both markers are highly expressed and that their expression on the cell surface is highly regulated.

Whereas SIV infection does not affect their differentiation profile and expression of HLA-DR and LFA-1, the proportion of $CD69^+$, $CCR5^+$ and $CXCR3^+$ cells is considerably increased in infected animals. Importantly, the durable increase of $CD69^+$ cells during SIV infection may be a marker of the generalized immune activation induced by HIV/SIV infection. This suggests also that this phenomenon takes place in the male genital tract as well as in the gut or in the lymphoid organs and suggests compartmentalizing of T cells between blood and semen.

The activation of semen T cells has been also described in HIV^+ men and a correlation between the proportion of $CD69^+$ T cells and SVL has been recently reported (Sheth *et al.*, 2012). We didn't found such a correlation, probably because of the small number of studied macaques. However, we observed a correlation between the percentage of $CD69^+$ $CD8^+$ T cells in blood and in semen. Such a correlation was also found in human, but concerned the proportion of $CD45RA^-CD38^+$ $CD8^+$ T cells (Gianella *et al.*, 2012).

Moreover, the percentage of $CCR5^+CXCR3^+$ cells was significantly increased in infected animals, reaching around 60% of both $CD4^+$ and $CD8^+$ semen T cells. This phenotype is typical of Th1 T cells, which tend to migrate towards tissues where inflammation takes place (Appay *et al.*, 2008). The increase of central memory, activated $CCR5^+$ $CD4^+$ T cells in the semen of infected individuals may impact on semen infectivity, since they are the principal target for HIV/SIV and can produce large amounts of virus (Veazey *et al.*, 2003). That may also explain the profound depletion of semen $CD4^+$ T cells in infected individuals.

3) Phenotype of semen antigen-presenting cells

As mentioned before, semen APCs (characterized here by their morphology and expression of HLA-DR and CD11b) are mostly macrophages, with a small proportion of DCs. They represent a heterogeneous population. The majority of them are $CD11b^+$ $CD14^{bright}CD163^{bright}$, a typical profile of activated macrophages. All express CD4 and a large proportion of them express CCR5. This is very similar to the macrophages resident in genital tissues like the female genital tract, the urethra and the foreskin (Ganor *et al.*, 2012; Hirbod

et al., 2010; McCoombe and Short, 2006; Miller and Shattock, 2003; Patterson *et al.*, 2002), whereas intestinal macrophages express much less CCR5 and are therefore less susceptible to HIV/SIV infection (Meng *et al.*, 2000; Smith *et al.*, 2003).

Furthermore, semen macrophages express in large proportions two integrins: LFA-1 and Mac-1. Both are involved in macrophage adhesion to epithelial cells and transmigration (Carreno *et al.*, 2002; Evans *et al.*, 2009; Jolly *et al.*, 2007). Surprisingly, the proportion of LFA-1$^+$ cells decreased in infected macaques, with a higher diversity among individuals than in uninfected macaques. Importantly, besides playing an important role in establishment of the virological synapse and HIV cell-to-cell transmission, LFA-1 facilitates HIV replication (Hioe *et al.*, 2001; Rudnicka *et al.*, 2009). In contrast, Mac-1 was found slightly increased in SIV$^+$ macaques. Further tests need to be done to increase the size of both groups of animals and confirm these observations.

Importantly, the presence of dendritic cells has not been reported in human semen yet. We identified a heterogeneous population of DCs, including pDCs. Although present in small numbers, it could be identified at all stages of infection in the three tested macaques. Importantly, DCs can be productively infected by HIV/SIV. As specialized antigen-presenting cells, DC can also carry and transmit viral particles *in trans* to other target cells. Further characterization of these cells needs to be done, especially to identify the nature of the BDCA3$^+$CD123$^-$ DCs (they may be Langerhans cells or stromal DCs, for example).

B. Isolation of semen HIV/SIV target cells and identification of infected cells

1) Identification of infected semen leukocytes

We developed a technique of cell sorting in order to isolate semen CD4$^+$ T cells and HLA-DR$^+$ macrophages. The purity of the sorted cell fractions was satisfactory. However, since we isolated semen macrophages on the basis of their morphology and HLA-DR expression, we can not exclude that a small proportions of DCs contaminated this fraction. However, since DCs are also HIV/SIV target cells, we considered that this eventual contamination was not problematic in our study.

Furthermore, quantification of SIV DNA copies by real-time PCR was performed directly on the cell lysates, thus avoiding DNA extraction and the inherent loss of material. Nevertheless, the number of DNA copies in most samples was under the limit of

quantification (30 copies). We tested the limit of detection of our technique using a plasmid containing SIV gag, and determined that we were theoretically able to detect 1 vDNA copy by testing the whole sample.

Given the small numbers of isolated cells, and the expected small proportions of infected cells, we performed our tests on the whole sorted fractions. Since our objective was to identify the productively infected cells, we decided to quantify the number of SIV DNA copies, reflecting the number of cells displaying provirus integrated in the cell genome. However, in absence of a nested PCR targeting integrated provirus, we can not exclude that the detected SIV DNA copies were not integrated within the host genome, but were circularized viral DNA (which represents however less than 10% of total vDNA). Further tests need to be done to investigate that.

We demonstrated that both semen $CD4^+$ T cells and macrophages can be infected, at all stages of infection. Unfortunately, because of the low numbers of sorted cells, we were not able to quantify the number of DNA copies per sorted cells in almost all samples. However, we assumed that the proportion of infected cells among the two fractions ($CD4^+$ T cells *versus* macrophages) was higher in $CD4^+$ T cells, given the fact that the number of sorted macrophages was always 10 to 10,000-fold higher than $CD4^+$ T cells. Moreover, the frequency of detection of vDNA copies was higher in sorted $CD4^+$ T cells than in macrophages, at all stages of infection. This is consistent with the observations made in other tissues in HIV^+ men and SIV^+ macaques.

In parallel to quantitative PCR on sorted semen $CD4^+$ T cells and macrophages, we identified SIV nef and gp41 by immunocytofluorescence in cytospun semen cells. We confirmed the presence of SIV infected cells (here positive for SIV proteins) among both cell types. Again, infected $CD4^+$ T cells were detected more frequently than macrophages.

Because of the small numbers of sorted cells, we didn't performed quantitation of the number of RNA copies. Such an information would have informed if the semen infected cells were latently infected, or in contrary, if they produced *de novo* particles. Both quantitative RT-PCR and *in situ* hybridization could be performed in order to complete our results.

2) Capability of semen leukocytes to produce infectious particles

We assessed semen $CD4^+$ T cells and macrophages from infected macaques by coculture with a cell line permissive to SIV infection, CEMx174 cells. Both cell types were able to transmit infection. Although few trials were able to be performed, we observed that

$CD4^+$ T cells could transmit infection more frequently at primary infection. On the contrary only macrophages succeeded to transmit infection at chronic stage.

This discrepancy may be due to the very low numbers of $CD4^+$ T cells that were sorted at chronic stage, rather than less efficient production of SIV by these T cells.

Also, cell-associated SIV transmission could be enhanced by risk factors such as inflammation, which is significantly increased during primary infection. The co-infection with an other STD may also increase the production of infectious virus by semen infected leukocytes.

C. In vivo *inoculation of sorted semen leukocytes*

In the last part of this work, we assessed the capacity of semen leukocytes to transmit infection *in vivo*. We first have chosen the rectal route, because of its higher transmission rate with cell free virus and from epidemiological data. Because of the small numbers of sorted cells, we pooled the sorted fractions from three different donors, and kept a small part of them to determine by PCR if infected cells were present in the inoculum. Putting together the immune cells of different animals may represent another limitation of our experiments. Indeed, it may induce cellular stress, aggregation and mortality, thereby limiting the infectivity of the inoculated cells. Moreover, despite the pool of the sorted cells from different donors, the total number of inoculated cells never reached the total leukocytes counts in an ejaculate under number conditions, and especially in leukocytospermic individuals.

A mean of 300,000 total leukocytes were inoculated at each experiment. Infected cells were detected in most of the samples, although in small proportions. None of the inoculated macaques became infected after three challenges.

HIV mucosal transmission is a rare event, and when it happens, the transmitter founder virus population is very small. This suggests that, most of the time, the mucosal barrier prevents viral invasion. In case of infection, the number of viral particles that succeed to cross these barriers is limited to 1 to 5. That is why the animal infectious dose used to infect animal models by mucosal exposure is generally high.

In a previous work, female macaques were challenged with 10^7 splenocytes containing 4.16×10^6 copies of vDNA. The numbers of DNA copies to successfully transmit infection by vaginal exposure to 50% of animals, and after a single challenge, was estimated at $6.69 \times 10^5 \pm 2.08$ copies (Salle *et al.*, 2010). In our present conditions, we barely collect 3×10^5 leukocytes, among which only a very small proportion of cells are infected. This may

explain the failure that we have encountered to establishing systemic infection after rectal challenge of sorted semen leukocytes.

However, the proportion of each leukocytes subset present in semen is very different to the inoculum of splenocytes, among which a majority of lymphocytes and very few macrophages are found. For now, we don't know the minimal number of infected semen $CD4^+$ T cells and macrophages that is needed to establish a systemic infection after mucosal exposure. This threshold remains to be determined yet.

We therefore decided to pursue our study with modifications in the protocol in order to optimize our experimental conditions.

First, we performed vaginal inoculations in female macaques treated with DMPA. Indeed, the volume of the macaque vagina is considerably smaller than rectum, so the inoculated cells are concentrated at a reduced mucosal surface and on contact with a thinner vaginal epithelium than under normal conditions.

Furthermore, we will perform twice weekly repeated challenges with a minimum of 5 challenges. Finally, the semen of four macaques will be collected during the first weeks of infection: at days 10, 14, 17, 21 and 28 dpi. As we mentioned it earlier, this period coincides with a leukocytospermia and a high frequency of the presence of infected $CD4^+$ T cells and macrophages. Besides, acute SIV infection induces a high seminal inflammatory state, which is susceptible to activate viral replication and transmission.

These experiments are currently in progress.

D. Identification of seminal factors susceptible to influence HIV/SIV mucosal transmission

1) Seminal cytokines and chemokines affected by SIV infection

We measured the seminal levels of 28 cytokines, chemokines and growth factors. As mentioned earlier, most of them were present at comparable levels than in human semen.

We identified 18 molecules that were increased in leukocytospermic semen (Figure 31). Among them, 10 were also affected by SIV infection: significantly increased in SIV^+ animals and/or correlated to PVL and/or SVL. Other molecules correlated to PVL and SVL, but were not increased by leukocytospermia.

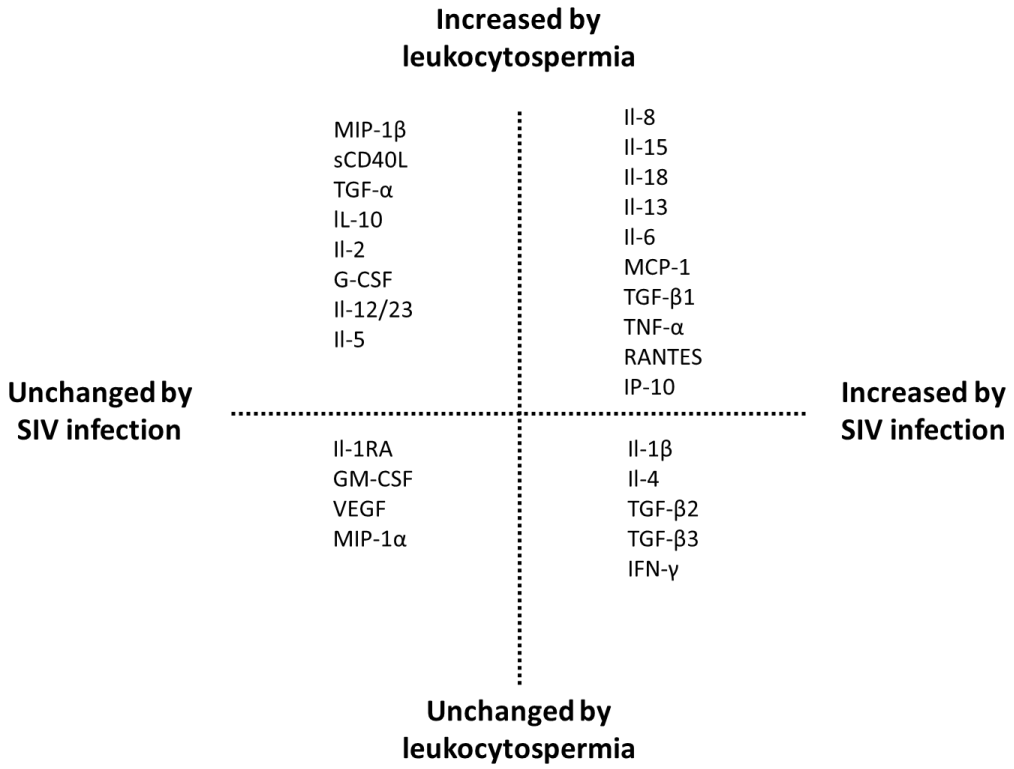


Figure 31 : Influence of leukocytospermia and SIV infection in seminal concentrations in 28 cytokines, chemokines and growth factors.

As discussed earlier, the inflammatory status induced by SIV infection, especially during acute infection, may favor the cell-associated virus transmission. First, pro-inflammatory cytokines, like IL-1β, IL-6, IL-15 or IL-18, trigger infected cells activation, and thereby can boost viral replication. Secondly, high levels of chemokines, like RANTES, MIP-1β and IL-8, may induce a migration of mucosal resident virus target cells towards the epithelial surface along a chemokine gradient.

The chemokines RANTES and MIP-1β possess potent anti-viral properties, notably by inhibiting by competition the fixation of viral particles to their co-receptor CCR5 (Agace *et al.*, 2000; Cocchi *et al.*, 1995; Lehner *et al.*, 2000; Lehner *et al.*, 1996). However, this activity may not influence cell-to-cell transmission.

Finally, the increased levels of TGF-β in the semen of infected individuals may also impact the host immune response. Indeed, this molecule display strong immunosuppressive activity and may thereby modulate the innate immune response to the pathogen (Politch *et al.*, 2007; Robertson *et al.*, 2009; Robertson *et al.*, 2002; Robertson and Sharkey, 2001).

2) Seminal adaptive immune response actors

We assessed the presence of two major actors of the adaptive immune response against HIV/SIV: SIV-specific IgG and $CD8^+$ T cells.

a. SIV-specific IgG levels in semen

Semen viral RNA load and SIV-specific IgG titers did not correlate, suggesting that the local humoral response fail to control viral shedding. This is consistent with the observation made on human patients where systemic antibody response to HIV following infection is poorly inducible and mostly ineffective, with the early B-cell response being largely directed against non-neutralizing epitopes of HIV envelope, and the later response being impaired by B-cell aberrant hyperactivation and abnormalities. Further neutralization assay needs to be done to investigate the neutralizing activity of semen SIV-specific IgG. Indeed, although a few studies reported the presence of neutralizing antibodies(Mestecky, 2007; Soderlund *et al.*, 2004)(Mestecky, 2007; Soderlund *et al.*, 2004), mostly IgA, seminal HIV-specific antibodies activity remains poorly described. Moreover, concentrations of SIV-specific IgA will be soon measured (in collaboration with Robin Shattock).

Interestingly, studies on HIV controllers reported a higher level of antibodies with ADCC activity than in progressor patients. Therefore it would be also interesting to evaluate ADCC activity in semen SIV-specific antibodies in infected macaques, and investigate whether it might be associated with local viral shedding.

b. Seminal SIV-specific $CD8^+$ T cell response

$CD8^+$ T cells play an important role in controlling HIV/SIV replication and disease progression. Moreover, the presence highly polyfunctional T cells in elite controllers and long-term nonprogressor HIV patients (Betts *et al.*, 2006; Dembek *et al.*, 2012).

The majority of macaques didn't display a detectable semen SIV-specific T cell response, with the exception of two animals. Both had a high proportion of IFN-γ, TNF-α and MIP-1β producing cells, with a majority of polyfunctional cells. Their responses were about 10-fold higher in semen than in peripheral blood. Importantly, this high SIV-specific response in semen was associated with a elevated local viral shedding. This suggests that the SIV-specific T cells do not significantly impact virus replication. Given the small number of studied macaques, no significant trend in the relation between specific T cell response and local viral shedding was reached. We therefore need to increase the number of animals in this study. Moreover, it would be highly interesting to assess if semen SIV-specific T cells are able to kill semen infected cells, and thereby to impact semen infectivity.

III. Perspectives

In this work, we tried to characterized finely the major SIV target cells that are present in the semen of infected animals, and demonstrated that they can be productively infected at all stages of SIV infection. Together with previous results obtained by our group, confirming that infected leukocytes are able to transmit infection after vaginal exposure and without inducing any damages in the exposed mucosa (Salle *et al.*, 2010), they provide strong evidence that infected semen leukocytes may play a role in HIV sexual transmission.

However, more work needs to be done to fully demonstrate our hypothesis.

A. Short-term perspectives

1) Continuation of the *in vivo* assay of semen leukocytes infectivity

At first, we need to pursue *in vivo* inoculations of sorted leukocytes from infected macaques. Importantly, we may need to increase the number of inoculated cells. The effect of seminal plasma on cell-associated virus transmission should also be investigated.

Secondly, if the *in vivo* infectivity of semen leukocytes is confirmed, it would be of major interest to determine which type of cells are able to transmit infection: $CD4^+$ T cells and/or macrophages. However, it would involve to collecting enough cells of each type, which could constitute a major limitation of our model.

Finally, by multiplying the experiments and the successful transmission events, we might be able to calculate the minimal dose necessary to produce infection and thereby the AID50.

2) Characterization of the SIV-specific adaptive response in semen

The work initiated here to fully characterize the SIV-specific adaptive response in semen of infected macaques needs to be completed by supplementary experiments.

The dosage of SIV-specific IgA will be performed in the semen of infected macaques at all stages of infection. We will also pursue further tests to assess neutralization and ADCC activity of semen antibodies targeting SIV proteins.

Similarly, we need to continue the initiated work on the characterization of SIV-specific T cell responses. We will increase the number of studied macaques, in order to perform statistical analysis. Moreover, we will develop another panel in order to study the Th2 response in $CD4^+$ T helper cells.

These investigations will maybe allow to identifying a potential influence of seminal viral shedding and infectivity.

3) Further characterization of semen leukocytes

To our knowledge, we described for the first time the presence of dendritic cells in the semen. Although we identified a population of plasmacytoid DCs, the other cells remain to be fully characterized.

Furthermore, we didn't investigate yet the presence of B cells and NK cells, two other major actors of the immune response against HIV/SIV.

4) Study of semen viral genetics

Evidence of a compartmentalization of HIV-1 variants between blood and semen, but also between viral particles and cell-associated virus in semen, has been reported in human (Coombs *et al.*, 1998; Gupta *et al.*, 2000; Paranjpe *et al.*, 2002; Zhu *et al.*, 1996). We would like to investigate such a compartmentalization in cynomolgus macaques. In particular, its dynamics during primary and chronic infection remains poorly investigated. Therefore, our experimental model may be highly interesting. Isolation of semen CD4+ T cells and macrophages would allow us to sequence separately the provirus and thereby investigate whether viral variants may or not be different in the two cell types.

In this perspective, sequencing of the virus will be performed in paired blood and semen samples:

- in semen and blood plasma,
- in semen and blood mononuclear cells.

Notably, tropism for the virus coreceptors (CCR5- *versus* CXCR4-tropic strains) will be investigated.

5) Dilution of the sorted semen leukocytes with seminal fluid

As we mentioned it earlier (see Introduction), the basic pH of seminal plasma has a buffering activity on vaginal acidic pH. That may play a protective role of the inoculated cells, since acidic pH immobilizes and kills leukocytes *in vitro* (Olmsted *et al.*, 2005).

Furthermore, we and other described that seminal plasma contains numerous factors susceptible to modulate leukocytes infectivity. Importantly, the high levels of pro-inflammatory and chemoattractant molecules (IL-8, MCP-1, RANTES, MIP-1β for example) are susceptible to induce an influx of virus target cells towards the mucosal epithelial surface and thereby increase the probability of a cell-to-cell contact between an inoculated cell and a host target cell.

Therefore, the adjunction of seminal plasma in the inoculum, diluted or not in PBS, might be important to consider for further experiments.

B. Long-term perspectives

If the cell-associated virus transmission is confirmed in vivo, two major questions will necessitate further research: by which mechanisms do the infected cells transmit infection, and how do we block this transmission?

1) Investigation of the mechanisms underlying cell-associated transmission

Previous work using labeled immune cells (PBMCs or splenocytes) demonstrated that, after vaginal exposure, some are able to migrated through the mucosal barrier and diffuse towards draining lymph nodes (Di Fabio *et al.*, 2001; Salle *et al.*, 2010; Zacharopoulos *et al.*, 1997). If we confirm that semen leukocytes are able to transmit infection *in vivo* after mucosal exposure, it would be on major interest to investigate whether they display the same migratory and dissemination properties.

Labeling the semen leukocytes with fluorescent molecules like CFSE and perform the same type of experiments than the cited groups would be feasible.

An alternative could be to label the cells with radioactive elements (18Fluor or 111In for example) and to track them *in vivo* by imaging (Positron Emission Tomography – PET –

or scintigraphy). Such technique would allow to investigating the precise dynamics of the potential migration of cells after exposure.

Moreover, we are going to develop in the next few months an *ex vivo* model with mucosal tissue explants (from macaque colo-rectal or cervico-vaginal tissue), to study the earliest steps of cell-associated transmission by semen leukocytes. We would like to investigate whether they can reproduce the different mechanisms of HIV transmission that have been described using such experimental model and reported here (see Introduction, Chapter II): a) formation of a virological synapse with either epithelial cells, followed by the transcytosis of viral particles through the epithelial surface, or with mucosal HIV/SIV target cells (DCs, macrophages or $CD4^+$ T cells); b) transmigration through the mucosa.

Furthermore, further tests need to be done to assess the effect of mucus in trapping semen leukocytes, as well as the influence of local pH on their motility and viability. Importantly, we must consider the buffering effect of seminal plasma.

2) Preventive strategies to block cell-associated transmission

If confirmed and characterized, HIV/SIV cell-associated transmission by semen leukocytes must be taken into consideration for further development of prevention strategies. Our findings in semen leukocytes expression of migration and adhesion markers may help to identify potential molecules (like LFA-1 and CCR5) which could be targeted with specific antibodies or competitive inhibitors.

The *ex vivo* explants model we will develop should help to test candidates molecules. Moreover, the system developed by the team of Prof. Deborah Anderson, consisting in a reconstituted human vaginal tissue, has proven its relevance and utility in this context. Further collaboration should be of major interest.

Finally, designing further experiments with a complete inoculum, which includes seminal plasma, cell-free and cell-associated virus, is one of our major objectives. This might provide powerful tools to improve our actual *in vivo* pre-clinical model to test new prevention strategies against HIV-1 sexual transmission.

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◆ **Summary :**

Semen is one of the major sources of HIV. We characterized the leukocytes subpopulations in the semen of cynomolgus macaques infected with SIV as a model of HIV/AIDS. All HIV/SIV target cells: $CD4^+$ T cells, macrophages and dendritic cells, are present and can be productively infected at all stages of SIV infection. Seminal inflammation correlated with local viral shedding, whereas adaptive immune response did not.

◆ **Key words :**

AIDS, HIV, SIV, macaque, sexual transmission, semen, cell-associated virus, mucosa

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