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Karunasinee Suphaphiphat

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Anti-viral immune response in the semen of cynomolgus macaques and inhibition of cell to cell transmission by broadly neutralizing antibodies in an SIV/SHIV model of infection

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Abbreviation list

HIV	Human immunodeficiency virus
FRT	Female reproductive tract
DCs	Dendritic cells
CAV	Cell-associated virus
GalCer	Galactosylceramide
ZO-1	Zonula occludens
TJ	Tight junction
LC	Langerhans cells
SIV	Simian immunodeficiency virus
HSPG	Heparan sulfate proteoglycans
MR	Mannose receptor
DCIR	DC immunoreceptor
PBMCs	Peripheral blood mononuclear cells
LNs	Lymph-nodes
ICAMs	Intercellular adhesion molecules
JAMs	Junctional adhesion molecules
LEEP-CAMs	Lymphocyte endothelial-epithelial cell adhesion molecules
E-cadherin	Epithelial-cadherin
IFN- γ	Interferon- γ
NK cells	Natural killer cells
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid

AIDS	Acquired immune deficiency syndrome
SP	Seminal plasma
SEM	Semenogelin proteins
PAP	Prostatic acid phosphatase
PSA	Prostate specific antigen
PGE	Prostaglandin E
Tregs	Regulatory T cells
WHO	World Health Organization
NSC	Non-spermatozoal cells
PMNs	Polymorphonuclear cells
HS	Heparin sulfate
MGT	Male genital tract
CMV	Cytomegalovirus
HTLV-I	Human T-lymphotropic virus type I
SEVI	Semen-derived enhancer of viral infection
WBC	White blood cell
ART	Antiretroviral therapy
Ab(s)	Antibody/Antibodies
SHIV	Simian human immunodeficiency virus
ADCC	Antibody-dependent cellular cytotoxicity
NABs	Neutralizing antibodies
FcR	Fc receptor
ADCP	Antibody-dependent cellular phagocytosis

ADCVI	Antibody-dependent cellular viral inhibition
non-NAbs	Non-neutralizing antibodies
NHP	Non-human primate
bNAbs	Broadly neutralizing antibodies
CD4bs	CD4 binding sites
MPER	Membrane proximal external region
V1/V2	Variable region 1 and 2
mAbs	Monoclonal antibodies
CoRbs	Coreceptor-binding sites
CD4i	CD4-induced
ATI	ART interruption
MOI	Multiplicity of infection
APCs	Antigen presenting cells
SAMHD1	Enzyme SAM domain and HD domain 1
MHC	Major histocompatibility complex
IgG	Immunoglobulin G
PrEP	Pre-exposure prophylaxis
PBS	Phosphate buffer saline
IC	Inhibitory concentration
AID50	Infect 50% of the animals
p.i.	Post-infection
BVL	Blood viral load
SVL	Seminal viral load

CVF	Cervico-vaginal fluids
ESN	Exposed-non infected
BV	Bacterial vaginosis

Chapter 1 Introduction

1.1 HIV-1 sexual transmission

1.1.1 Current situation

More than 30 years since the first reported case in the early of 1980s [1], Human Immunodeficiency virus (HIV)-1 infection has continue to be one of the major public health issue. In 2017, an estimated 37 million people were living with HIV worldwide and 53% of them resided in eastern and southern Africa regions. Despite the downward trend of new HIV-1 infections in 2017, the number of new cases was estimated to be 1.8 million and almost half of them were women [2,3].

Unprotected sexual contact represents the main HIV-1 transmission route [4–6], with the efficiency of transmission varying based on the type of exposure. The expected range of per-contact act is shown in **Table 1**. Among sexual exposure, a receptive anal intercourse has the highest risk per act of HIV transmission [7]. However, in developing countries, the primary route of HIV-1 dissemination is through heterosexual coitus [8]. In general, male to female through penile-vaginal transmission is greater than female to male transmission. Oral intercourse is less likely to result in HIV-1 transmission.

	Transmission routes	Per act transmission risk
Parental exposure	Blood transfusion	92.50%
	Needle-sharing injection drug use	0.63%
	Percutaneous needle stick	0.23%
Sexual exposure	Receptive anal intercourse	1.38%
	Insertive anal intercourse	0.11%
	Receptive penile-vagina intercourse	0.08%
	Insertive penile-vagina intercourse	0.04%
	Receptive oral sex	Low
	Insertive oral sex	Low
Vertical transmission	Mother-to-child transmission	22.60%

Table 1: Estimated per-act probability of acquiring HIV-1 from an infected source, by exposure routes.

Adapted from Patel *et al.*, 2014 [7].

1.1.2 Structure of the female reproductive tract and related impact on HIV-1 mucosal transmission

The female reproductive tract (FRT) separates into 2 compartments including the upper FRT (endocervix, uterus and fallopian tubes) and the lower FRT (vagina and ectocervix) (**Figure 1**) [9]. The exposed region during sexual intercourse is the lower FRT [6]. However, a multilayered stratified squamous epithelium of vagina and ectocervix provides better physical barrier for the incoming virus than endocervix that lined with a single layered of columnar epithelium [6,10]. Endocervix and junction zone between ectocervix and endocervix are considered as the vulnerable site for HIV-1 heterosexual transmission due to their mucosa structure allowing close contact to intraepithelial leukocytes and leukocytes at the submucosa [4,5,11,12].

At the mucosa level, several immune cells including CD4⁺ T cells, macrophages and dendritic cells (DCs) which express CD4 and CCR5 co-receptors are considered as

target cells for HIV-1 infection [4,13]. Either HIV-1 cell-free virus or cell-associated virus (CAV) can migrate through the mucosal epithelium, by paracellular passage or transcytosis, through micro-abrasion or laceration occurring during coitus [4]. After penetration, HIV-1 encounters and directly infects susceptible CD4⁺ T cells, macrophages and DC [14]. Additionally, DC, Langerhans cells (LC) and fibroblasts mediate the viral transmission by transferring the virus to CD4⁺ T cells; chiefly in trans-infection mode [4].

The susceptible cells that are present throughout the lower FRT are CD4⁺ T cells, DC and macrophages, whereas the target cells that populate within the epithelium of the uterine lumen are CD4⁺ CCR5⁺ T cells, DC-SIGN⁺ dendritic cells, Langerhans cells and macrophages [15,16]. The resident immune cells can promote HIV-1 acquisition by up taking of virions across the epithelial barrier [11]. Simian immunodeficiency virus (SIV) intravaginal challenge in macaque have demonstrated that SIV can pass the vaginal mucosa within one hour and after 18 hours post infection, the infected cells can be found in the draining lymph nodes [17]. Moreover, an *ex vivo* models of both ectocervical and endocervical tissues of human have shown successful HIV-1 infection [18,19].

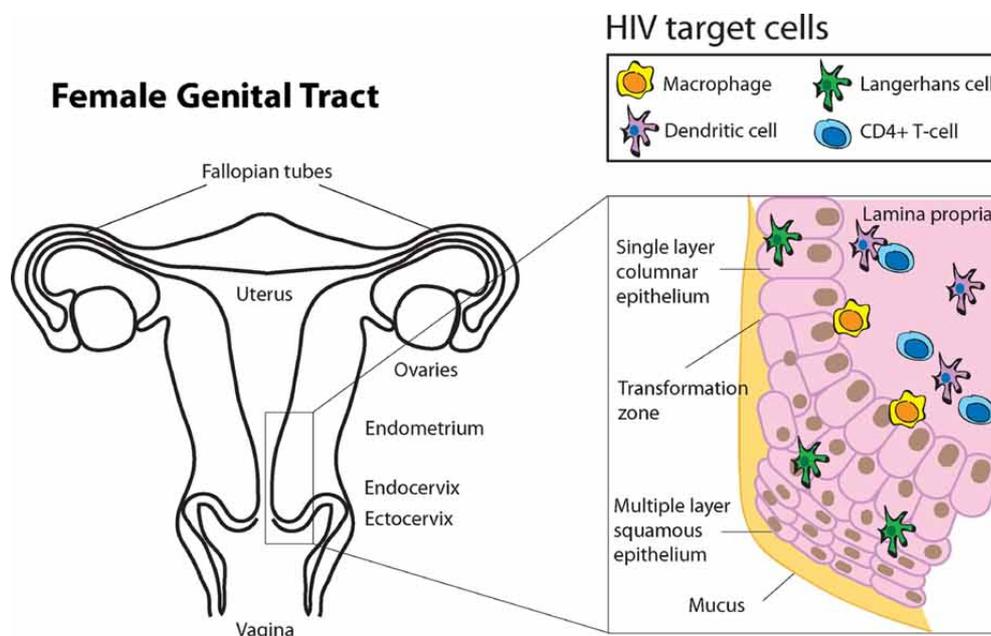


Figure 1: Female reproductive tract.

Adapted from Arien *et al.*, 2011 [5]

1.1.3 Mode of HIV-1 sexual transmission

Numerous *in vitro*, *ex vivo*, and *in vivo* studies have contributed to the understanding of the mechanisms by which HIV-1 can overcome the physical and chemical barriers of the genital, and to a lesser extent, the anal and colorectal mucosa, so that the virus reaches its target cells present in the submucosa. Several mechanisms for the passage of HIV-1 through the mucous membranes have thus been proposed depending on the site of the infection and the nature of the virus, i.e. whether it is in the form of free particles (Cell-free virus) or whether it is present inside infected cells (Cell-Associated virus) (**Figure 2**).

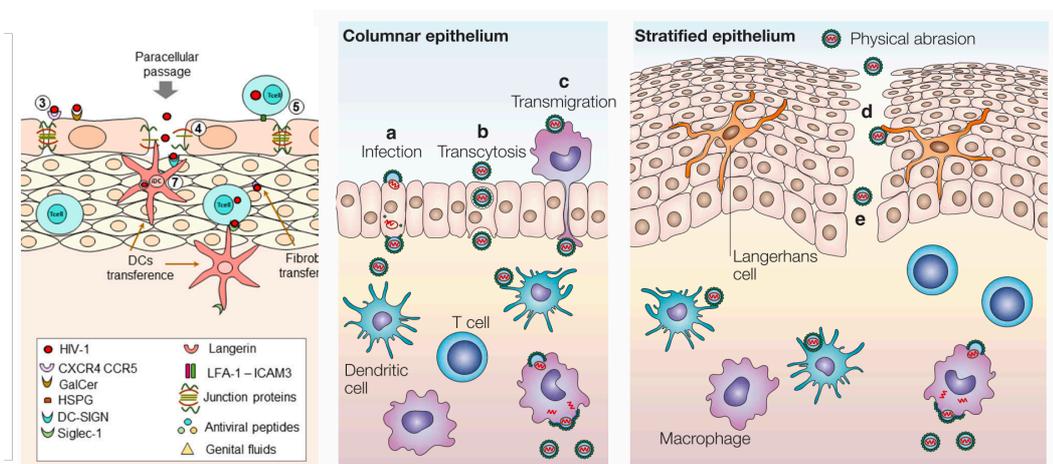


Figure 2: Mechanism of HIV-1 mucosal transmission.

Adapted from Gonzalez *et al.*, 2019 and Shattock & Moore, 2003 [4,6]

a) Mechanism of HIV-1 sexual transmission at the mucosa

i) Paracellular passage

Paracellular passage is the process that causes a loss or disruption of tight junctions between epithelial cells. The resulting gap formation that occurs allows the passage of the virus to reach the submucosa (**Figure 2**) [20–22]. This process is able to arise in almost all mucosal types [4]. The appearance of breaches at mucosal level might be due to a mechanical cause during the sexual act, especially at the level of the colonic mucosa [23–25]. However, several other factors may promote or cause the appearance of breaches, including inflammation of the mucosa associated with

a pre-existing infection, the virus itself or the use of lubricants, microbicides or spermicides on phase I trials [23,26–28].

Inflammation of the mucosa induced by bacterial or viral infection results in an influx of immune cells and pro-inflammatory cytokines, which can alter the epithelial barrier and thus make the sub-epithelial mucosal cells accessible to other pathogens, including HIV-1 [23,24].

The virus itself is also capable of altering the colorectal epithelial barrier as well as the endometrial and cervico-vaginal barriers. Nazli *et al.*, using both *in vitro* and *ex vivo* models have shown that exposure to HIV-1 particles alters the integrity of a colorectal epithelial monolayer and of the primary epithelial monolayer models of endometrium and cervix [29]. Either the HIV-1 co-receptors CCR5, CXCR4 and the galactosylceramide (GalCer) expressed on the epithelial cells were able to bind the HIV-1 gp120. This resulted in decreased transepithelial resistance, a decrease in occluding, claudins and the cytoplasmic protein zonula occludens (ZO-1) expression and an augmentation of Ca²⁺ and MAPK and P13K pathways which led to ZO-1 and claudins/occludin destabilization, tight junction (TJ) disruption, surface protein internalization and disruption of the epithelial monolayer [21,30–32]. The disruption of mucosal epithelium could be triggered by host antiviral mediators. Neutralization experiments have shown that the envelope protein of the virus (gp120) generates an alteration of the epithelial barrier by the secretion of TNF by epithelial cells [33]. Moreover, the activation of the apoptosis pathway could disrupt the epithelial cells by the addition of caspases resulting from degradation of claudins, occludin and ZO-1 proteins [33].

Several studies on phase I clinical trials have shown that lubricant gels, microbicides, or spermicides normally recommended for vaginal use can cause an alteration of colorectal epithelium [26,34], a loss of adherent junctions and TJ between epithelial cells [28], and consequently an increase in mucosal permeability [27].

Despite the fact that infection with HIV-1 through a break is the easiest, fastest, and most efficient mode of passage of the virus since free virions or infected

cells pass directly through the mucosa to contact the resident target cells in the lamina propria, the impact of this process *in vivo* remained unidentified.

However, not all mucosa presents the same susceptibility to the formation of breaches and the virus can use various other mechanisms of passage to cross an intact epithelial barrier.

ii) Transcytosis

Transcytosis is the process that makes use of the vesicular/endosomal transportation of the cells to carry the viral particle into the intracellular compartment (**Figure 2**). The process is characterized by the polarized transport of viruses within epithelial cells, from the apical to the basal pole, without productive infection of the host cell. After translocation, the virus is delivered to the external basal space where their target cells reside. The virus can directly infect susceptible lymphocytes or be carried and transferred by DC and Langerhans cells (LC) [4]. The process is found mainly in columnar epithelial cells [35–37] and squamous epithelial cells in pseudo stratified epithelium [38–40] at oral, intestinal, vaginal and endometrial epithelial cells [39,41]. Indeed, in the genital and colorectal mucosa this mechanism was described for the first time *in vitro* (I407, HT-29, CaCo2 and endometrial HEC-1 colorectal lines) for HIV-1 by Bomsel *et al.* in 1997 [37]. Transcytosis of HIV-1 has since been demonstrated and confirmed in various models of polarized cultures of immortalized (I407), colon (HT29, Caco2, T84), endometrial (HEC1) and monolithic cell monolayers of primary vaginal and endocervical cells [40,42–46]. Moreover, transcytosis has also been observed in *ex vivo* colorectal explants exposed to HIV-1 free viral particles or to infected leukocyte cells [46,47]. The mechanisms have not been fully defined and the role of the viral envelope is still debated. Some studies mention a necessary binding of gp120 to HSPG receptors (Heparan sulfate proteoglycans) [40] and to the gp340 receptor [48] known to be expressed by vaginal epithelial cells, endocervical and colorectal mucosa. Conversely, other studies propose an independent mechanism of the viral envelope [49] since occurring even with virus devoid of viral envelope, in models of vaginal and colorectal epithelial lineages. In the case of the passage of viral particles from infected cells, this

mechanism seems to depend on the establishment of a virological synapse. Interestingly, transcytosis between infected cells and mucosal epithelial cells was found to be more potent than transcytosis between free viral particles and mucosal epithelial cells due to the formation of virological synapse between HIV infected CD4⁺ T cells and the epithelial cells [50].

Not only paracellular passage but also transcytosis could be activated by the interaction with viral protein and pro-inflammatory cytokines [4]. Combination of paracellular passage and transcytosis could facilitate viral transmigration *in vivo*, however transcytosis *in vivo* has not been formally demonstrated.

iii) Capture by immune cells

The capture of viral particles by the cells of the immune system present in submucosal and / or intraepithelial is a potential mechanism for passage of the virus through the different types of mucosae (**Figure 2**). Indeed, a mechanism used by the virus for accessing subepithelial target cells is capture by DCs or LCs, which are a subtype of DCs that are found specifically in the pluristratified epithelia. LCs differ from DCs in that they specifically express Langerin (CD207) and reside in the epidermis and in most mucosal epithelia, while DC-SIGN⁺ DCs mainly reside in submucosa [51]. Although CD4⁺ T cells are the main HIV-1 target cells, at the genital epithelium DCs are thought to be one of the first immune cells encountering the virus at the submucosa as they are located within outermost genital epithelial layers compared to the CD4⁺ T cells [52]. DCs have cytoplasmic extensions called dendrites that allow them to capture viral particles within the mucosa but also at the level of the lumen. Indeed, our group previously showed that intestinal lamina propria CD11c⁺ DCs extend transepithelial dendrites and/or migrate across a tight epithelium to capture luminal HIV-1 virions both *in vitro* and *ex vivo* [46]. Interestingly, DCs migration was dependent by the interaction between the viral envelope and the CCR5 expressed by DC. Consequently, X4-tropic viruses cannot reach the sub-mucosa using this mechanism [53]. This may in part explain the preferential transmission of R5-viruses at mucosal level.

Two main mechanisms have been described for DC's role in viral dissemination (**Figure 3**) : (i) *cis-infection*: the virus binds to the cell membrane molecules CD4 and CCR5, resulting in productive infection of DCs [54]; (ii) *trans-infection*: HIV-1 particles can be internalized by DC or can be bound to DC surface receptors like DC-SIGN without being productively infected. Besides DC-SIGN other receptors can, to a lesser extent, bind HIV-1 and mediate the *trans-infection* pathway such as the Mannose receptor (MR), DC immunoreceptor (DCIR) or GalCer which are predominantly expressed on immature DC [54,55] or to Siglec-1 molecule, expressed by mature DC [56]. The virions bound to the surface of DCs maintain their infectious capacity for several days until reaching a susceptible cell, like the CD4⁺ T cells [46,57,58]. HIV-1 was shown to replicate in vaginal DC more efficiency than in skin LC or blood-derived DC [53,59–61]. This is possibly due to the lack of Birbeck granules in DCs, whereas in LC HIV-1 captured by langerin is internalized into Birbeck granules and partially degraded [51]. Moreover, vaginal DCs preferentially support the replication of R5-tropic strain than X4-tropic strain [53]. This event may explain why R5-tropic strain are preferentially transmitted during mucosal acquisition.

Similar to DC, HIV-1 was shown to bind to Siglec-1 in macrophages leading to the formation of compartments containing viral particles that could subsequently trans-infect CD4⁺ T cells [62].

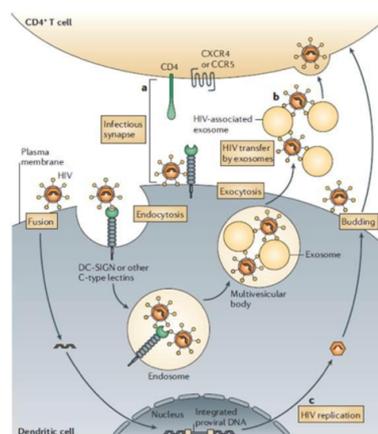


Figure 3: HIV-1 dissemination mediated by DCs.

a. and b. *trans*-infection; c. *cis*-infection. Adapted from Wu & KewalRamani, 2006 [63].

iv) Transmigration of infected cells

HIV-1 infected leukocytes can migrate through epithelial cells without any damage of the mucosal barrier to propagate HIV-1 infection (**Figure 2**) [64]. When the transmigration occurs, the infected leucocytes transfer infection to permissive cells such as activated CD4⁺ T cells and macrophages. *In vivo* and *in vitro* experiments have proved that leukocytes infected with SIV or HIV-1 are able to migrate across the genital epithelium. Using *in vitro* models of polarized monostratified epithelial cells, the transmigration of monocytes and to a lesser extent of CD4⁺ T cells has been shown [65,66]. The same is true for macrophages (derived from blood monocytes) and peripheral blood mononuclear cells (PBMCs) [67]. Using explant culture models of the human cervix, Maher *et al.* microscopically observed the infiltration of seminal leucocytes in the outermost layers of the pluristratified epithelium of the ectocervix, but the absence of infiltration into the endocervical epithelium [68]. Kolodkin *et al.*, demonstrated the transmigration of PBMC through the colorectal barrier into explants of human colon within 1 hour of exposure [69]. These observations have been confirmed *in vivo* in humans where radio-labeled autologous cells have been introduced intra-rectally and their distribution in the colon has been followed by imaging up to 24 hours post inoculation [70]. Moreover, our group showed in macaque that after intravaginal inoculation of labeled leukocytes, cells were found in the peripheral lymph-nodes (LNs) 2 days post inoculation [71].

The underlying mechanism of apical-basal transmigration and the adhesion molecules involved are still undefined. Various adhesion molecules present on genital macrophage and T cells are an indispensable factor allowing leukocyte transmigration from apical-to-basal epithelium. Integrins of the $\beta 2$ family, which correspond to the molecules comprising the subunit CD18: LFA-1 (CD18 / CD11a), Mac-1 (CD18/CD11b) and ITGAX (CD18/CD11c) are adhesion molecules predominantly present on the surface of macrophages, monocytes and T cells, and which participate to their transmigration through mucosal epithelia [72]. In semen collected from SIV infected macaques, LFA-1 on macrophages and T cells [73] enabled to counteract with intercellular adhesion molecules (ICAMs) on mucosal

epithelium which resulted in triggering the viral infected cells crossing epithelium [74]. The Junctional adhesion molecules (JAMs) is also involved in the transmigration mechanism. In TJs, JAM-A bound to LFA-1 [75], whereas JAM-C bound to Mac-1 in desmosomes [76]. Both molecules have been found in vaginal and cervical epithelium [72]. Thus, they are probably associated with an infiltration of macrophages in genital mucosa. Moreover, the cell trafficking could be influenced by the Lymphocyte endothelial-epithelial cell adhesion molecules (LEEP-CAMs). This adhesion protein is expressed in the vaginal mucosa, bound to T cells and play a role in maintaining intraepithelial lymphocytes [77]. Additionally, epithelial-cadherin (E-cadherin) is a molecule driving lymphocyte adhesion and transmigration. It's a junctional structure protein of the vaginal, cervical and intestinal epithelium and contribute to maintain the epithelial integrity [72].

The mechanism is also influenced by chemokine and inflammatory mediators which are highly expressed in vaginal mucosa and submucosa [12,78]. The SDF-1 chemokine, which is highly present in the semen of HIV-1 infected subject [79], potentially activate LFA-1 on semen leukocytes [80]. Furthermore, under inflammation, vaginal and cervical mucosa generate not only high level of cytokines such as SDF-1 α and IL-8 but also RANTES, MIP-1 α and MIP-1 β [81], which could recruit leukocytes [82]. In inflammatory conditions, ICAM-1 expression could be upregulated by interferon- γ (IFN- γ) generated by T cells and natural killer cells (NK cells) [83].

b) Source of transmitted virus: cell-free vs cell-associated

HIV-1 is present in female genital secretions and semen not only as cell free virions but also as infected leukocytes (semen composition will be further discussed in **section 1.2.1**) [4,5,84]. While several studies based on *in vivo* and *in vitro* models demonstrated that CAV is more potent to transmit the infection than cell-free virus [5,85,86], CAV has been largely overlooked. There is still very little comparative data between transmission by infected cells versus that with free virus in humans, and their specific contribution is still debated. Thanks to a mathematical model, it is

estimated that cell-to-cell transmission is 1.4 times more effective than free virus transmission and contributes to 60% of new viral infections [87].

Several studies have sought to determine the source of the transmitted virus by analyzing the sequences of viral ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), both in donor genital secretions and in the blood of newly infected individuals. These studies have shown that the virus found in the blood of newly infected persons was in some cases closer in sequence to the viral DNA found in the infected cells of the donor's genital secretions, and in others closer to the viral RNA derived from the free viral particles [85,88,89]. The simplest interpretation of these observations is that the source of the virus may be variable from one transmission to another, and that both the free virus and the infected cells play a role in the transmission of HIV-1.

In humans, *in vivo* inoculation of colloidal particles of HIV-1 size and leukocytes showed that they co-localized after several hours in the sigmoid colon and in the vagina, according to the route of rectal or vaginal inoculation, respectively [70]. Despite these similar migration capabilities, *in vivo* macaque studies have shown that cell-to-cell transmission is the primary means of transmitting SIV at the vaginal and colorectal level [69,71]. Our group has demonstrated indeed, that the inoculation of infected leukocytes could establish systemic infection without any mucosal abrasion. Cynomolgus macaques treated with depo-provera were intravaginally inoculated with SIVmac251 infected splenocytes labeled with CFSE. Interestingly, using *in situ hybridization*, the labeled cells were detected in the tissue of vagina and iliac LN after 21 hours of inoculation and in axillary LN after 45 hours of inoculation, indicating the rapid dissemination of the infected cells [71]. There is no report, up to date, of transmission initiated at mucosal level by semen cells, which would be more physiologically relevant.

In vitro, HIV-1 transcytosis through different epithelial cell lines (I407, HT-29, Caco-2, HEC-1, ME-180) is much more efficient when initiated by infected cells than by free virus particles [65,69]. The observation of transcytosis of free virus requires an inoculum (in units of p24) from 100 to 1,000 times greater than with infected cells

[45], if one wants to have a number of viral particles having crossed the barrier enough to generate a new infection. Infected cells also demonstrated greater ability to induce infection following transmigration through an epithelial barrier compared to free virus, as demonstrated by Van Herrewege *et al* [67].

In conclusion, it is now well established thanks to *in vitro*, *ex vivo* and *in vivo* models, that HIV-1 transmission by infected cells is more effective in initiating a new infection than cell-free virus and, according to the models used, it can be 10 to 1,000 times more effective [90,91].

The facilitation of HIV-1 dissemination by CAV may result from the close contact between infected cells and susceptible target cells via the process of membrane protrusion and cell engulfment or virological synapse (**Figure 4**) [92]. Indeed, it has been demonstrated in different cellular models that contact between cells increases transmission of the virus by concentrating receptors implicated in transmission at contact points between cells [93]. At the level of these contact zones, a larger quantity of viral particles than that found naturally around a target cell is released in a directional manner, thus increasing the multiplicity of infection to which the target cell is subjected [94]. Upon contact with a target or epithelial cell, the membrane of the infected cell is re-arranged, with on one side the recruitment of receptors and molecules necessary for endocytosis or transcytosis of viral particles, and on the other side the recruitment of membrane's molecules that will be carried away by the virus during its budding, and which will facilitate its future entry into new target cells [95,96].

Finally, the vast majority of preventive strategies used to date have been developed to target free viral particles only. The omission of strategies targeting infected cell transmission probably reflects the lack of knowledge of the scientific community on this subject, but also the technical difficulties of studying this mode of transmission. However, it is vital to downscale the mechanisms that underlie this mode of transmission in order to define new and effective prevention approaches that do not target HIV-1 in its free form. Approaches to block cell-to-cell transmission will be further discussed in **chapter 1.3.5**.

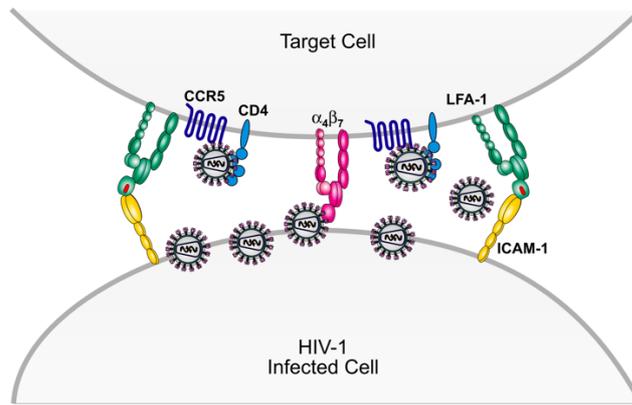


Figure 4: HIV-1 transmission through virological synapse formation.

Adapted from Cicala *et al.*, 2010 [97].

1.2 The role of semen in HIV-1 mucosal transmission

The main route for HIV-1 dissemination is during unprotected sexual intercourse. Consequently, semen represents the main vector of HIV-1 dissemination worldwide [8,98]. Considering the importance of semen in HIV-1 infection, the comprehensive understanding of this viral carrier can possibly contribute to the end of the Acquired immune deficiency syndrome (AIDS) pandemic.

Semen is a very rich biological fluid whose primary function is to ensure the reproduction of the species. It is composed of an acellular fraction, the seminal plasma (SP), which represents approximately 95-98% of the total volume, and a cell fraction composed mostly of spermatozoa (**Figure 5**). In addition to its role in the protection, transport and survival of spermatozoa, SP is able to modulate the immune response of the FRT for fertilization and embryo implantation [99].

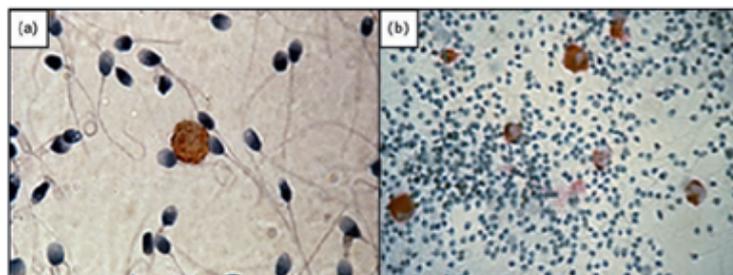


Figure 5: Human semen smear.

Seminal leukocytes were detected by immunohistochemistry (a) CD4⁺ T cells; (b) macrophages. Adapted from Politch *et al.*, 2014 [100].

1.2.1 Composition of semen

a) Physical and cellular characteristics

As mentioned, semen is a complex combination of semen cells and seminal fluid. SP contains a wide range of compounds such as inorganic ions, organic acids, sugars, lipids, steroids, amino acids, polyamines and proteins with superior buffering capacity than most other body fluids [101]. The classic pH of a healthy man's sperm is between 7.4 and 8.4 [102], which constitute a more alkaline environment than those present in the FRT [103]. This property of SP allows to neutralize the acidity of the FRT [104], which allows the survival of spermatozoa in this environment for fertilization. Unfortunately, one of the undesirable side effects of this buffering capacity is to allow free virus particles and infected cells contained in the sperm to survive, thus promoting the risk of infection [105]. These aspects will be further explained in **chapter 1.2.3**.

Compared to early puberty, the adolescent semen is characterized by an increased volume and number of spermatozoa and an improved ability to liquefy [106]. In healthy men, the volume of semen is between 2-5 mL per ejaculation, containing approximately 25-100 millions of viable and motile spermatozoa per a milliliter of semen [107]. The rheological properties of sperm are changed drastically after ejaculation. In fact, very quickly the gelatinous coagulum is liquefied, under the action of coagulation factors originating from the vesicles. The main components of the coagulum are semenogelin proteins 1 and 2 (SEM1 and SEM2) very abundant in sperm (20 mg/mL) and fibronectin. During liquefaction, the coagulum is transformed into a fluid material by proteolytic cleavage of specific sites of the seminogens by the so-called liquefaction factors: Prostatic Acid Phosphatase (PAP), Prostate Specific Antigen (PSA), originally synthesized at the level of the prostate. Liquefaction occurs 5 minutes after ejaculation *in vivo* and 20 to 30 minutes *in vitro* (reviewed in [108]).

b) Seminal plasma

Seminal plasma is not just a transporter of spermatozoa to the FRT but it encompasses various signaling molecules that temporally modulate FRT status [8].

This fraction of the ejaculate contains various bioactive reagents (listed in **Table 2**), including immunomodulatory, proinflammatory and growth factors that can contribute to a successful implantation in healthy couples [109]. This protein-rich fraction contains 25-55 mg/mL of proteins, including enzymes such as protease, esterase, phosphatase, but also prostaglandin E (PGE), fibronectin, polyamine and proteins playing a role in the immune system such as complement molecules and immunoglobulins (**Table 2**) [11,106]. The different components originated from testis, epididymis and accessory glands [98,106]. Moreover, a wide array of cytokine expressions in SP constitute a unique environment that is different from other mucosa and blood. Namely, TGF- β (~100 μ g/mL) and PGE₂ (~1 – 80 ng/mL) are the main cytokines present in semen [98,110–112]. Both molecules are the effective immunosuppressive cytokines that could suppress leukocyte activation (e.g. NK cells, macrophages and DCs) [113,114]. TGF- β is present in 3 isoforms (TGF- β 1, TGF- β 2 and TGF- β 3) and could be activated from latent to active forms by proteases and acid pH in the vagina [110]. The cytokine has been demonstrated to be involved in inducing regulatory T cells (Tregs) differentiation and downregulating NK cells activity, resulting in immune tolerance of the FRT [110].

Other types of cytokines present in the semen have inflammatory, regulatory, adaptive and hematopoietic properties. Presence of soluble HLA-G in semen possibly suppressed NK cells from entering cytotrophoblast [115]. A sperm coating glycoprotein - CD52g may be able to prevent anti-sperm immunity and infertility [116]. SDF-1 may play a role in leukocyte recruitment involved in the immune defense of the vaginal mucosa following insemination [109]. Also MCP-1, IL-8, Fractalkine, GM-CSF, IL-7 and IL-15 cytokines at inflammatory sites are associated with the recruitment, maturation and proliferation of immune cells including monocytes, T-cells, B-cells, DCs and NK cells [117–119]. In healthy subjects, concentration of these cytokines were 5-fold higher in semen than in plasma [120].

Seminal plasma components	Concentration
Immunomodulatory factors	
TGF-β1	
Total	219.3±13.4 ng/ml ^a
Bioactive (% total)	2.3±0.4 ng/ml ^a (1.2%)
TGF-β2	
Total	5.3±0.7 ng/ml ^a
Bioactive (% total)	0.25±0.04 ng/ml ^a 5.3%)
TGF-β3	
Total	172.2±32.8 ng/ml ^a
Bioactive (% total)	3.5±1.2 ng/ml ^a (1.8%)
PGE-1	7.0±6.0 µg/ml ^a
PGE-2	14.0±11.0 µg/ml ^a
PGF-1a	1.0±0.7 µg/ml ^a
PGF-2a	2.0±2.0 µg/ml ^a
IL-7	2,365.8 (1,109.5-3,985.5) pg/ml ^b
IL-15	27.8 (8.8-45.4) pg/ml ^d
Fractalkine	802.9 (333-1,636) pg/ml ^d
HLA-G	82 (29-1,161) U/ml ^c
Chemokines	
MCP-1	3.3 (0.3-81.5) ng/ml ^b
IL-8	1.6 (0.4-14.7) ng/ml ^b
SDF-1α	5.1 (ND-18.0) ng/ml ^b
Proinflammatory cytokines	
TNF-α	1.5 (ND-40.3) pg/ml ^b
GM-CSF	1.5 (ND-1,190.6) pg/ml ^b

Table 2: Principle components of human seminal plasma.

a. Mean ± SE; b. Geometric means (range); c. Median (range); d. Median (interquartile range). Adapted from Anderson & Politch, 2015 [109] and Olivier *et al.*, 2014 [120].

c) Semen cells

According to the World Health Organization (WHO), semen from healthy men contains at least 20 millions of spermatozoa per milliliter of semen [107] and 50% of them show forward motility [121]. In addition to spermatozoa, the semen cellular fraction include immature germ cells, epithelial cells and leukocytes, which in total account for less than 15% of semen cells [121,122].

Immature germ cells are the major population of non-spermatozoal cells (NSC). They have been categorized as spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. The number of immature germ cells increase in case of men who have spermatozoa less than 6 million per milliliter. Two types of epithelial cells are found in semen. Squamous epithelial cells, originated from excretory duct, possibly indicators of bacterial infection or inflammation. Another type of epithelial cells arise from seminal vesicles and are associated with inflammation of seminal vesicles [121].

Leukocytes are normally present in semen, where they represent about 13% of the NSC. They are probably involved in the elimination of abnormal and degenerating spermatozoa. Among leukocytes, granulocytes (or polymorphonuclear cells (PMNs)) are by far the most abundant cells since they are estimated to represent between 50 and 60% of the total population. Monocytes / macrophages come in second place and represent 20 to 30% of the semen leucocytes. Cytotoxic T lymphocytes CD4 and CD8 [122,123], each half present and accounting for about 5% of leukocytes, are also found [124]. Some studies have reported the minute presence of a B cell population [125,126], as well as dendritic cells in the sperm of macaques [127]. In humans, the presence of this population is more anecdotal because it concerns almost exclusively men suffering from chronic inflammation [128].

Semen leukocyte concentration varies significantly between individuals but should not exceed 1×10^6 /mL as recommended by WHO [107]. Otherwise, we talk of "leukocytospermia". This condition occurs often during infection or genital inflammation, mostly asymptomatic, and affects 5 to 10% of the healthy male population [129]. This prevalence can be as high as 24% in men with HIV-1 infection [72,100]. In these leukocytospermic men, the increase in leucocyte concentration affects all populations, namely granulocytes, monocytes/ macrophages and T cells [130]. This increase in the number of seminal leukocytes is probably due to the exacerbated release of these cells from the epithelium, an alteration of the integrity of the epithelial barrier and an attraction of these cells at the site of inflammation [122].

Very few studies have investigated the phenotype of semen leukocytes (**Figure 6**). A few studies in humans and a previous study of our group in macaques, have reported the expression on CD4 T lymphocytes of activation markers such as the IL-2 receptor (CD25), CD69, as early activation marker and HLA-DR, as a late activation marker [73,100,126,131]. Also the expression of various other surface markers, such as CD103 (mucosal marker), has been found in CD4 cells, but in a more heterogeneous manner, suggesting that only a fraction of the proliferating lymphocytes have a classical mucosal profile [122]. CD103 is a marker expressed by a vast majority of intraepithelial lymphocytes (95%), whereas it is present on less than 2% of blood cells. On the other hand, in the macaque, CD4 T cells express the HIV coreceptors CCR5 and CXCR4 [73]. In general, among the CD4⁺ T lymphocytes, there are naive and memory (effector and central) populations that can be identified by expression profiles (CD4⁺ CD45RA⁺ and CD4⁺ CD45RO⁺, respectively). In sperm, the majority of CD4 T cells have a memory phenotype [73,132]. It should be noted that these proportions are different from those found in the blood since lymphocytes with a naive phenotype constitute about half of the cell population. This means that these lymphocyte populations will not have the same ability to respond to antigens and from the point of view of HIV infection, they do not exhibit the same sensitivity. Memory lymphocytes are more susceptible to infection than naive lymphocytes [133]. Also CD8⁺ T cells exhibit an activated phenotype, demonstrated by the expression of the TIA-1 activation marker, a granule-associated protein found in cytotoxic CD8 lymphocytes [122].

The phenotype of monocytes / macrophages and dendritic cells has so far been poorly documented. Dendritic cells in human sperm have an immature phenotype (CD80⁻CD86⁺ CD83^{low} CCR6⁺ CCR7⁻CD14⁺) [128]. Although monocytes and macrophages represent the second most abundant leukocyte population behind granulocytes, no study in humans has finely characterized them. Characterization of semen monocytes and macrophages in the macaque model has been instead performed by our group [73]. These cells strongly express CCR5 and are additionally endowed with migration and adhesion factors such as the LFA-1 and Mac-1 molecules.

Although the origin of leukocyte is uncertain, it has been reported that, in normal semen, the epididymis and rete testis are the sources for lymphocytes and macrophages, whereas prostate and seminal vesicles are the sources for PMNs. On the other hand, in leukocytospermia, the increased number of leukocytes was associated with the genital tract inflammation and their origin is possibly from prostate [134–136].

Semen is obviously not a simple carrier of spermatozoa but a complex biological fluid, involving immunomodulatory and pro-inflammatory molecules and containing target cells of HIV-1. Not surprisingly, semen can contribute to affecting HIV-1 transmission. This will be precisely the subject of **Chapter 4.1** of this manuscript

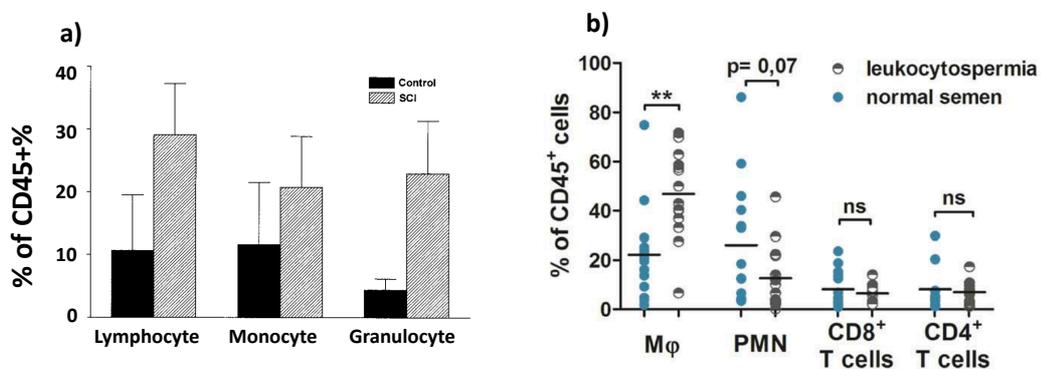


Figure 6: Phenotype of seminal leukocytes.

a) in human semen and b) in cynomolgus macaque semen. Adapted from Basu *et al.*, 2002 and Bernard-Stoecklin *et al.*, 2013 [73,126].

1.2.2 Semen during HIV infection

a) Presence of HIV in semen: what's its origin

As previously mentioned, HIV-1 is present in semen both as cell-free virions and infected cells. Many studies have reported that heparin sulfate (HS) served as an ancillary attachment factor for HIV-1 in DCs, macrophages and epithelial and endothelial cells [137–141]. HS could recognize HIV-1 depending on four HS binding domains, including the V2 and V3 loops, the C-terminal domain and the CD4-induced bridging sheet of the gp120 [142,143]. Spermatozoa could bind to virions through HS and transfer infection to macrophages, CD4+ T cells and DCs [98,144]. In spite of this,

spermatozoa-associated virus was speculative as a source of HIV-1 in semen because several studies failed to observe viral DNA contents integrated into spermatozoa, indicating inability to support viral replication [122,145–148]. The major source of seminal HIV-1 infected leukocytes are possibly T cells and macrophages. HIV provirus was detected more frequently in T cells than in macrophages [122], however, no correlation was observed between the number of seminal T cells and viral DNA in semen [149]. The association between leukocytospermia and the presence of HIV in semen remained to be investigated.

The shedding of virus in semen arose from the male genital tract (MGT). MGT organs, that are releasing HIV-1 virions and infected leukocytes, are mainly the accessory glands, including epididymis, prostate and seminal vesicles (review by [149]). The accessory gland may be the main contributor of HIV-1 virions and infected cells (review by [135]). Analysis of the HIV RNA from male genital fluids, such as secretions from urethra and prostate, displayed that prostate and epididymis were the main source of virus [150]. As the vasectomy had little influence on the level of HIV RNA, the epididymis may not serve as a source of virus [151]. The prostate and seminal vesicle may play a role as viral source [149] as they are the main source of seminal plasma [152], accounting for 60% by seminal vesicles and 30% by prostate [136]. Additionally, they are more susceptible to infection than other accessory glands (review by [152]).

Although testis is unlikely to be the main origin of the virus, it could not be excluded [135]. Testis is the least HIV-1 infected area compared to other parts of the MGT due to the blood-testis barrier [153]. According to the availability of susceptible cells in testis, the infected testis leukocytes possibly migrate across the epithelium of rete testis to seminal lumen then the virus may be release via Sertoli cells (review by [135]). Model of SIV infected macaques revealed that testis could be productively infected during primary infection and asymptomatic chronic infection [153]. The testis's infected cells were macrophages and T cells, as reported in men [134,154] and in macaques [155]. Moreover, testis act as a viral sanctuary, due to a limited access to drugs by the blood barrier and the drug efflux pumps (such as ABC

transporter) [156,157]. Consequently, testis must be taken into consideration for effective HIV-1 therapies.

Several evidences indicate that viral strains and infected cells present in semen do not originate solely from blood but would originate at least in part within the MGT. During the acute phase of infection, viral RNA (corresponding to free virus) and viral DNA (corresponding to infected leucocytes) in semen have sequences extremely similar to those from virus present in the blood (review in [135]). However, during the chronic phase genetic differences between HIV strains in the blood and in the sperm emerge and free viral particles present in the semen constitute a population distinct from that found in the blood [134,158]. This indicates the existence of an independent local viral replication, as well as a restricted exchange of virions and infected cells between the two compartments, allowing the parallel evolution of various virus populations within the body. In addition, the sequences of DNA and viral RNA in infected sperm cells may differ from those of virions, suggesting a different origin of virions and infected cells (review in [135]). Whitney *et al.* (2011) reported that in SIV infected rhesus macaques the viruses in blood and semen were similar during early infection then were distinguished after the peak of viremia, indicating that anatomic compartmentalization occurred at early time point [159]. Anderson *et al.*, proposes a number of non-exclusive and variable mechanisms allowing sperm contamination by HIV over time, namely: (i) direct importation of virus from blood; (ii) clonal amplification of viral blood strains in infected cells infiltrating the male genital tract; (iii) local replication in cells resident in the MGT leading to a distinct viral evolution [72].

At early stage of infection, semen containing high level of HIV-1 RNA was potentially infectious in parallel with leukocytospermia and elevated inflammation markers, which led to leukocyte recruitments [72,73,152]. Semen at first week of HIV-1 infection could efficiently transmit infection through sexual intercourse [149]. At chronic phase, a lower risk of HIV-1 transmission was observed due to decreasing not only blood viral load but also seminal viral load [135]. The shedding of both virions and infected cells continued to be detectable for months to years after

starting ART [160–162]. Halfon *et al.* (2010) have reported that, under ART, HIV-1 RNA in semen could be detected in only 3-5% of HIV-1 seronegative patient indicating the lower risk of sexual transmission [163].

The presence of HIV-1 in semen was commonly found in chronic stage of infection and leukocytospermia but it could be isolated either chronic infection or leukocytospermia [164,165]. The HIV-1 persistence in semen was irrelevant to the number of CD4⁺ or CD8⁺ T cells [166–168] and possibly intermittent, which was unrelated with plasma viral load [169–171]. The level of persistent virus in semen may be influenced by co-infection with sexually transmitted diseases, such as cytomegalovirus (CMV), chancroid, syphilis, gonorrhoea and Chlamydia infections [172–174]. The large regions of membrane protein on CMV and human T-lymphotropic virus type I (HTLV-I) were similar to CD4. This resemblance may contribute to the higher susceptibility to HIV for the CMV and HTLV-1 infected leukocytes [175].

b) Modulation of semen parameters in HIV-infected patients

HIV-1 infection has an effect on several physical and cellular parameters of semen. These effects are mostly detected during the chronic phase of infection, in which not only spermatozoa are affected (reduced motility, lower number of spermatozoa, and/or increased abnormal morphology) but also physical characteristics are changed (decreased ejaculated semen volume, increased seminal pH and round cell amount). Some of the semen alternations may result from ART. Indeed, ART may impact on several metabolic and endocrine function of testis and MGT, resulting in anomalies of spermatozoa [176].

In comparison with blood, several immunomodulatory mediators, including cytokines (IL-1 α , IL-7, IL-8, MIP-3 α , MCP-1, MIG, IP-10) and chemokines (SDF β 1 and TGF- β), are enriched in semen not only in healthy men but also in HIV-1 infected men [129,177–179]. The acute phase of the infection is characterized by a higher level of pro-inflammatory cytokines and chemokines compared to non-infected or chronic HIV-1 infected subjects (for review see [180]). Overexpression of pro-inflammatory cytokines / chemokines in the seminal plasma of infected men alters

the cytokine network and may impair the ability of the immune system to respond to HIV-1 infection [120,178]. It should also be taken into account that this cytokine network evolves dynamically according to the different stages of viral infection, as described by Vanpouille *et al.* [181]. A correlation between pro-inflammatory cytokine levels and viral load in semen has been reported in several studies. Thus, a positive correlation between a seminal viral load and levels of IL-1 β [182] RANTES [183], IL-6, IL-8, IFN γ [184] and IL-17 [185] was described in HIV-1⁺ patients. In addition, these variations in cytokine concentrations could have numerous consequences since, for example, it has been shown that high concentrations of pro-inflammatory cytokines promote the expansion and activation of the immune cells of the exposed mucosa. For instance, endometrial epithelial cells exposed to SP from acutely HIV-infected men, produced higher level of pro-inflammatory cytokines (IL-1 α , IL-6, and TNF- α), which increased HIV-1 replication in CD4⁺ T lymphocytes [186]. Consequently, the modulation of semen factors possibly has an effect on viral propagation during HIV-1 sexual transmission in the FRT.

Finally, HIV-1 infection may modulate the phenotype of semen cells, such as macrophages, CD4⁺ and CD8⁺ T cells. In a previous study our group has characterized semen macrophages and CD4⁺ T cells in SIV-infected macaques [73]. Infection determined an increase in the number of CD69⁺ CD4⁺ T lymphocytes which presented an activated phenotype. Moreover, we reported a decrease in the number of cells positive for LFA-1 and an increase in the number of cells positive for Mac-1, suggesting that the virus may modify the adhesion capacity of the cells that it infects. The modification of the dynamics and activation state of semen CD8⁺ T cells in SIV infected macaques will be the argument of the **chapter 4.1** of this thesis.

1.2.3 Role of semen in HIV-1 transmission

Although *in vivo* studies show that SP is not necessary for the establishment of infection [71,187], a number of studies have demonstrated that the various components of SP may affect, facilitate or inhibit, HIV-1 transmission.

a) Facilitation of HIV-1 transmission by semen

Semen may favor viral propagation in the FRT by changing the vaginal pH. HIV-1 infectivity is stable at pH 5-8 but is reduced by 25% at pH 4.5 [188]. The pH of the SP is 7.4-8.4 with buffering properties to bring the acidic vaginal pH back to a very fast neutral pH (30 seconds) and maintain this neutral or even basic pH for 2 hours [102,189]. Because free HIV-1 particles and infected leukocytes are sensitive to acid pH, they are therefore likely to be inactivated by vaginal acidity. This buffering action of the SP, allowing the protection of spermatozoa, has the undesirable side effect of granting some protection to the virus, thus favoring infection [190]. Moreover, as mentioned in the previous paragraph, semen exposure induces a strong inflammatory response of the FRT mucosa, resulting in the disruption of mucosal barrier and the infiltration of HIV-1 key targets, as well as recruitment of immune cells (neutrophils, macrophages and T-lymphocytes) to cervix [191–193]. For instance, the migration of LCs, stimulated by CCL20 secretion via NF κ B signaling pathway in vaginal epithelium was under the influence of SP [193].

Semen-derived enhancer of viral infection (SEVI) is an amyloid fibril found in semen that help the virus to attach to the surface of susceptible target cells [194]. The potential role of SEVI to facilitate HIV-1 transmission has been reported in *in vitro* and *ex vivo* assays and in rat models [194–199]. SEVI enhancing effect responded in a time- and dose- dependent manner and was not strain-specific. Indeed, *in vitro* experiments demonstrated that the infection of PBMCs, macrophages and DCs with R5-, X4- and dual tropic HIV-1 strains was increased by SEVI. Productive HIV-1 infection could start with 1-3 virions in the presence of SEVI [194]. hCD4/hCCR5 transgenic mice treated with SEVI before challenge showed 5- time increase in HIV-1 YU2 cDNA in the spleen compared to non-SEVI treated group [194].

The function of semen to suppress immune responses in the FRT has an impact not only for successful fertilization but also for HIV-1 transmission facilitation. However, the underlying mechanisms driven by SP are not well determined. TGF- β and PGE₂, which are highly expressed in semen, have a role to suppress immune cells

activation (e.g. neutrophils, NK cells and macrophages) and to promote DCs differentiation and T regulatory response [200].

SP contains complement molecules that can be activated by HIV-1 particles (regardless of the tropism of the strain). This activation leads to the cleavage of the protein of complement C3. The opsonization of viral particles by C3a fragments formed as a result of cleavage may favor the infection of cells expressing the complement receptor such as epithelial cells, macrophages, DCs and CD4⁺ T cells [201]. SP of HIV-positive individuals also contains antibodies against the viral envelope that can opsonize free virus particles. The formation of these complexes promotes transcytosis of the virus via interaction with the neonatal Fc receptor expressed by epithelial cells by concentrating the virus at the cell membrane [202]. This phenomenon is also observed with neutralizing antibodies supposed to have a protective role against infection [203,204].

Finally, seminal plasma may abrogate the activity of HIV-1 microbicides by electrostatic interaction. In the presence of SP anionic polymer microbicides (such as cellulose sulfate and carrageenan) displayed 4-73 times reduction of antiviral activity [205]. The cationic polyamines and polyanions of SP may be involved in the electrostatic interaction with the drugs [206,207].

b) Inhibition of HIV-1 transmission by semen

Semen has been reported to be involved in inhibition of HIV-transmission as well. SP could hamper the capture of HIV-1 mediated by DC-SIGN expressed in DCs and prevent transfer of infection to T cells [208]. The clusterin, present at high level (0.4-15 mg/mL) in SP, was identified as the main ligand of DC-SIGN [98]. In addition, SP reduced the expression of CD4 and CXCR4 on lymphocytes, despite an increase of CCR5 expression was observed [209].

Cationic polypeptides in SP, including 52 discovered peptides such as semingelins, fibronectine etc, have shown an anti-HIV-1 activity. The interaction was observed even using very low concentration (3,200 folds) of SP but the action was temporarily (\leq 24 hours) [210]. Also reactive oxygen species generated by

seminal leukocytes and spermatozoa [211] possibly play a role in intrinsic antiviral activity. SP could also enhance the integrity of FRT epithelium, resulting in better function of the physical barrier [8]. In the context of cell-to-cell transmission, Lawrence *et al.* showed in a polarized model of HEC-1 cells that the SP has an inhibitory effect on the transmigration of infected leukocytes across the epithelial barrier. This effect is associated with increased expression of the ZO-1 junction protein between epithelial cells and an increase in leukocyte adhesion to epithelial cells [66]. An earlier study had already shown an increase in the adhesion capacity of a lymphocyte line on the cervical and intestinal epithelial barriers (MT180 and I407 non-polarized lines respectively) exposed to SP [212]. Thus, SP seems to be able to play an inhibitory or promoting role to cell-to-cell adhesion depending on the cell types involved.

1.2.4 Immune responses affected by semen during HIV-1 infection

The factors in semen that influenced HIV-1 progression were associated with co-infection and other invading pathogens and with high level of leukocytes, indicative of genital tract inflammation. Leukocytospermia in semen could be found not only in HIV-1 seronegative individuals with genital tract inflammation but also in HIV-1 seropositive subjects [122]. White blood cell (WBC) in semen of HIV-1 infected men ($1.0E+05$ cells per milliliter of semen) are generally present in lower number than in uninfected men ($2.4E+05$ cells per milliliter of semen). Normally, $CD4^+$ and $CD8^+$ cells are present in semen ($5.7E+03$ $CD4^+$ T cells and $1.9E+03$ $CD8^+$ T cells per milliliter of semen) [131]. In the semen of infected men, the number of macrophages remained stable but the number of $CD4^+$ and $CD8^+$ T cells was undetectable [131]. After treatment with antiretroviral drugs, the seminal leukocyte population was brought back to be the same level as non-infected individuals [131]. Leukocytes played a role not only as immunomodulator but also as a viral carrier, particularly macrophages and T cells that have been shown to contain HIV-1 provirus [122]. As a consequence of antiretroviral therapy (ART), the restored immune cells in semen may result in increasing the risk of HIV-1 transmission [131].

In addition to the changes on an array of cytokines/chemokines and leukocytes, already discussed in the previous sections, HIV-1/SIV-specific antibodies are present in abundant quantities and high frequencies (81-100%) in the semen of HIV⁺ men and SIV⁺ macaques, and are mostly IgG [213–215]. Those antibodies (Abs) may exert different antiviral activities. Neutralizing HIV-specific IgA are present in the semen and vaginal washes from HIV-1 exposed and seronegative sex-workers [203,216–218]. Moreover, several studies have shown that neutralizing IgG could prevent macaques infection following intravenous or vaginal inoculation of simian human immunodeficiency virus (SHIV) [219–222]. Furthermore, Parsons *et al.* (2016) previously demonstrated that HIV-1-specific antibodies in SP could mediate antibody-dependent cellular cytotoxicity (ADCC) responses *in vitro* [223]. Although HIV-specific Abs may prevent or partially control mucosal HIV-1/SIV entry [213,224], the ability of HIV-1 specific antibodies in semen to modulate HIV-1 transmission remain inconclusive.

1.3 Antibodies against HIV-1

1.3.1 Mechanism of protection mediated by antibodies: neutralization versus Fc- mediated effector functions

The Ab response may be exploited for HIV-prophylaxis or therapy. Abs may act at different stages of the viral cycle, as shown in **Figure 7** [225–227]. Indeed, binding of Abs to a free virus results in: a) aggregation of virions which decreases the size of the virus inoculum; b) blocking by steric hindrance of the interaction between the viral receptor and the cellular receptors; c) blocking the fusion step between the viral envelope and the cell membrane; d) inhibition of post-attachment events such as decapsidation, assembly and budding/release of virions.

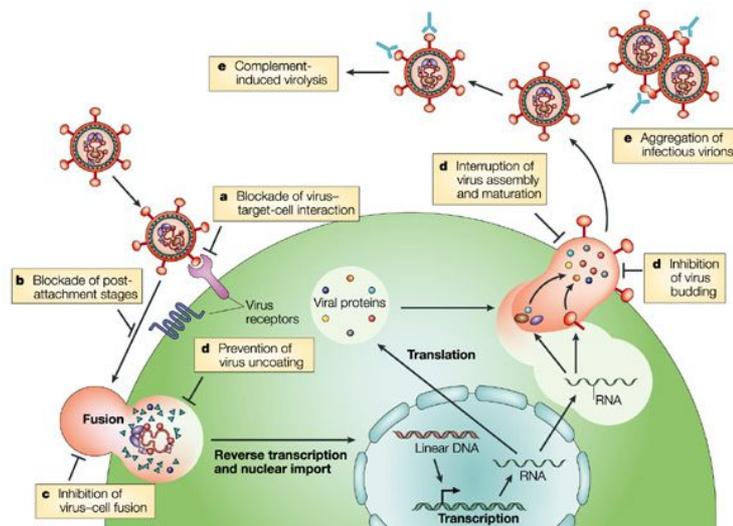


Figure 7: Antibody action during the HIV-1 replication cycle.

Adapted from Zolla-Pazner, 2005 [226].

Viral entry into target cells is triggered by the interaction between the trimeric envelope glycoprotein and the CD4 receptor expressed by target cells (review in [228,229]). Neutralizing antibodies (NAbs) are directed against conserved regions of the HIV-1 envelope gp120 and gp41 [227] and hamper the entry of the virus (**Figure 8**). The development and mode of action of NAbs will be discussed more in details in the next paragraph.

The mechanisms that contribute to protection and control of viremia by Abs are not limited to neutralization of free virions, but also by Fc-mediated effector functions. Abs can engage the Fc receptor (FcR) present on the surface of innate effector cells (such as NK cells and phagocytes, among others) to stimulate antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular viral inhibition (ADCVI) [230,231]. Additionally, Abs can induce complement-dependent cytotoxicity through their Fc domain (**Figure 8**). In humans, five FcRs have been identified: FcRI (CD64), FcRIIa and b (CD32), FcRIIIa and b (CD16) [232]. These different receptors are characterized according to their affinity for IgG (review in [233]).

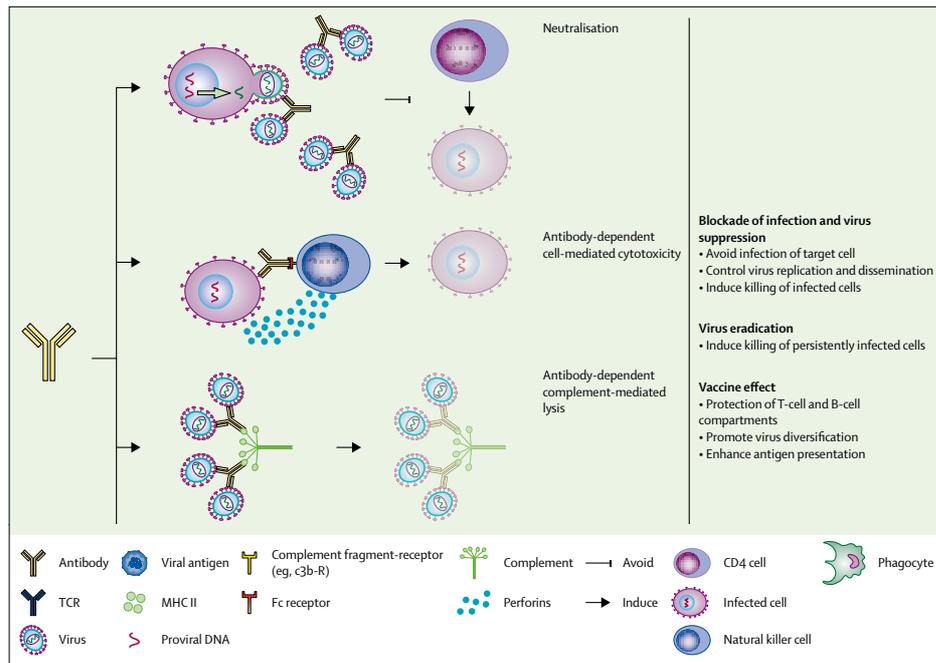


Figure 8: Antibody mechanism of action against viral infection.

Adapted from Jaworski & Cahn, 2018 [234].

ADCC against HIV-1 infection is a mechanism by which effector cells such as NK cells, monocytes, or neutrophils are able to lyse infected cells (review in [235,236]). This activity requires the interaction between the infected cell and the effector cell via specific anti-HIV-1 antibodies. The antibodies must therefore bind to the envelope epitopes expressed on the surface of the infected cell via its Fab part and to the effector cells through the Fc portion [232,237]. This double interaction results in the release of cytotoxic granules (granzymes and perforins) responsible for the lysis and destruction of the infected cells [238–240] (**Figure 9**). NK cells are the main effector immune cells that mediated the lysis of Ab-bound infected cells. The potential protection by ADCC depends on NK cell distribution, maturation and activation in each individual (review in [241]). Due to the highly dynamic conformational change of the HIV-1 envelope, the window of opportunity of Ab to mediate protection through ADCC function is restricted to only few hours (review in [242]).

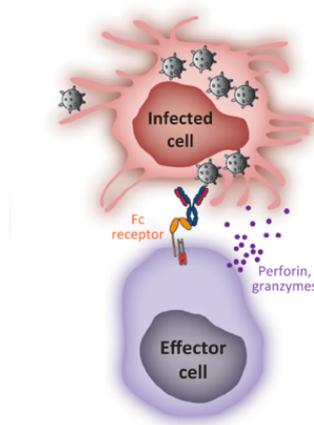


Figure 9: Facilitation of infected cell clearance mediated by anti-HIV-1 bNAbs through Fc dependent.

Adapted from Mouquet, 2014 [243].

The analysis of the RV144 vaccine trial has pointed out that specific anti-HIV non-neutralizing Abs (non-NAbs), specially Abs directed against the V1/V2 loop of the HIV-1 envelope, contribute to the moderate protection observed [244]. Several studies done in non-human primate(NHP) and humanized mouse models reported the contribution of Fc-mediated effector functions in controlling viremia [245,246]. Moreover, HIV-1 specific ADCC antibodies are associated with lower viral load, higher CD4-cell counts, and improved clinical outcomes [247,248]. Bruel and colleagues reported that broadly neutralizing antibody (bNAbs) targeting the CD4-binding site, the glycans/V3 and V1/V2 loops on gp120, or the gp41 moiety, eliminated infected cells in the presence of NK cells through ADCC [249].

While neutralization can prevent viral infection in the first place, the engaging of the FcR on innate effector cells can interfere with the establishment of the viral reservoir, as demonstrated in some studies of passive infusion of bNAbs (administered either pre-exposure or post-exposure) in NHPs [250,251]. Using a mathematical model Lu and colleagues [252] showed that administration of the bNAb 3BNC117 in chronically infected individuals not only accelerated the clearance of free virus particles but also directed the clearance of persistently infected cells. Then, using a humanized mouse model of HIV-1 they proved that the elimination of HIV-1-infected CD4 cells by 3BNC117 was dependent on a mechanism that involved

Fc receptor engagement [252]. These results shown that, besides preventing viral acquisition by killing HIV-1-infected cells, bNAbs might be more successful in suppressing viremia during chronic infection and have the potential to induce viral eradication.

1.3.2 Development and structure of anti-HIV broadly neutralizing antibody (bNAbs)

a) Development of bNAbs in HIV-1 infected patients

During HIV-1 infection, the humoral immune response develops within one week after the detectable viremia and is characterized by the appearance over time of different types of Abs [243,253,254]. Initially, it is detected as antigen-antibody complexes. A few days later, free Abs against gp41 are observed, followed, a few weeks later by Abs against gp120. These Abs mainly target the non-neutralizing side, so they have no effect on viremia and do not exert selective pressure on the virus [253]. They may be produced by pre-existing memory B cells that cross-react with gp41 epitopes. Nevertheless, these Abs, via their Fc fragment, can contribute to antiviral activity by inducing virion phagocytosis or lysing infected cells via an ADCC mechanism [255–257]. The first neutralizing Abs against "autologous" virus appear after two to three months. The selection pressure induced by these Abs lead to the emergence of escape variants, resistant to neutralization [235]. These early autologous neutralizing Abs are unable to neutralize virus variants isolated from other HIV-infected patients [258–263]. Heterologous neutralizing Abs appears 2-4 years after primary infection in about 20% of chronic HIV-1 infected subjects [264]. Of note, approximately 1% of them (called "elite neutralizers") develops neutralizing Abs that neutralized cross-clade HIV-1 virus, called bNAbs [265]. Although the virus envelope shows extraordinary diversity, we now know that there are highly conserved epitopes, which are the target of largely neutralizing antibodies, and which constitute sites of vulnerability to the humoral response [235,257].

bNAbs have unique features including the changes on heavy chain domain, sulfate tyrosines, long and hydrophobic CDR-H3's and extensive hypermutation [266,267]. A hammer like structure created by the long CDR3 regions on

immunoglobulin heavy chain could promote the binding efficacy to HIV-1 envelope [268–270]. Moreover, the hypermutation, especially in HCDR3 region, could be detected in up to 32% of bNAbs, indicating the complex maturation required to generate bNAbs [271–273]. The reason of the ability in some individuals to develop bNAbs is not well defined but it may be related to the duration and the level of viral infection. Due to the fact that not all subject could generate bNAbs, the underlying factor is possibly related to both virus and host [235]. Interestingly, it has been reported that viral subtype could affect the breadth and potency of humoral immune response. HIV-1 positive subjects who are infected with subtype C or A possibly generated bNAbs with higher breadth and potency than the subjected infected with subtype B [274–277].

b) HIV-1 epitopes that are target of bNAbs

By the effort of many researchers worldwide, the characteristics and the target epitopes of potent bNAbs have been disclosed. bNAbs target few conserved regions of the HIV-1 envelope spike, called “sites of vulnerability” including the CD4 binding site (CD4bs) on gp120, glycan shield regions - V1/V2 loop and V3 loop on gp120, the membrane proximal external region (MPER) on gp41 and the bridging region between gp120 and gp41 (**Figure 10**) [243,278].

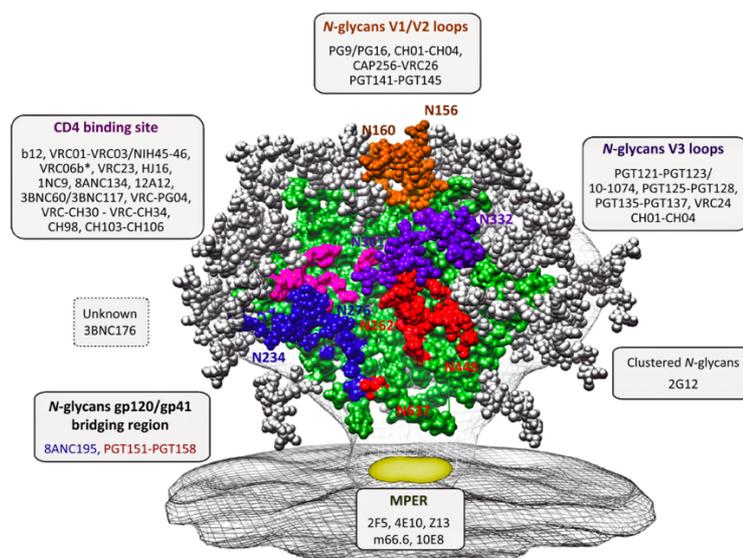


Figure 10: Structure and bNAb recognition of HIV-1 envelope spike.

Adapted from Mouquet, 2014 [243].

(A) A number of bNAbs recognize the **CD4-binding sites** (CD4bs) on gp120, which is the conserved domain of gp120 concealed among glycan shield. To attain broaden potency, bNAbs need to develop specifically to fit in this restrict access. The category of the anti-CD4bs bNAbs is divided into two groups – VH gene restricted Abs, such as VRC01, 3BNC117, 8ANC131 and IOMA, and CDR-H3 loop dominant Abs, such as HJ16 and CH103. Of note, VRC01 class bNAbs (e.g. VR01 Ab) that used CD4 mimicry has astonishingly efficacy, neutralizing 98% of HIV-1 isolated strains *in vitro* [279–284];

(B) the variable region 1 and 2 (**V1/V2**) has been proven to be another site of the spike that is vulnerable to neutralizing antibodies. More precisely, the Ab could recognize a quaternary epitope at the trimer apex of V1/V2 and N-linked glycans at position N156-160. PG9, PG16, and PGDM1004 are representatives of V1V2-directed neutralizing monoclonal antibodies (mAbs) that generally compete with one another for antigen [285–287];

(C) **N-linked glycan-containing epitopes** form a site of spike vulnerability, including the gp120 V3 loop, and several nearby glycans, 2G12, PGT128, and PGT121/10-1074 represent the bNAbs that recognize this region [288–290];

(D) there are also bNAbs that target the **coreceptor-binding sites** (CoRbs) on gp120. The exposure of this site, also known as the CD4-induced (CD4i) site, is (as its name suggests) induced by conformational changes that occur as a result of the binding of CD4 to gp120. Antibodies targeting this region are called CD4-induced antibodies, and include 17b, X5, m36, 48d, and E51 [291–293]. Based on the length of the IgG heavy chain CDR, most full-size CD4i Abs do not have high antiviral potency because full-size Abs targeting CD4i-specific epitope is sterically restricted during viral entry [294].

(E) The **membrane-proximal external region** (MPER) of gp41 contains highly conserved residues that also play a critical role in the virus fusion process. Ab in this group used their long CDR H3 loop to bind a linear sequence, that is located in the highly conserved region of MPER, and some of them could bind to a lipid component from viral membrane. Only 671–683 AA as a linear epitopes of this MPER are

recognized by potent neutralizing mAbs [295,296]. These bNAbs include 4E10, 2F5, Z13, and 10E8 [297–299]. Although previous studies show that combination of 2F5 and 10E8 protects rhesus against SIV infection, 10E8 still exhibits most potent and broad neutralizing activity compare with other MPER active antibody targeting gp41 of HIV-1. It can neutralize about 92% of circulating HIV-1 isolates *in vitro* (review in [300]).

(F) Finally, the most recent group of bNAbs is directed against the **bridging region of gp120-gp41**. The main target epitopes were trimer-specific, ranged between gp120 and gp41 including glycan (review in [301]). However, their targets were not always overlapping. One Ab in the group – 8ANC195 was not only capable to bind gp120 monomer but also recognize various stage of conformational changes of pre-fusion trimer [302]. Ab members of the group include 35O22, PGT151, 8ANC195, 3BC315/3BC176, CAP248-2B [303–307].

1.3.3 Antibody protection studies in animal models against cell-free virus challenge

Initial studies have tested the prophylactic passive transfer of first generation bNAbs in macaque models to protect them from SHIV infection. For instance, combination of 2F5 (anti-MPER Ab) and 2G12 (anti-gp120 glycan Ab) bNAbs together with HIVIG showed that protection in macaques against a pathogenic SHIV virus is possible via both intravenous and mucosal routes [308,309]. Similarly, the combination of 2F5, 2G12 and F105 (anti-CD4bs) NAbs displayed effective protection against both systemic SHIV challenge in mature animals and oral SHIV challenge in neonate animals [221]. Our group evaluated the efficacy of a triple combination of 2F5, 4E10 and 2G12 bNAbs combined in a microbicide gel against vaginal SHIV challenge. The Ab combination, prepared at a concentration of 20 mg/g of HEC gel for each Ab (60 mg in total) protected 70% (10 out from 15) animals [222]. The use of single Ab was also protective. The anti-CD4bs Ab b12 has been used in multiple SHIV protection studies that have helped in understanding both the mechanism as well as durability of protection provided by bNAbs [310]. Following studies using individual Abs, including 2F5, 4E10 and 2G12 showed protection against mucosal

SHIV challenge, which further supported the concept of antibody-mediated protection [230,311]. While the first generation bNAbs were efficacious to inhibit cell-free infection against either intravenous or mucosal SHIV challenge, nevertheless the amount of bNAb that was needed to get effective protection was high (infusion dose >20 mg/mL and Ab concentration in plasma higher than 100 µg/mL). Those concerns together with the lack of information on lifelong protection did not support their usage in human population.

The identification of newer generation bNAbs with potencies 10 to 100 folds greater than earlier bNAbs has renewed interest in their potential clinical use [312]. Indeed, the new generation of highly potent bNAbs display better efficiency to suppress viremia for longer time by using lower concentration of Ab than the first generation of bNAbs [243]. For example, the anti-V3 glycan Ab PGT121 conferred sterilizing immunity against mucosal SHIV_{162P3} virus in all animals when administered 5 and 1 mg/Kg, with corresponding average Ab serum concentrations of 95 µg/mL and 15 µg/mL, respectively. Infusion with 0.2 mg/Kg of Ab resulted in 60 % protection [313]. Following studies using other second-generation bNAbs, including anti-CD4bs - VRC01, VRC07-53, 3BNC117 and anti V3 glycan 10-1074 have shown successful protection *in vivo* [313–319].

Besides being used to prevent viral acquisition, bNAbs have been evaluated also for therapeutic properties. Ab-mediated immunotherapy demonstrated promising results in animal models using individual bNAbs or Ab combinations in presence and absence of ART [243]. The administration of PGT121 in SHIV_{162P3} infected rhesus macaques at chronic stage of infection suppressed the viremia to undetectable level in 18 macaques for 35 to >100 days. Three out of eighteen macaques displayed no emergent viral rebound. Moreover, reduction of HIV-DNA was observed in PBMCs, lymph nodes and the mucosa of gastrointestinal tract [320]. Single use or combination of 10-1074 and 3BNC117 in chronically SHIV-AD8 infected macaques temporary suppressed blood viremia (4-7 days) but viruses resistant to 10-1074 emerged. The treatment with Ab combination at 36 months post-infection exhibited a better outcome demonstrated with only one out of five macaques

showing viral rebound [321]. The combination therapy of bNAbs (single infusion with VRC07-523 and PGT121 at day10 post-infection) with ART (at day 21 post-infection) in macaques infected with SHIV_{162P3} showed efficacy comparable with ART alone [322].

Moreover, bNAbs immunotherapy have been evaluated in acute infected macaques. The combination of PGT121 and VRC07-523 bNAbs was used to orally treat neonatal macaques 1 day after SHIV_{162P3} oral inoculation. In the treated group, viral DNA was detected at low level in mucosa and lymph nodes after 24 hours of treatment. On day 14 post-infection, no detectable viremia was observed in treated macaques [250]. In another study using adult macaques infected with SHIV-AD8 via intrarectal or intravenous routes, combination of 10-1074 and 3BNC117 was given at day 3 post-infection. This treatment inhibited virus replication for 48-177 days after which viral rebound was observed [323]. Based on the success of protection studies in animal models, bNAbs have been explored for their efficacy in human clinical trials.

1.3.4 bNAbs used in human clinical trials

Most of the earlier studies have been focused on Ab treatment of HIV-1 infected individuals with some limited success. 2G12, 4E10 and 2F5 bNAbs used either singularly or in combination before ART in infected individuals have failed to control viral rebound, resulting from the fast emergence of escape variant [324,325]. However, preclinical data with second-generation bNAbs re-invigorated this area of research and several bNAbs are currently investigated in clinical trials (**Figure 11**).

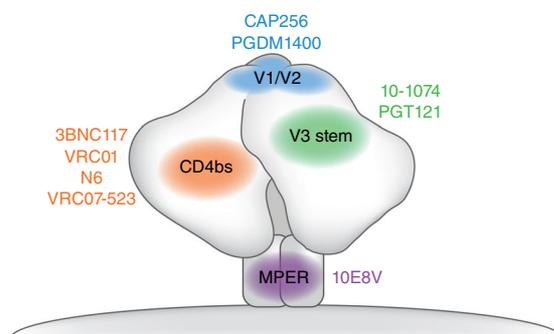


Figure 11: Second generation bNAbs tested in clinical trials.

Adapted from Caskey *et al.*, 2019 [326].

Indeed, studies using second-generation bNAbs, including 3BNC117, VRC01 and 10-1074 reported better outcomes [327–330]. Primarily, 3BNC117 monotherapy was assessed in 11 infected patients receiving a single Ab administration. The treated subjects (10 out from 11) showed a reduction of plasma viral load (mean = 1.48 log₁₀ copies/mL) and a viremia suppression for 28 days after treatment [327]. In addition, infected cell clearance was observed [252,331]. Interestingly, 10-1074 bNAbs displayed similar potency to what reported for 3BNC117 with the exception that the incidence of viral rebound was higher [326]. The clinical trial with VRC01, which target the same site as 3BNC117, was less efficacious than those using 3BNC117 [329,332]. The adapted bNAbs monotherapy regimen used in ART interruption (ATI) have shown interesting outcomes. 3BNC117 treatment undergoing ATI displayed low level of viral rebound and of emergence of resistant variants. Few participants could maintain the viral suppression until the Ab concentration went below 20 µg/mL [333]. In VRC01 clinical trial, repeated administrations of VRC01 in ATI contributed to viremia suppression for 4 weeks [332]. Interestingly, the clinical potential of bNAbs corresponds with their efficacy against primary HIV-1 isolates and is directly related to the protection in animal models [326].

The trials of bNAbs monotherapy have exhibited the drug-liked effect of bNAbs. The current combination of ART that are directed against different target are more efficient and led to lower emergent resistant variants. Applying the similar idea of using a combination of ART, bNAbs immunotherapy has been further used in combination [326]. Although, the clinical trial on combination of 10-1074 and 3BNC117 showed better potential than the monotherapy did, the treatment could not completely suppress viremia in the participants with high viral load at starting treatment [334]. In the following trial, the similar bNAbs combination was infused in the infected subjected at 2 days before ATI then 3 and 6 weeks after ATI. The results showed successful viremia suppression for long period [335].

However, as a threat, viral resistance is still present in HIV-1 clinical therapeutics, and HIV-1 has been shown to develop resistance to NAbs as well.

In conclusion, bNAbs have proven their protective potential against cell-free infection. However, as HIV transmission occurs also through infected cells present in biological fluids, it is extremely important to define the role and the protective efficacy of bNAbs in the context of cell-associated transmission.

1.3.5 Role of bNAbs in prevention against cell-associated virus transmission

Although it could represent the predominant mode of viral spread *in vivo*, bNAbs prevention of CAV transmission has not been studied the same depth as cell-free infection. Indeed, almost all *in vitro* neutralization assays and *in vivo* protection experiments have been performed using cell-free virus inoculum. The *in vitro* studies performed have produced conflicting results regarding the potency of bNAbs to block cell-free versus CAV transmission [336–344], possibly due to varied experimental system used by different laboratories [341]. Differences were seen across virus strains and Abs epitopes, and a substantial variability can be attributable to whether the assay system used acutely transfected or chronically infected donor cells, cell lines or primary cells, lab-adapted strains or transmitter/founder viruses [341,345]. However, there is today a general agreement that bNAbs exert a reduced efficacy against CAV, evidenced by the much higher concentration or bNAbs combination required compared to cell-free virus inhibition. This might be due to several mechanisms (**Figure 12**), such as: steric hindrance at the virological synapse, the increased multiplicity of infection (MOI) observed in CAV transmission, the different conformation of the viral envelope during cell-free and CAV transmission (and this might affect some epitopes more than others as well as vary between genetically diverse env) and to the stability of viral envelope-Ab complexes (for a recent review see [346]). Finally, the exposition of some neutralizing epitopes may be limited if membrane fusion occurs within endosomal compartments in the target cell [347].

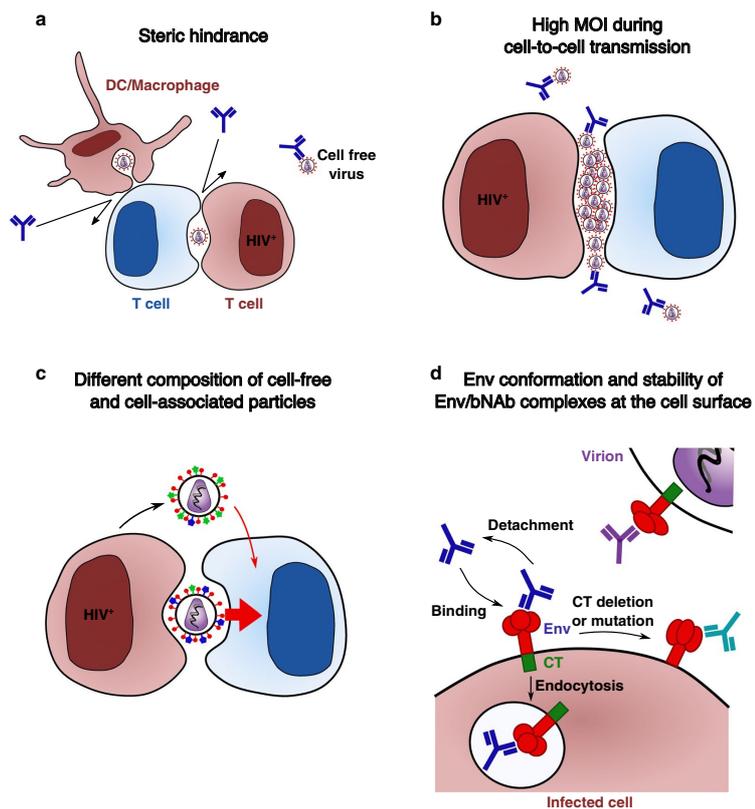


Figure 12: Potential mechanisms explaining the increased resistance of cell-associated HIV-1 to bNABs-mediated neutralization.

Adapted from Dufloo *et al.*, 2018 [346].

Experiments performed using the “first generation bNABs”, such as 2F5, 4E10, b12 and 257-D, produced conflicting results and no clear pattern could be determined [348–350]. As for cell-free viral inhibition, the development of “second generation bNABs” has permitted a more comprehensive examination of the mode of cell-to-cell virus inhibition by bNABs. A study by Abela *et al.* indicated that the targeted epitope may influence the efficacy of a given Ab [336] and that CD4bs Abs lost efficacy during CAV transmission. The relative neutralization resistance in intercellular assay systems was confirmed by other studies [339,340,344], however in one of them [339] Abs directed against the CD4bs and the V3 loop were the most active to inhibit transmission between T cells. A recent study by Li *et al* [338] showed that a function motif in the gp41 appears to contribute to the loss of potency and magnitude of multiple bNABs during cell-to-cell transmission. In the case of DCs/T cells transmission, the group of Moog showed that bNABs inhibit HIV-1 transfer from

primary DCs and pDCs to autologous CD4⁺ T cells [61,351]. Antibody-conferred inhibition via Fc has been observed in case of transferring HIV-1 infection from antigen presenting cells (APCs) to surrounding T cells, which may be related to the FcRs presence on the surface of DCs and macrophages. The FcR-conferred protection required the binding of FcRs to Abs [61,341]. Interestingly, in comparison with anti-gp41 bNAbs, the anti-gp-120 bNAbs were not only more potent in Fc-mediated protection but also more efficient in preventing infection transmission from either macrophages or DCs to T cells [61,337]. An increasing number of observations are also pointing to the fact that possibly a combination of bNAbs is necessary to efficiently inhibit CAV transmission. In *in vitro* studies no single Ab was able to inhibit all strains tested [340] and a combination of PG9 and VRC01 was more effective during cell-to-cell transmission compared to single Abs [344].

It is important to note that the relevance of *in vitro* studies to the *in vivo* efficacy of the same Ab to inhibit cell-to-cell versus cell-free transmission is an understudied area. Up to date only one study evaluated the efficacy of bNAbs to inhibit CAV transmission *in vivo* in macaques. The authors used SHIV_{162P3}-infected splenocytes to intravenously challenge pigtail macaques and infused the animals with the anti-V3 bNAb PGT121 [352]. Partial protection from infection was observed and a delay in peak viremia or a delayed viremia was reported for not protected macaques [352].

Finally, bNAbs could mediate Fc effector functions that may block HIV-1 CAV transmission. The Fc domain presented on Ab is recognized by FcR receptor on various immune cell types to trigger the mechanism of antibody-mediated inhibition which possibly occurred in case of bNAbs and inhibited also the CAV transmission. For instance, bNAbs were able to recruit NK cell via ADCC to kill HIV-1 infected cells [249,353,354]. Moreover, bNAbs could activate ADCP and the complement pathway. For CAV transmission, bNAbs and non-NABs mediated ADCC function relied on the accessibility of viral envelope protein on the cell surface. There are evidences that to obtain optimal protection *in vivo*, bNAbs need Fc-mediated immune responses to be involved [245,246,355]. Although, bNAbs may not provide completely neutralization

activity against CAV, bNAbs mediated Fc function behaves like the additional mechanism to direct against infected cells. Lu *et al.* (2016) demonstrated that bNAbs could eliminate HIV-1 infected cell and trigger the Fc mediated protection in humanized mice. Infusion with either 3BNC117 alone or the combination of 3BNC117 and 10-1074, performed 12 hours before the transfer of infected cell, could reduce not only the percentage of infected cells but also the level of CAV HIV-1 DNA compared to the control mice [356]. Similar results were observed when using CD4⁺ T cells infected with primary HIV-1 isolated strains. Furthermore, the clearance of HIV-infected cells *in vivo* possibly depended on the interaction of Fc γ R on effector cell and Fc domain on 3BNC117 Ab [356]. Their observations highlight the important function of Fc γ R mechanism mediated by bNAbs to eliminate HIV-1 infected cells *in vivo*.

In addition, bNAbs may be related to the removal of latent infected cells. In humanized mice the administration of a triple combination of 3BNC117, PG16 and 10-1074 early after infection prevented the establishment of latent reservoir [357].

Additional studies are necessary to understand why some bNAbs are less efficient against CAV infection and why this varies with different viral strains and cell types. At the present there is limited evidences regarding which *in vitro* assay better predicts *in vivo* results. Moreover, all the *in vitro/ex vivo* studies published have made use of *in vitro* infected cells to evaluate the efficacy of bNAbs to inhibit cell-to-cell transmission. Data making use of the relevant donor cells found in the semen of infected subjects are missing. The ability of bNAbs to inhibit cell-to-cell transmission initiated by semen cells will be the subject of the **chapter 4.2** of this manuscript.

1.4 Macaque models: similarities and differences to humans

NHP species including African monkeys and apes are natural SIV host which typically do not develop disease following the infection [358,359]. On the other hand, macaque species which are non-natural SIV host, can develop the infection with certain strains of SIV, resulting in high viral load, progressive CD4⁺ T cells depletion and progression to AIDS [358,359]. As the disease progression in macaques

resembles to HIV-1 infection in humans [358,360], the macaque models are routinely used as animal models for HIV-1/AIDS research [358–361].

Interestingly, the key pathogenic features of HIV-1 infection in human can be observed in SIV infected macaques [358,359,361,362]. Both HIV-1 and SIV have similar key features of virus persistence including: integration of viral DNA into the target cell genome [363,364] with similar preference integration sites [365], response to interferons favoring HIV/SIV DNA persistence [366], inducing latent HIV/SIV without co-engagement of T-cell receptors by costimulatory signals [367]. Both the infected human and macaque distribute the infected leukocytes containing viral DNA and RNA sequences to peripheral blood, LNs and at mucosal sites [368–370]. Cytotoxic T lymphocytes are unable to clear infected cells in long term because of resistance mutation in HIV-1/SIV [371,372]. Those similarities highlight the sharing of reservoir dynamics between HIV-1 infected patients and SIV infected macaques [361].

While sharing some key pathogenic aspects, there are however some differences between HIV-1 infection in humans and SIV infection in macaques. For instance, SIVmac is more virulent than HIV-1 [373]. In SIV⁺ macaques, the set point of viral load is 10 to 100 folds higher than in HIV⁺ subjects. The disease progression to AIDS generally occurs within 1-2 years and up to 40% of macaques develop AIDS more quickly (< 1 year) [373,374]. Moreover, another difference between HIV-1 and SIV infection regards their interaction with the enzyme SAM domain and HD domain 1 (SAMHD1) [361]. SAMHD1 is a HIV-1 restriction factor [375] which antagonize with the viral accessory protein Vpx present in some SIVs (particularly those that are used in the macaques) [376,377]. As SAMHD1 may play a role in blocking viral infection by interfering with a specific step of viral replication [358,361], it also displays a dissimilarity between HIV-1 infection of human and SIV infection in macaque models [361].

The immune response to antigens in NHPs, for both adaptive and innate immune response, are very close to those in humans [358,360]. However, there are interspecies differences. While the major histocompatibility complex (MHC) region

maps to chromosome 6 of humans which is composed of one copy of *HLA-A*, *HLA-B* and *HLA-C* per haplotype, the MHC class I region in macaques is more expanded and complex, containing multiple *MHC-A* and *MHC-B* genes per chromosome [360]. The number and variability of antigen specific T cells that recognize individual MHC-peptide complexes are quite similar in NHPs and humans [360]. Moreover, immunoglobulin loci in macaques are more diverse than in humans [360]. Although both humans and macaques have four IgG subclasses, including IgG1, IgG2, IgG3 and IgG4, the macaque IgG subclasses lack the sequence diversity regions that are important for the FcγR interaction in human IgG1 [378]. Indeed, the subclasses of macaque IgG are much less structurally and functionally diverse group of proteins than those found in humans [379]. For examples, IgG3 of human has a more elongated hinge than those of macaque [378,379]. As a consequence, a shorter hinge in the macaque probably renders it less flexible than its human counterpart [378]. This increased flexibility of the human hinge region is thought to confer the enhancement of HIV-1 neutralizing potency [380,381]. Members of the FcγR family between humans and macaques share several common traits [378,379]. However, the main key difference has been recognized for FcγRIII which is not present on macaque neutrophils [382–384] and for FcγRII which has a higher level of expression on macaque neutrophils [382,384]. Due to the importance of Fc-dependent antibody function, the insight on the interspecies variability of IgG and its receptors between humans and NHPs may increase the accuracy of preclinical observation to convert to success of clinical therapeutics.

Although the macaque models have some limitations, they can be used to establish “proof of concepts” for mucosal transmission, physiopathology and many therapeutic approaches of HIV-1/AIDS such as Pre-exposure prophylaxis (PrEP), ART and microbicides [359,362]. These animals are still suitable for HIV-1 research as there is no ideal animal model available.

Chapter 2 Objectives

As emphasized in the introduction, the empirical evidences and interests on CAV transmission mediated by semen from HIV seropositive subjects are growing. Due to the infectious potential of CAV, it is important to better understand the dynamic modulation of semen during HIV-1 infection and to find the optimum strategies to prevent viral dissemination. In this thesis project, I used *in vitro* assays and the NHP model of infection of cynomolgus macaques to answer to specific questions. In specific, the aims of the project are to investigate the immune responses in semen during SIV infection and to evaluate the potency of bNAbs to block CAV transmission. The two aims are divided in 2 studies.

In the first study, we assessed dynamic changes of the immune response in the semen of SIVmac251 infected cynomolgus macaques. We investigated SIV-specific innate and adaptive responses in semen and compared to systemic responses, including CD8⁺ T cells responses and SIV specific humoral responses (*in vitro* neutralization and FcγRIIIa binding activity), to evaluate their potential role in controlling viral shedding. Moreover, we measured levels of cytokines, chemokines and growth factors in seminal plasma in order to define the association between the inflammatory state of semen, cell activation and potential risk of transmission.

In the second study, we assessed the efficacy of a panel of bNAbs against CAV transmission. We developed an *in vitro* CAV transmission assay using either TZM-bl or human PBMC as target cells and SHIV_{162P3}-infected splenocytes and CD45⁺ semen leukocytes as donor cells. The panel of bNAbs was selected to target different epitopes of HIV envelope, including 1st generation bNAbs (2F5, 4E10, 2G12 and b12) and 2nd generation bNAbs (10-1074, PGT128, PGT151, 3BNC117, N6, PGDM1400, PG16 and 10E8). A first screening of bNAbs efficacy to inhibit SHIV_{162P3} transmission was done using splenocytes and a reduced panel was confirmed with semen cells. On the base of the results obtained *in vitro*, we have selected an anti-V3 directed bNAb to be used in a future study to block CAV transmission *in vivo* in cynomolgus macaques.

Chapter 3 Experimental approach

3.1 The cynomolgus macaque model for HIV research: use of the pathogenic strains SIVmac251 and SHIV_{162P3}

Three species of macaques, including rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*) and cynomolgus macaques (*Macaca fascicularis*), have become widely used and accepted models for AIDS research [358–361].

In this work we used cynomolgus macaques imported from Mauritius. The macaques used descend from a small founder population with only 7 MHC haplotypes identified, indicating a limited MHC diversity and contributing to a certain homogeneity in infection profiles, particularly during primary stages [385–387]. Nevertheless, some studies have reported heterogenic profiles in viral replication control in chronic infected Mauritius cynomolgus macaques, which was similarly reported in HIV⁺ men [388–390]. SIV infection in cynomolgus macaques is generally characterized by lower set point viral loads and slower progression of CD4⁺ T cells decrease than Indian and Chinese rhesus macaques [358]. The slower disease progression of cynomolgus macaques compared to rhesus macaques may be preferable as it mimics a closer situation compared to HIV infection and AIDS in humans.

The SIV genome is slightly different from the HIV-1 genome. For instance, SIVmac presents an accessory gene *vpu* instead of *vpx* found in HIV-1 (**Figure 13**). SIVmac251, an uncloned strain displaying high genetic diversity like HIV-1, is widely used for mucosal challenges, with rectal, vaginal or intravenous exposure and to study the pathogenesis of infection [358,391]. The virus stocks, commonly used in our laboratory, have been titrated *in vivo* and used many times for mucosal challenges [389,391–393]. 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 [391].

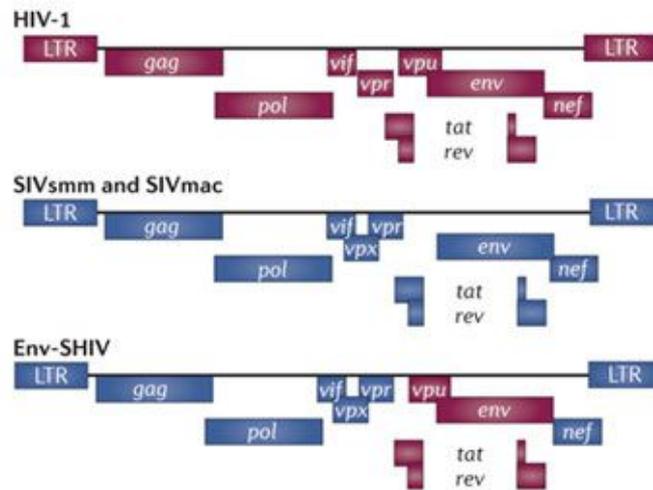


Figure 13: Genetic difference between HIV-1, SIVmac and Env-SHIV strains.

Adapted from Hatzioannou & Evans, 2012 [358].

Thus, Mauritius cynomolgus macaques infected with SIVmac251 represent a valuable animal model that allowed us to study the immune response in semen during SIV 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.

Although SIV virus is useful to study pathogenesis of AIDS, the difference between the SIV env protein and the HIV-1 env protein contributed to the non-cross neutralization activity of anti-HIV-1 bNAbs to SIV [312,394]. Thus, chimeric strains of SHIV were constructed. Generation of SHIV (**Figure 1**) is mostly created by replacing the SIVmac239 genes of *rev*, *tat* and *env* with the HIV-1 genes of *rev*, *tat*, *vpu* and *env*. A case in point is SHIV_{163P3} – R5 tropic SHIV – that is a useful strain to evaluate treatments aiming to prevent HIV-1 transmission such as NAbs [358].

Indeed, we used SHIV_{162P3} virus to evaluate the efficacy of bNAbs against CAV transmission. Progressive examination of bNAb candidates in clinical trials require pre-clinical studies thus, it is important to investigate their efficacy in vigorous animal model like NHP. The bNAbs potency in preventing cell-free infection has been evaluated often in NHP models. However, their protective potential in the context of CAV transmission is not well defined and only one study has been published so far [352].

Despite of the fact that a few studies have evaluated the bNAbs potency against CAV, some research teams have successfully established CAV transmission models in NHP. Early efforts have shown successful CAV transmission in rhesus macaques after intravenous inoculation. However, the initiation of infection following mucosal inoculation was more difficult [395]. Despite this, using SIV⁺ CAV as challenge stocks, Kaizu *et al.* and our group have achieved to transmit infection following vaginal inoculation in cynomolgus macaques [71,396]. Kolodkin-Gal *et al.* demonstrated rectal transmission following SIV⁺ CAV inoculation in rhesus macaques [69]. It is interesting to note that the viral inoculum used in the studies of Kaizu *et al.*, Sallé *et al.* and Kolodkin-Gal *et al.* were in a range of the expected virus per ejaculation of HIV-1 infected men, indicating the main implication of CAV model. Those studies supported our project to evaluate the efficacy of bNAbs in CAV mucosal transmission model (vaginal route) in cynomolgus macaques using splenocytes as infected cells, a model that has been successfully set up in our laboratory.

3.2 Semen sampling by electro-ejaculation

The collection of semen samples by sexually mature animals has been performed using a technique called electro-ejaculation [397], which consists in an electronic stimulation of the prostate. It is a non-invasive method which is largely considered as reliable, reproducible and safe. However, it differs from the electro-stimulation technique, which consists in stimulating the penis with electrodes, without anesthesia [397]. The sampled volume and the concentration in spermatozoa might be reduced by electro-ejaculation compared to electro-stimulation.

In the lab we have successfully set up the protocol to collect semen by intrarectal electrostimulation of the prostate using a probe (12.7 mm diameter) lubricated with a conductor gel and an AC-1 electro-ejaculator (Beltron Instrument, Longmont, USA). Sequential stimulation was performed with a pattern of six cycles, nine stimulations of 2 s for each cycle, followed by a tenth stimulation lasting 10 s. The voltage was increased every two cycles (1-3 V for the first two, 2-4 V volts for

series 3-4, 6-8 V for series 5-6). If a complete ejaculum was not obtained after six cycles of stimulation, a seventh cycle at 7 to 10 V was performed. Complete ejaculum (volume ranging between 0.1 and 0.5 mL) was immediately diluted in 1.2 mL phosphate buffer saline (PBS) and centrifuged.

3.3. Establishment of *in vitro* neutralization assays to predict the *in vivo* protective potential of bNAbs

Neutralization activity is regarded as a crucial contributor for HIV-1 protection [312]. There are growing evidences that strategies to optimize bNAbs-mediated protection from CAV transmission are needed. NHP models represents valuable systems for testing such strategies [395]. In addition, complementary *in vitro* assays that accurately predict the capacity of bNAbs to inhibit cell-to-cell spread *in vivo*, can contribute to optimize those strategies. As discussed earlier in this manuscript, many groups established *in vitro* experimental systems of CAV transmission, which however were highly variable in terms of target cells and donor cells used and viruses utilized [345,398]. Consequently, divergent results across studies were obtained [336–344]. There is no clear evidence displaying which *in vitro* assay closely reflect the CAV transmission *in vivo* and confer protection by bNAbs [395]. To reduce this variation and better predict the *in vivo* protection conferred by bNAbs on the base of *in vitro* results, one approach might be to use a common CAV stock to screen bNAbs *in vitro* and in parallel *in vivo* in NHP models.

Therefore, we set up an *in vitro* experimental system to assess bNAbs-mediated inhibition of CAV transmission, using splenocytes collected from SHIV_{162P3} infected macaques at the peak of infection. This same stock of cells will be employed in *in vivo* challenge studies in cynomolgus macaques.

Chapter 4 Results

4.1 First article (Submitted): Innate and adaptive anti-SIV responses in macaque semen: implications for infectivity and risk of transmission

4.1.1 Summary

In the first article, we aimed to investigate the anti-SIV innate and adaptive immune response in semen of cynomolgus macaque during SIVmac251 infection. We conducted a comprehensive study on a total of 53 animals. We analyzed viral shedding and seminal cytokine/chemokine profiles. Moreover, we characterized the phenotype of semen CD8⁺ T leukocytes and the SIV specific CD8⁺ T cell response using *ex vivo* stimulation of semen cells with SIV gag peptides and the intracellular staining with antibodies targeting CD45RA, IL-2, IFN- γ , TNF and MIP-1 β . To determine Ab response, we quantified SIV-specific Ab titers in the semen of macaques at different stages of infection and performed an *in vitro* neutralization assay and ELISA based Fc γ RIIIa binding assay to evaluate the potential function of SIV-specific Ab.

In macaque semen, we observed that many cytokines were affected by SIV infection. Several pro-inflammatory (IL-8, IL-15, IL-18, IFN- γ , IP-10 and RANTES) and the immunomodulatory molecule IL-13 were found significantly increased during the primary infection compared to uninfected macaques and several of them predicted seminal and /or blood viral load. This inflammatory environment might recruit target cells and modulate immune cells. Indeed, we provide evidence of a high level of preexisting activated CD69⁺ CD8⁺ T cells in the MGT, which is further elevated upon SIV infection. In parallel, we observed upregulation of CCR5⁺ CXCR3⁺ CD8⁺ T cells, typical of a Tc1 phenotype, which is consistent with the capacity of those cells to migrate to inflammatory tissues. Neither SIV-specific CD8⁺ T-cell responses nor humoral responses controlled seminal viral shedding. The neutralization activity was generally weak but specific, as assessed using a VSV-pseudotyped virus as control. In 67 % of the infected macaques, we were able to detect Fc γ RIIIa-binding Abs in semen, which is indicative of ADCC. Interestingly, Fc γ RIIIa-binding activity in semen positively correlated with SIV-specific Ab titers and seminal viral load.

Thus, failure to control viral replication in SIV-infected semen is related to a general inflammation and immune activation, which possibly mirrors what happen in the MGT and which could lead to enhanced HIV-1/SIV transmission

Overall, our study highlights the complex interplay between immune activation and inflammation and the role might play in modulating semen infectivity and risk of transmission. These observations are relevant to our understanding of natural immunity to HIV-1. A better comprehension of the mechanisms that regulate immunity in the MGT may contribute to improving the efficacy of currently available prevention measures and lead to the identification of new determinants of HIV-1 transmission.

4.1.2 Manuscript

Innate and adaptive anti-SIV responses in macaque semen: implications for infectivity and risk of transmission

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

Study conception and design: RL and MC. Acquisition of data: KS, SS, CG, and BD. Analysis and interpretation of data: KS, SS, CG, NB and MC. Drafting of the manuscript: KS and MC. Critical revisions: KS, SK, BW, MH, RL and MC.

Keywords

SIV, Cytokines, Antibodies, ADCC, CD8+ T cells

Abstract

Word count: 195

HIV-1 infection is transmitted primarily by sexual exposure, with semen being the principal contaminated fluid. However, HIV-specific immune response in semen has been understudied. We investigated specific parameters of the innate, cellular and humoral immune response that may affect semen infectivity in macaques infected with SIVmac251.

Serial semen levels of cytokines and chemokines, SIV-specific antibodies, neutralization and FcγR-mediated functions and SIV-specific T-cell responses were assessed and compared to systemic responses across 53 cynomolgus macaques.

SIV infection induced an overall inflammatory state in the semen. Several pro-inflammatory molecules correlated with SIV virus levels. Effector CD8+ T cells were expanded in semen upon infection. SIV-specific CD8+ T-cells that expressed multiple effector molecules (IFN-γ+MIP-1B+TNF+/-) were induced in the semen of a subset of SIV-infected macaques, but this did not correlate with local viral control. SIV-specific IgG, commonly capable of engaging the FcγRIIIa receptor, was detected in most semen samples although this positively correlated with seminal viral load.

Several inflammatory immune responses in semen develop in the context of higher levels of SIV seminal plasma viremia. These inflammatory immune responses could play a role in viral transmission and should be considered in the development of preventive and prophylactic vaccines.

Contribution to the field

Sexual HIV-1 transmission occurs primarily by contaminated semen, however little is known about the innate and adaptive anti-viral immune response taking place in the semen, that may limit or enhance viral transmission. We show in a non-human primate model that acute SIV infection is associated with increased levels of pro-inflammatory and immunomodulatory molecules (IL-8, IL-13, IL-15, IL-18, IFN-γ, RANTES and IP-10) which predicted viral load and potentially recruit target cells and modulate immune cells. In parallel, we observed upregulation of CCR5+ CX3CR1+ CD8+ T cells, typical of a Tc1 phenotype, which is consistent with the capacity of those cells to migrate to inflammatory tissues. Although a subset of infected macaques developed SIV-specific CD8+ T-cells that expressed multiple effector molecules and antibodies capable of engaging the FcγRIIIa receptor, neither SIV-specific CD8+ T-cell responses nor humoral responses controlled seminal viral shedding. Thus, failure to control viral replication in SIV-infected semen is related to a general inflammation and immune activation, which possibly mirrors what happens in the male genital tract and which could lead to enhanced HIV/SIV transmission. Overall, our study highlights the complex interplay between immune activation and inflammation and the role it might play in modulating semen infectivity and risk of transmission.

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Ethics statements

Studies involving animal subjects

Generated Statement: The animal study was reviewed and approved by Comité d’Ethique en Experimentation Animale de la Direction des Sciences du Vivant au CEA.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

In review

Data availability statement

Generated Statement: The datasets generated for this study are available on request to the corresponding author.

In review

1 **Innate and adaptive anti-SIV responses in macaque semen:**
2 **implications for infectivity and risk of transmission**

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31 **Key words: SIV, cytokines, antibodies, ADCC, CD8+ T cells**

32

33 **Abstract**

34 HIV-1 infection is transmitted primarily by sexual exposure, with semen being the principal
35 contaminated fluid. However, HIV-specific immune response in semen has been understudied.
36 We investigated specific parameters of the innate, cellular and humoral immune response that
37 may affect semen infectivity in macaques infected with SIVmac251.

38 Serial semen levels of cytokines and chemokines, SIV-specific antibodies, neutralization and
39 Fc γ R-mediated functions and SIV-specific T-cell responses were assessed and compared to
40 systemic responses across 53 cynomolgus macaques.

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42 molecules correlated with SIV virus levels. Effector CD8⁺ T cells were expanded in semen
43 upon infection. SIV-specific CD8⁺ T-cells that expressed multiple effector molecules (IFN-
44 γ ⁺MIP-1 β ⁺TNF^{+/-}) were induced in the semen of a subset of SIV-infected macaques, but this
45 did not correlate with local viral control. SIV-specific IgG, commonly capable of engaging the
46 Fc γ RIIIa receptor, was detected in most semen samples although this positively correlated with
47 seminal viral load.

48 Several inflammatory immune responses in semen develop in the context of higher levels of
49 SIV seminal plasma viremia. These inflammatory immune responses could play a role in viral
50 transmission and should be considered in the development of preventive and prophylactic
51 vaccines.

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53
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In review

55 **Introduction**

56 More than 80% of new HIV-1 infections worldwide occur during sexual intercourse,
57 involving mucosal transmission of the virus [1–3]. Mucosal and genital tract tissues constitute
58 the major source of HIV-1 contaminating fluids. Semen, which contains both cell-free particles
59 and infected cells [4–6], represents the main vector of HIV-1 dissemination, illustrated in part
60 by transmission occurring more frequently from men to men and women than women to men
61 [7]. Transmission of HIV-1 has been correlated with seminal viral load (SVL) and stage of
62 infection [8,9], but the mechanisms of transmission have not been fully addressed. To establish
63 an infection via the genital mucosa, HIV-1 in semen must overcome substantial immunological
64 and physiological barriers to cross the epithelium and infect underlying target cells. Several
65 factors are known to affect semen infectivity and enhance the risk of HIV-1 transmission to a
66 partner, such as concomitant genital infections and inflammation, natural antimicrobial
67 molecules (β -defensins, secretory leukocyte peptidase inhibitor –SLPI-, lysozyme,
68 lactoferrin), semen-derived amyloid fibrils [10,11], cytokines (IL-7) [12], and male
69 circumcision [13–17]. However, very little is known about the actors of the innate and HIV-
70 specific adaptive immune response present in semen that may limit or enhance viral
71 transmission. Indeed, most studies on anti-HIV-1 responses in the genital tract have focused on
72 women, whereas knowledge about male genital tract (MGT) immunity against HIV-1 is still
73 limited. Improved knowledge of the role of immunoglobulins (IgG) and cellular responses
74 present in semen may help in the development of more pertinent strategies in the field of HIV-
75 1 transmission prevention.

76 Although the role of cell-mediated immunity in reducing HIV-1 replication and killing
77 infected cells has been fully established [18], very few studies have assessed the presence of
78 HIV-specific CD8⁺ T cells and their functionality in semen [19–22]. Sheth *et al.* reported that
79 HIV-specific CD8⁺ T cells in semen do not correlate with reduced local virus shedding [23].
80 However, only a subset of HIV-specific CD8⁺ T cells (polyfunctional cells) are most important
81 in controlling viremia [24] and, it is still unclear whether the presence of HIV-specific
82 polyfunctional cytotoxic cells in semen have an impact on the local shedding of viral particles
83 or infected cells and HIV-1 transmission.

84 HIV-specific antibodies (Abs) may prevent or partially control mucosal HIV-1/SIV entry
85 [25,26]. In macaques, systemic and mucosal application of neutralizing IgG prevents infection
86 after intravenous or vaginal inoculation of SHIV [27–30]. HIV-specific non-neutralizing Abs
87 may also play a protective role through antibody-dependent cell-mediated cytotoxicity
88 (ADCC), induction of viral aggregation, or prevention of viral uptake [31–34]. HIV/SIV-
89 specific Abs, mostly IgG, have been reported to be present in abundant quantities and at high
90 frequencies (81-100%) in the semen of HIV⁺ men and SIV⁺ macaques [26,35,36]. However,
91 their ability to neutralize the virus and induce ADCC has been little investigated [37,38].

92 Here, we focused on the innate and adaptive immune response in the semen of SIV-infected
93 macaques that we used as a model of HIV-1 infection and AIDS. We defined the dynamics of
94 SVL, cytokine levels, the SIV-specific Ab response, and CD8⁺ T cell levels during acute and
95 chronic infection. An understanding of the humoral and cellular immune function in semen and
96 its effect on local viral replication in the cynomolgus macaque model should provide a
97 framework for developing immunological interventions to prevent sexual transmission of HIV-
98 1.

99 **Materials and Methods**

100

101 **Ethical statement and animals housing**

102 Cynomolgus macaques (*Macaca fascicularis*), weighing 5 to 11 kg, were imported from
103 Mauritius and housed according to European guidelines for animal care (Journal Officiel des
104 Communautés Européennes, L 358, December 18, 1986 and new directive 63/2010). All work
105 related to animals was conducted in compliance with institutional guidelines and protocols
106 approved by the local ethics committee (Comité d’Ethique en Experimentation Animale de la
107 Direction des Sciences du Vivant au CEA under numbers: 2015032511332650(APAFIS#373);
108 10-062; 12-103).

109 **Infection and sample collection**

110 Adult males were infected by intravenous or intrarectal inoculation of 50-5,000 animal
111 infectious doses 50% (AID50) of SIVmac251 isolate [39]. Semen and blood were collected
112 from sedated animals following 5 mg/kg intra-muscular injection of Zoletil®100 (Virbac,
113 Carros, France).

114 Electroejaculation was performed by intrarectal electrostimulation of the prostate using a
115 probe (12·7 mm diameter) lubricated with a conductor gel and an AC-1 electro-ejaculator
116 (Beltron Instrument, Longmont, USA), as previously described [40]. Sequential stimulation
117 was performed with a pattern of six cycles, nine stimulations of 2 s for each cycle, followed by
118 a tenth stimulation lasting 10 s. The voltage was increased every two cycles (1-3 V for the first
119 two, 2-4 V volts for series 3-4, 6-8 V for series 5-6). If a complete ejaculum was not obtained
120 after six cycles of stimulation, a seventh cycle at 7 to 10 V was performed. Complete ejaculum
121 (volume ranging between 0·1 and 0·5 ml) was immediately diluted in 1·2 ml phosphate buffer
122 saline (PBS) and centrifuged.

123 Blood samples were collected into BD Vacutainer® Plus Plastic K₃EDTA tubes for plasma
124 viral load quantification and BD Vacutainer® Plus Plastic SST tubes for serum preparation (BD
125 Biosciences, Le Pont de Claix, France).

126 **Seminal plasma and cell preparation**

127 Seminal plasma samples were isolated from total semen immediately after collection by
128 spinning 15 min at 775 x g. After separation from seminal plasma, seminal cells were diluted
129 in 14 ml complete medium, consisting of RPMI-1640 Glutamax medium (Invitrogen, Carlsbad,
130 USA) supplemented with a mixture of penicillin, streptomycin, and neomycin (Invitrogen) and
131 10% FCS (Lonza, Allendale, USA), and kept at room temperature for a maximum of 1 h. Cells
132 were then centrifuged 10 min at 1,500 x g, filtered through a 70-µM sieve and washed with 5
133 ml PBS supplemented with 10% FCS.

134 **Quantification of blood and semen RNA viral load**

135 Blood plasma was isolated from EDTA blood samples by centrifugation for 10 min at 1,500
136 x g and cryopreserved at -80°C. Seminal plasma was maintained on ice for a maximum of 1 h
137 and cryopreserved at -80°C. Blood viral RNA was prepared from 250 µL cell-free plasma using
138 the Nucleospin 96 RNA kit (Macherey Nagel GmbH&Co KG, Düren, Germany), according to
139 the manufacturer’s instructions. Retro-transcription and cDNA amplification and quantification
140 were performed in duplicate by RT-qPCR using the Superscript III Platinum one-step
141 quantitative RT-PCR system (Invitrogen, Carlsbad, USA). RT-PCR was performed as
142 previously described [39]. The quantification limit (QL) was estimated to be 111 copies/ml and
143 the detection limit (DL) 37 copies/ml. Semen viral RNA was prepared from 500 µL seminal
144 plasma using the QIAamp Ultrasens Virus kit (Qiagen, Courtaboeuf, France), according to the
145 manufacturer’s instructions. Quantitative RT-PCR was performed under the same conditions as
146 above, with a QL of 37 copies/ml and a DL of 12·3 copies/ml.

147 **Cytokine quantification in semen**

148 The concentration of IL-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23, IL-13,
149 IL-15, IL-17, IL-18, sCD40L, GM-CSF, G-CSF, TGF α , IFN γ , MIP-1 α , MIP-1 β , MCP-1, TNF
150 and VEGF were measured in seminal plasma using the Milliplex[®] Map Non-Human Primate
151 Cytokine Magnetic Bead Panel - Premixed 23-plex (Merck Millipore, Darmstadt, Germany).
152 Levels of RANTES and TGF- β 1, 2, and 3 were measured using monoplex and a 3-plex
153 Milliplex kits. Assays were performed in duplicate using 25 μ L seminal fluid. CXCL10/IP-10
154 was quantified using Quantikine ELISA human IP-10 immunoassay kit (R&D Systems, MN,
155 USA). Samples were thawed at room temperature and centrifuged for 10 min at 1,500 x g to
156 harvest all cellular components. Immunoassays were performed according to the
157 manufacturer's instructions. Data were acquired using a Bio-Plex 200 instrument and analyzed
158 using Bio-Plex Manager Software, version 6.1 (Bio-Rad, Hercules, USA).

159 **Phenotypic characterization of semen leukocytes**

160 Staining was performed in parallel on whole blood samples. All staining, except that of
161 whole blood assays, were performed after saturation of Fc receptors in healthy macaque serum
162 (in-house production) for 1 h at 4°C. Amine-reactive dye Live/dead[®] Fixable Blue (Life
163 Technologies) was used to assess cell viability and exclude dead cells from the analysis. Cells
164 were stained with monoclonal Abs for 30 min at 4°C, washed in PBS/10% FCS and fixed in
165 CellFIX[™] (BD Biosciences). Three different antibody panels were used. Panel 1, targeting
166 semen leukocyte subpopulations and T-cell activation, consisted of anti-CD45 PerCp (clone
167 B058-1283), anti-CD3 V500 (clone SP34-2), anti-CD4 PE-Cy7 (clone L200), anti-CD11b
168 Alexa Fluor 700 (clone ICRF44), anti-HLA-DR APC-H7 (clone G46-6), anti-CD69 FITC
169 (clone FN50), and anti-CD95 APC (clone DX2) (all BD Biosciences, Franklin Lakes, USA);
170 anti-CD8 V450 (clone BW138/80; Miltenyi Biotec GmbH, Bergisch Gladbach Germany); and
171 anti-CD28 (clone 25-0289-73; CliniSciences, Nanterre, France). Panel 2, targeting CCR5 and
172 CXCR3 expression on T cells, consisted of the same Abs used in panel 1 plus anti-CCR5 APC
173 and anti-CXCR3 FITC (IgG1, clone 1C6) (both BD Biosciences). Panel 3, targeting LFA-1 and
174 Mac-1 expression on T cells, consisted of the same Abs used in panel 1 plus anti-CD11a PE
175 (IgG1, clone HI111) and anti-CD18 APC (IgG1, clone 6.7) (both BD Biosciences).
176 Corresponding isotype controls of CCR5, CXCR3 CD11a and CD18 were used at the same
177 concentrations as the reference antibody. Acquisition was performed on a BD LSRII equipped
178 with four lasers (355, 405, 488 and 633nm) and analyzed using Flowjo v7.6 (Tree Star,
179 Ashland, OR).

180 ***Ex vivo* stimulation and intracellular CD45RA, IFN- γ , TNF, MIP-1 β , and IL-2 staining**
181 **of peripheral blood and semen mononuclear cells**

182 Briefly, 1x10⁶ peripheral blood mononuclear cells (PBMCs) were incubated for 1 h at 37°C
183 in 5% CO₂ with medium alone, staphylococcus enterotoxin B (2 μ g/100 μ L), or a commercial
184 pool of SIV gag peptides (89 peptides from p15 and p27, ProteoGenix, Schiltigheim, France)
185 at a concentration of 0.2 μ g/100 μ L, in the presence of the co-stimulatory Abs CD28 (clone
186 L293, IgG1) and CD49d (clone L25, IgG2b). Semen cells were split into two vials and
187 incubated for 1 h with medium alone or the SIV gag peptide pool/co-stimulatory Abs. Brefeldin
188 A (BD Biosciences) was then added (1 μ g/100 μ L) and the samples were incubated for 4 h,
189 permeabilized, and stained with combinations of CD45-PerCp (clone D058-1283, IgG1), CD3-
190 APC-Cy7 (clone SP34-2, IgG1), CD8-V500 (clone RPA-T8, IgG1), CD45RA-PE-Cy7 (clone
191 L48, IgG1), CD154-FITC (clone TRAP1, IgG1), IL-2-APC (MQ1-17H12, IgG2a), MIP-1 β -PE
192 (clone D21-1351, IgG1), TNF Alexa Fluor 700 (clone Mab11, IgG1), and IFN- γ -V450 (clone
193 B27, IgG1) (BD Biosciences). All Abs used in this panel were from BD Bioscience. A positive
194 response by PBMCs was considered to be SIV-specific if: 1) the response by Gag-stimulated
195 cells was at least two-fold higher than that of the unstimulated control and 2) the frequency of

196 the CD8⁺ SIV-specific response was > 0·1%. A positive response by semen was considered to
197 be specific if: 1) more than 500 CD8⁺ T cells were acquired and 2) the frequency of positive
198 cells was of > 1%.

199 **Quantification of SIV-specific Ab titers in blood and semen**

200 Blood serum was isolated from SST blood samples by centrifugation for 10 min at 1,500 x
201 g and cryopreserved at -80°C. Seminal plasma was isolated as described above. ELISA was
202 performed using the Genscreen HIV-1/2 kit, Version 2 (Biorad, Marnes-la-Coquette, France),
203 according to the manufacturer's instructions, which detect IgG, IgM and IgA. A reference
204 positive serum from a SIVmac251-infected cynomolgus macaque was used as a standard. The
205 titer was calculated based on a cut-off calculated separately for blood and semen samples using
206 the mean value of samples from uninfected or immunized monkeys. The maximal titer was
207 fixed at 8,000, extrapolated from the standard scale.

208 **TZM-bl neutralization assay**

209 Viral titrations were performed in TZM-bl cells as previously described [41]. We used a cut-
210 off value of 2·5-times the background relative luminescence units (RLUs) when quantifying
211 positive infections in the TCID assays, according to the guidelines for the TZM-bl assay. The
212 TCID₅₀ was defined as the reciprocal of the viral dilution resulting in 50% positive wells (Reed-
213 Muench calculation). A standard inoculum, corresponding to a virus dilution that yields
214 approximately 300,000-500,000 RLU equivalents (+/- 15,000 RLUs), was used for the
215 neutralization assay to minimize virus-induced cytopathic effects while maintaining the ability
216 to measure a 2-log reduction in virus infectivity. Both plasma and seminal plasma samples were
217 heat-inactivated at 56 °C for 30 min before use in the neutralization assay. Samples were
218 prepared by serial two-fold dilution, starting from a concentration of 1/40 and 1/160 for plasma
219 and seminal plasma, respectively. The diluted samples were incubated with viral supernatant
220 (SIVmac251) for 1 h. Then 10,000 TZM-bl cells were added to each well. The plate was
221 incubated for 48 h and the luciferase activity measured. Infections were carried out in culture
222 medium containing 15 µg/ml DEAE dextran (diethylaminoethyl; Amersham Biosciences,
223 Fairfield, Connecticut, USA). Additionally, we used HIV-1-based vesicular stomatitis envelope
224 glycoprotein G (VSV-G)-pseudotyped virus as a control for specificity of the neutralization
225 activity, as the virus enters cells in a CD4 receptor-independent manner. The viral vector
226 expressing luciferase was constructed by substituting the *nef* gene sequence of the HIV-1_{NL4-3}
227 genome with the firefly luciferase gene [42]. A similar neutralization assay protocol was used
228 for the experiment with the VSV-G pseudotyped virus as that used for the experiment with the
229 SIVmac251 virus, but in the absence of DEAE dextran. The viral input was 8·75 ng/ml, yielding
230 a comparable RLU value to that obtained with SIV mac251. Neutralization titers are the
231 antibody concentration at which RLUs are reduced by 50% relative to the RLUs in the virus
232 control wells after subtraction of the background RLUs in the control wells with only cells
233 (IC₅₀). By analogy, the IC₇₅ and IC₉₀ were defined as the concentration of Abs able to decrease
234 the percentage of infected cells by 75% and 90%, respectively. The IC₅₀, IC₇₅, and IC₉₀ were
235 calculated using a linear interpolation method [43]. For calculating the mean, a value of greater
236 than the highest Ab concentration used was recorded if 50% inhibition was not achieved. In
237 contrast, if 50% inhibition was not achieved at the lowest Ab concentration used, a two-fold
238 lower concentration than that value was recorded.

239 **ELISA-based rsFcγRIIIa dimer-binding assay**

240 The protocol of the ELISA-based IgG assay using soluble FcγR dimer has been previously
241 described [44]. Briefly, ELISA plates (MaxiSorp plates; Nalgene Nunc, Rochester, NY) were
242 coated overnight at 4°C with SIVmac251 gp130 recombinant protein (NIBSC, England) diluted
243 to 1 or 5 µg/ml in PBS (to test blood plasma and seminal plasma Abs, respectively), as well as

244 no Ag as a negative control. HIVIG (#3957; National Institutes of Health AIDS Reagent) was
245 used at 5 µg/ml to normalize the results across all plates. Coated plates were subsequently
246 washed with PBS containing 0.05% Tween 20 (Sigma Aldrich) and blocked with blocking
247 buffer, consisting of PBS containing 1 mM EDTA and 1% BSA (both from Sigma- Aldrich),
248 for 1 h at 37°C. SIV⁺ serum and seminal plasma samples were diluted 1/50 in blocking buffer
249 and incubated in the gp130-coated wells for 1 h at 37°C. Then, purified dimeric macaque
250 rsFcγRIIIa-biotin (I158 allele) was added to the plate at a concentration of 0.1 µg/ml and the
251 plates incubated for 1 h at 37°C. Horseradish peroxidase (HRP)-conjugated streptavidin
252 (Thermo Fisher Scientific) was added and the plates incubated for another 1 h at 37°C. After
253 washing, the color was developed using 3,3', 5,5-tetramethylbenzidine (TMB) (Life
254 Technologies), followed by the addition of 1 M HCl stop solution. The absorbance at a
255 wavelength of 450 nm was recorded as OD. The no-Ag values were subtracted from each Ag
256 sample and the resulting values normalized to 5 µg/ml HIVIG. A positive signal was defined
257 as an OD higher than the mean + 3 x SD of the OD obtained using sera and seminal plasma
258 from macaques before infection.

259 **Data visualization and statistical analysis**

260 All data visualization and statistical analysis were carried out using GraphPad Prism 8.1
261 software (GraphPad software, La Jolla, USA), except for the pie chart of the SIV-specific CD8
262 T-cell response, which was generated using Spice software (NIH, Bethesda, USA). The
263 nonparametric Spearman rank correlation test was used to investigate the relationship between
264 parameters. The nonparametric Mann-Whitney test was used to compare different groups of
265 macaques and the nonparametric Wilcoxon rank sum test and paired t-test were used to compare
266 data from the same macaques at various time points before and after SIV infection. P values of
267 0.05 or lower in two-tailed tests were considered significant, *p < 0.05, **p < 0.01, ***p <
268 0.001, ****p < 0.0001.

269

270 Results

271

272 SIV infection induces pro-inflammatory and immunoregulatory cytokines in semen

273 Seminal plasma levels of inflammatory cytokines may have a major impact on viral shedding
274 in semen, both of cell-free and cell-associated virus and affect the state of the recipient's cells
275 in the FRT and rectum, increasing the risk of mucosal HIV-1 transmission. We measured these
276 parameters in seminal plasma samples of macaques intravenously infected with the high dose
277 of 5,000 animal infectious dose 50% (AID₅₀) of SIVmac251 virus to define the relationship
278 between cytokine composition and viral seeding of the MGT.

279 Blood viral load (BVL) strongly correlated with seminal viral load (SVL) ($r = 0.671$, $p <$
280 0.0001 , $n = 25$, **Figure 1A**), confirming our previously published results [40], as well as those
281 reported for HIV-1-infected patients [6,45–47]. Viral load peaked at 10 days post infection
282 (dpi), followed by a rapid decrease and stabilization in both compartments at three months post-
283 infection (**Figure 1D-E**).

284 We measured the seminal plasma levels of 28 cytokines and chemokines in a transverse
285 study on three independent groups of macaques at various stages of infection: before infection
286 ($n = 12$), acute infection (10-14 dpi, $n = 10$), and chronic infection (> 1 year pi, $n = 16$). VEGF,
287 the active form of TGF- β 1-3, MCP-1, and IL-8 were present at high levels (mean $> 1,000$ pg/ml)
288 in the seminal plasma of uninfected macaques (**Figure 1B**). The levels of several pro-
289 inflammatory cytokines (IL-8, IL-15, IL-18, IFN- γ , and RANTES) and the immunomodulatory
290 molecule IL-13 were significantly higher in the seminal plasma of macaques during the primary
291 infection (Mann-Whitney test, $p = 0.0015$, 0.0006 , 0.0138 , 0.0002 , 0.0206 , and 0.0358 ,
292 respectively) than that of uninfected macaques (**Figure 1C and Table 1**). These observations
293 were confirmed in 10 macaques longitudinally followed until 170 dpi (**Figure 1D, E**). Cytokine
294 levels were elevated at 10 to 14 dpi, with a peak at 28 dpi, followed by a reduction. We observed
295 the same profile for IL-4 and IL-1 β . Accordingly, seminal plasma levels of IL-1 β , IL-4, IL-8,
296 IL-15, IL-18, and RANTES positively predicted SVL and BVL (**Table 1**). Neither IL-13 nor
297 IFN- γ levels predicted viral shedding (**Table 1**). Indeed, following the peak during the acute
298 phase, IL-13 levels progressively increased from three months pi and IFN- γ did not disappear
299 during the follow-up period (**Figure 1C, E**). Finally, there was a positive correlation between
300 TGF- β 1 levels and SVL and BLV, whereas TGF- β 2 and 3 levels predicted only BVL (**Table**
301 **1**). Interestingly, semen concentration of IP-10 steadily increased during the first month of
302 infection and levels were not stabilized even in the chronic phase (Mann-Whitney test, $p =$
303 0.0120 , 0.003 , 0.0005 , 0.0022 , 0.0201 and <0.0001 for 10, 28, 29, 35, 160 and 315 dpi,
304 respectively. **Figure 1F**), suggesting a role for IP-10 in lymphocytes trafficking to mucosal
305 tissues during HIV/SIV infection.

306 In conclusion, SIV infection was associated with inflammation in seminal plasma, especially
307 during the first weeks of infection, with pro-inflammatory molecule levels correlating with
308 seminal viral shedding.

309

310 Semen CD8⁺ T cells have a mucosal-like phenotype and are highly activated following SIV 311 infection

312 We then performed an in-depth characterization of the phenotype and dynamics of T-cell
313 populations in blood and seminal plasma during SIV infection. The proportion of semen T cells
314 was strongly affected by SIV infection, with rapid, significant, and durable depletion of CD4⁺
315 T cells ($p = 0.0047$ at 14 dpi, Mann-Whitney test), which was paralleled by an increase in CD8⁺
316 T cells among total CD45⁺ leucocytes (**Figure 2A**). As expected, CD4⁺ T-cell depletion also
317 occurred in peripheral blood (**Figure 2B**). These data confirm that macaques infected with
318 SIVmac251 recapitulate the observations reported for HIV-1⁺ patients [35].

319 Most of the cytokines which showed higher levels in the seminal plasma of SIV⁺ macaques
320 are known to induce T-cell activation. We thus phenotyped the semen CD8 T cells.

321 In macaques, CD95 and CD28 are commonly used in flow cytometry analysis to
322 discriminate naïve (T_{nv}, CD95⁻CD28⁻) from central memory (T_{cm}, CD95⁺CD28⁺) and effector
323 memory (T_{em}, CD95⁺CD28⁻) T cells [40]. In uninfected macaques, semen CD8⁺ T cells were
324 all of the memory phenotype (CD95⁺), with a mean of 59.4% ± 12.9 T_{cm} cells, 38.2% ± 11.2
325 T_{em} cells and 2.4% ± 2.9 T_{nv}, whereas in the blood naïve cells represented 25.6% ± 15.9 of
326 total CD8⁺ T cells (**Figure 2C**). The proportion of T_{cm} cells was higher in the semen than
327 blood, whereas the proportion of T_{em} cells was slightly higher in the blood (Mann Whitney
328 test, $p < 0.0001$ and 0.0173 respectively, for T_{cm} and T_{em}) (**Figure 2C**). There was no
329 difference between non-infected and SIV⁺ macaques in the proportion of each CD8⁺ T cell
330 subset for either seminal plasma (**Figure 2D**) or blood (**Figure 2E**).

331 The expression of CD45RA by memory cells (**Figure 2F**) is considered to be a marker of
332 end stage differentiation, as reviewed in [48]. We observed a lower proportion of CD45RA⁺
333 CD8⁺ T cells in the semen than blood of uninfected macaques (mean of 8.81% ± 6.39 vs
334 86.00% ± 3.70, **Figure 2F**). In SIV⁺ macaques, the proportion of semen CD45RA⁺ cells did
335 not significantly change in either compartment (mean of 5.6% ± 2.6 and 70.1% ± 31.1, **Figure**
336 **2F**).

337 We then analyzed the expression of the chemokine receptors CCR5 and CXCR3, which are
338 effector cell markers. Cells expressing such markers may originate from the MGT rather than
339 the blood. CCR5⁺CXCR3⁺ T cells have been associated with IFN- γ production and a T_{cl} profile
340 [49,50]. Moreover, these molecules likely facilitate the highly-directed migration of effector T
341 cells to tissues expressing a gradient of their ligand(s), such as cervico-vaginal tissues and the
342 gut mucosa [51–53]. At steady state, 22.0% ± 11.7 of semen CD8⁺ T cells were CCR5⁺CXCR3⁻
343, 73.0% ± 11.2 CCR5⁻CXCR3⁺, and 17.8% ± 9.9 CCR5⁺CXCR3⁺ (**Figure 2G**). SIV infection
344 induced a significant increase in the proportion of CCR5⁺CXCR3⁻ and CCR5⁺CXCR3⁺ cells
345 (Mann-Whitney test, $p = 0.0022$ for both subsets, mean of 67.2% ± 12.1 CCR5⁺CXCR3⁻ and
346 53.7% ± 11.4 CCR5⁺CXCR3⁺ cells; **Figure 2G**). This suggests that most semen CD8⁺ T cells
347 in SIV⁺ macaques display a T_{cl} profile. This is consistent with the capacity of these T cells to
348 home to inflammatory tissues [54]. Then, we analyzed the expression of LFA-1 integrin, formed
349 by the α -chain CD11a and the β -chain CD18, which is a major marker of adhesion and
350 transmigration of T cells [55]. Semen CD8⁺ T cells displayed high expression of this integrin,
351 with 100% of the cells positive for CD11a and a mean of 40.33% ± 24.56 LFA-1⁺ cells.
352 Although not significant, SIV infection slightly increased the proportion of LFA-1⁺ cells (mean
353 of 54.50 ± 24.61, Mann-Whitney test, $p = 0.3823$) but not that of CD11a⁺CD18⁻ cells (**Figure**
354 **2H**), which is consistent with a previously activated memory phenotype.

355 We further characterized T_{cm} and T_{em} CD8⁺ T cells by analyzing the expression of CD69
356 and HLA-DR, two T-cell activation markers that are commonly elevated on CD8⁺ T cells during
357 HIV/SIV infection. In uninfected macaques, the percentage of CD69⁺ CD8⁺ T cells was much
358 higher in the semen than blood (12.22- and of 5.85-fold higher for in the T_{cm} and T_{em} subsets,
359 respectively; **Figure 3A**), indicating a preexisting high level of activated T cells in the MGT.
360 During infection, the proportion of CD69⁺ cells increased in the semen of SIV⁺ animals for both
361 the T_{cm} (Mann-Whitney test, $p = 0.0022$) and T_{em} ($p = 0.0001$) subsets (**Figure 3B**), whereas
362 it was higher in the blood for only T_{em} cells (Mann-Whitney test, $p = 0.0061$, **Figure 3C**).
363 Such a durable increase in the proportion of CD69⁺ cells was confirmed in eight infected
364 macaques that we followed longitudinally up to 150 dpi (**Figure 3D**). Moreover, the proportion
365 of CD69⁺ cells in both T_{cm} and T_{em} CD8⁺ cells correlated between blood and semen (**Figure**
366 **3E-F**). The percentage of HLA-DR⁺ CD8⁺ T cells was also higher in the semen than blood
367 (3.56- and 2.58-fold for the T_{cm} and T_{em} subsets, respectively; **Figure 3G**), confirming the
368 activation state of semen cells. There was a trend with SIV infection to increased percentage of

369 Tcm HLA-DR⁺ CD8⁺ cells in semen (**Figure 3H**), that was significant in blood (Mann-Whitney
370 test, $p = 0.0052$, **Figure 3I**).

371 Thus, at steady state, semen CD8⁺ T cells display an activated memory profile typical of
372 mucosal T cells [56,57]. SIV infection induces considerable changes in their phenotype, as well
373 as in their dynamics. We then studied the SIV-specific CD8⁺ T-cell response to obtain a better
374 insight into their function.

375 To analyze the subset of T cells that recognized SIV Gag, we performed a five-hour *ex vivo*
376 stimulation experiment with a pool of SIV *Gag* peptides on semen cells and PBMCs to assess
377 and quantify the presence of SIV-specific CD8⁺ T cells and assessed the expression of IFN- γ ,
378 TNF, IL-2, and MIP-1 β (**Figure 4A, D**). There was no significant expression of IL-2, neither
379 in cells from the blood or semen (not shown). There was a clear SIV-specific CD8⁺ T-cell
380 response at 128 dpi for three of seven macaques studied (#31044, #30690, and #BC461) in both
381 seminal plasma (**Figure 4B, C**) and blood (**Figure 4E, F**). In seminal plasma, we observed four
382 types of SIV-specific cytokine-producing CD8⁺ T cells: mostly highly polyfunctional IFN- γ
383 γ^+ MIP-1 β^+ TNF⁺ cells (6%, 1.04%, and 0.63% positive cells for #30690, #31044, and #BC461,
384 respectively), followed by IFN- γ MIP-1 β^+ TNF⁻ (1.2%, 1.92%, and 0.44% positive cells for
385 #30690, #31044, and #BC461, respectively), IFN- γ^+ MIP-1 β^+ TNF⁻ (0.69%, 2.47%, and 0.87%
386 positive cells for #30690, #31044, and #BC461, respectively), and IFN- γ^+ MIP-1 β^- TNF⁻ cells
387 (0.82%, 0.59%, and 0.6% positive cells for #30690, #31044, and #BC461, respectively)
388 (**Figure 4B, C**). The SIV-specific response of all these macaques displayed the same dominant
389 profiles in blood (**Figure 4E, F**): mostly IFN- γ^+ MIP-1 β^+ TNF⁻ cells (0.3%, 0.19%, and 0.2%
390 positive cells for #30690, #31044, and #BC461, respectively), followed by IFN- γ^- MIP-1 β^+ TNF⁻
391 (0.24%, 0.21%, and 0.04% positive cells for #30690, #31044, and #BC461, respectively), and
392 highly polyfunctional IFN- γ^+ MIP-1 β^+ TNF⁺ cells (0.16%, 0.13%, and 0.05% positive cells for
393 #30690, #31044, and #BC461, respectively). The proportion of these SIV-specific cells was
394 much lower in blood than in semen. We still detected a polyfunctional SIV-specific response
395 in semen CD8⁺ T cells of animals #31044 and #BC461 at a late stage of infection (413 dpi),
396 although at a lower proportion (**Figure 4C**). The proportion of IFN- γ^+ , TNF⁺, and MIP-1 β^+
397 cells in neither compartment (blood or semen) correlated with the control of viral RNA loads
398 (data not shown).

399 In conclusion, most of the SIV-specific cells of both compartments displayed high
400 polyfunctionality, with concomitant expression of at least two of the three cytokines IFN- γ ,
401 MIP-1 β , and TNF. The SIV-specific response we observed in semen CD8⁺ T cells was not
402 associated with reduced SIV shedding. However, in the semen, the macaques with the highest
403 SIV-specific responses in CD8⁺ T cells displayed the highest SVL (data not shown). The same
404 trend was observed in blood, with the exception of one macaque (#30742), which had the
405 highest proportion of cells positive for the three cytokines and the lowest BVL (data not shown).

406 **Analysis of SIV-specific antibody titer and functions in the semen reveals a role for ADCC** 407 **in the modulation of semen infectivity**

408 The presence of Abs with neutralization activity or those able to activate Fc-mediated
409 functions could have a large impact on the infectivity of semen. We therefore analyzed these
410 functions in paired blood and semen samples from six macaques longitudinally followed up to
411 148 dpi (**Figure 5A**). Interestingly, a strong positive correlation was observed between SIV-
412 specific Ab titers and viral load in semen ($r = 0.8218$, $p = 0.0056$) but not blood (**Figure 5B**
413 and data not shown), suggesting that there may be local Ab response in response to virus
414 replication. The profile of SIV-specific Ab titers among individuals was highly diverse (**Figure**
415 **5C**). Ab titers were detected at low levels 51 dpi in blood and semen (mean titer of $57.15 \pm$
416 42.17 and 41.08 ± 56.34 , respectively). At the chronic stage (148 dpi), Ab titers were
417 significantly higher (Mann-Whitney test, $p=0.0317$) in blood (mean titer of $3,920.22 \pm$
418 $3,378.05$) than in seminal plasma ($1,500.75 \pm 3,195.91$; **Figure 5C**). During the chronic stage,

419 the most elevated titers in the seminal plasma were observed for two macaques (#30717 and
420 #21362R) that displayed constant high viral shedding in blood and semen (“high shedder”)
421 (**Figure 5A-C**). However, the sensitivity of the ELISA we used to measure antibody titers was
422 higher in blood serum than in seminal plasma, which induced a higher background and possibly
423 an underestimation of the level of semen immunoglobulins. We did not observe a correlation
424 between anti-SIV specific Ab titers in the two compartments ($r = 0.5106$, $p = 0.1351$, data not
425 shown), consistent with observations in HIV⁺ subjects, suggesting that the origin of semen IgG
426 is different from that of blood IgG, being possibly produced in the MGT [26]. It is possible that
427 such specific responses can neutralize SIV, since neutralizing Abs (NAbs) produced in the
428 MGT may modulate the per-act risk of sexual transmission. We assessed the NAb response
429 against the challenge virus SIVmac251, a tier 2 virus, using blood serum and seminal plasma
430 at 148 dpi. Seminal plasma is known to have a toxic effect on cell. Thus, we performed
431 preliminary experiments to assess semen toxicity on TZM-bl cells (data not shown). Based on
432 the results obtained, we used semen at a starting dilution of 1/160 (versus 1/40 used to assay
433 blood serum) to avoid nonspecific inhibition and/or cell death. We detected NAbs in the blood
434 of five of the six macaques and in the seminal plasma of only one (#30742) (**Figure 5D**). The
435 responses were generally weak in both compartments, but specific, as assessed using a VSV-
436 pseudotyped virus as a control (data not shown).

437 As the NAb response was very weak, we then sought to evaluate whether Abs with ADCC
438 functions were present in the seminal plasma samples. The cross-linking of FcγRs by Abs is a
439 process essential to initiate ADCC responses. A novel ELISA-based IgG assay, using a soluble
440 recombinant FcγRIIIa dimeric protein, recapitulates this process *in vitro*. As previously
441 described, recombinant FcγR engagement by Abs correlates with measures of functional ADCC
442 using cell-based assays [44,58]. Thus, we assessed the ability of SIVmac gp130-bound Abs
443 present in the blood and semen of infected macaques to engage soluble recombinant FcγRIIIa
444 as a surrogate of ADCC activity. Abs able to engage FcγRIIIa emerged at 51 dpi in the blood
445 of six/six macaques relative to baseline values (mean OD ± SD = 11 ± 5.89) (paired t test, $p =$
446 0.04) and significantly increased by 148 dpi ($p = 0.0044$) (**Figure 6A**). FcγRIIIa-binding Abs
447 were present in the semen of three/six animals at 51 dpi, relative to baseline values (mean OD
448 ± SD = 1.5 ± 1.29), at high titers in macaque #30717 and at low titers in animals #21362R and
449 #29965. By 148 dpi, the titers increased in the three macaques and FcγRIIIa-binding Abs
450 became detectable in a fourth animal (#30569) (**Figure 6A**). FcγRIIIa-binding Abs correlated
451 with total anti-SIV Ab titers in both blood and semen ($r = 0.8546$, $p = 0.0007$ in blood and $r =$
452 0.8333 , $p = 0.0083$ in semen; **Figure 6B**). Unexpectedly, viral load and FcγRIIIa-binding Ab
453 titers positively correlated in the semen, although the correlation was borderline significant ($r =$
454 0.6093 , $p = 0.0669$) (**Figure 6C**). On the contrary, there was no correlation in the systemic
455 compartment ($r = -0.2308$, $p = 0.4708$ data not shown). Indeed, the two “high shedder” animals
456 (#30717 and #21362R) also had the highest semen FcγRIIIa-binding Ab titers (**Figure 5A and**
457 **6A**). This suggests that continuous antigen stimulation is necessary to evoke Abs with a
458 potential ADCC function.

459

460 Discussion

461 HIV/SIV infection is associated with increased level of cytokines in the periphery and some
462 of them are even more elevated in the seminal plasma of infected individuals [23,40,59–64].
463 Here, we report the first extensive study evaluating the effect of SIV infection on the seminal
464 cytokine/chemokine network. Many molecules correlate with BVL and/or SVL, including pro-
465 inflammatory molecules (IL-1 β , IL-8, IL-15, IL-18, RANTES) and immunomodulatory
466 cytokines (IL-13 and TGF- β). These molecules are known to modulate immune-cell activation
467 and migration profiles, as well as to initiate T-cell effector functions [65]. They may both
468 influence the leukocytes that are present in the donor's semen and the partner's mucosal tissue,
469 thereby influencing the efficacy of viral transmission. For example, the immunosuppressive
470 properties of TGF- β could be detrimental in the context of sexual transmission of HIV-1
471 because it would divert cell-mediated immune responses. When deposited and activated in the
472 female reproductive tract (FRT), TGF- β present in semen elicits a transient pro-inflammatory
473 response, with the consequent influx and activation of macrophages, dendritic cells, and
474 neutrophils [66]. Our results further confirm and extend previous reports [60,61,67] that SIV
475 infection in the MGT has a profound effect on semen composition, leading to altered cytokine
476 profiles that can modulate HIV/SIV replication, promote viral shedding, and cause local target-
477 cell activation. Moreover, these observations highlight the need of HIV-1 vaccine studies to
478 consider the potential impact of semen exposure on the mucosal immune system.

479 The highly inflammatory environment we describe in the semen of SIV-infected macaques
480 possibly mirrors what happens in the MGT and can potentially modulate the activation status
481 of immune cells. Indeed, semen CD8⁺ T cells displayed an activation profile, with a higher
482 proportion of CD69⁺ and HLA-DR⁺ cells than in peripheral blood. In addition, the proportion
483 of CD69⁺ cells was higher in SIV-infected macaques in both compartments, particularly in
484 effector cells, consistent with the results of previous studies conducted on HIV⁺ individuals
485 [68] and SIV⁺ macaques [69]. Furthermore, in this study, the proportion of CD69⁺ central
486 memory and effector memory CD8⁺ T cells in the blood correlated with that in the semen of
487 SIV⁺ macaques, indicative of generalized immune activation in SIV-infected macaques and
488 suggesting that systemic immune activation could be used to predict levels in the genital tract.
489 SIV-infected macaque CD8⁺ T cells displayed high proportions of CCR5⁺ CXCR3⁻ and
490 CCR5⁺CXCR3⁺ cells, a phenotype associated with the inflammatory homing Tc1 effector
491 profile [51,52]. The increased expression of CXCR3⁺ is paralleled by high levels of its ligand
492 IP-10, supporting a role for the chemokine in effector cells recruitment to mucosal sites.
493 Altogether, these observations strongly suggest that semen CD8⁺ T cells are of mucosal origin
494 and undergo substantial modifications in their activation profile and effector function in
495 infected animals. The role of such effector T cells in the semen and whether their excretion is
496 active or passive are still unclear. Interestingly, activated, cytotoxic (TIA-1⁺) CD3⁺ T-cell
497 infiltrates have been reported along the MGT (mostly in the prostate, seminal vesicles, and
498 epididymis) of SIV-infected macaques displaying high viremia [70]. Semen CD8⁺ T cells may
499 originate from these cellular infiltrates of the various semen-producing organs and therefore
500 reflect the inflammatory status within the MGT.

501 Cell-mediated immunity plays a major role in controlling HIV-1 replication in humans and
502 SIV replication in macaques [18,40]. Moreover, the presence of highly polyfunctional T cells
503 has been reported in elite controllers and long-term nonprogressor HIV patients [71,72]. Here,
504 we investigated the SIV-specific CD8⁺ T-cell response in semen and its potential role in
505 HIV/SIV transmission. The three animals that displayed a detectable SIV-specific T-cell
506 response had a high proportion of IFN- γ , TNF and MIP-1 β producing cells and mostly
507 polyfunctional cells. Remarkably, these responses were approximately 10-fold higher in semen
508 than peripheral blood. Importantly, such a high SIV-specific response in semen was associated
509 with elevated local viral shedding. Although the numbers of animals in this study with SIV

510 Gag-specific T cell responses is small, this suggests that the SIV-specific T cells in semen do
511 not control replication of the virus. Moreover, although the modest numbers did not allow
512 statistical analysis nor the possibility to draw specific conclusions, some points warrant
513 discussion. The CD8⁺ T cell response in the blood during acute HIV or SIV infection increased
514 following the increase in viral load and there was an inverse correlation between viral load and
515 the CD8⁺ T cell response during primary infection [73–75]. High CD4⁺ T-cell and CD8⁺ T cell
516 IFN-γ responses during early infection in Cynomolgus macaques were associated with better
517 long-term viral control [39]. During chronic infection, polyfunctional HIV-specific CD8⁺ T-
518 cell responses were maintained in nonprogressors [71]. The importance of CD8⁺ T-cell
519 responses in controlling viral replication was shown a long time ago by *in vivo* depletion of
520 CD8⁺ T cells in SIV-infected macaques [76,77]. In our cross-sectional study, we did not observe
521 the same profile in the semen. On the contrary, the CD8⁺ T-cell effector function appeared to
522 have no effect on local viral shedding. Although these studies are technically demanding due
523 to accessing semen and the modest numbers of cells recovered, future studies could analyze
524 responses to a broader range of SIV proteins to understand the breadth of the T cell immunity.

525 Humoral immunity in semen is likely to be important in HIV/SIV transmission. We found
526 that the dynamics of viral RNA loads and SIV-specific Abs in the blood and semen of
527 SIVmac251-infected macaques were similar to those reported for humans [6,47,78]. SIV-
528 specific Abs were detected in all semen samples, generally at lower levels than in serum,
529 confirming the observations previously reported for HIV-1 infected subjects [79]. However,
530 viral RNA loads did not correlate with SIV-specific Ab titers, neither in the blood nor semen.
531 In both cases, these results suggest that the local humoral response failed to control viral
532 shedding. This is consistent with observations made in human patients, whose systemic
533 antibody response to HIV following infection is mostly ineffective, with an early B-cell
534 response being largely directed against non-neutralizing epitopes of the HIV envelope. The
535 response during late stages is impaired by aberrant B-cell hyperactivation [80–82]. Here, we
536 report the presence of Abs that neutralize the autologous virus in five of six serum samples and
537 in one of six semen samples from macaques at 148 dpi. Thus, NAbs seem to develop more
538 easily at the systemic level than at the mucosal site. The only macaque (#30742) with a weak
539 NAbs response in semen also had the strongest neutralizing response in serum. Both serum and
540 semen from these macaques did not inhibit infection by a VSV-pseudotyped virus, indicating
541 that although weak, the observed response was specific. SIVmac251 is a “difficult to neutralize”
542 virus, so the lack of neutralization observed is not surprising. However, we cannot exclude that
543 the detection of NAbs in the semen may have been underestimated and that semen dilutions
544 below 1/160 could inhibit infection.

545 Although a protective role against HIV-1 acquisition has been reported for Abs present in
546 genital secretions, env-specific IgG present in semen may instead facilitate mucosal
547 transmission of HIV-1. Indeed, a proportion of HIV virions may be coated with IgG in semen
548 and form an immune complex that can cross the mucosal epithelium, as previously reported for
549 anti-HIV broadly NAbs [83]. The presence of Abs in genital secretions, such as semen, should
550 be considered in the design of prevention strategies, as it could impede attempts to provide
551 immune-based prophylactics and/or vaccines. However, we cannot exclude that the efficiency
552 of vaccine-induced or passively transferred Abs might be reduced in prophylactic approaches
553 by the presence of virion-Ig complexes.

554 Studies have reported the presence of Abs with ADCC activity in cervico-vaginal fluids from
555 HIV-1 infected women with lower genital viral load [84,85], in HIV controllers [86] and in
556 chronically HIV-1-infected individuals who did not transmit infection to their heterosexual
557 ESN partners [87]. However, little is known about ADCC activity in the MGT and Fc-mediated
558 antibody functions have been mostly overlooked in semen. Only one study reported the
559 presence of ADCC Abs in the semen of HIV-1-infected individuals [38]. The presence of

560 ADCC-mediating Abs has not been previously evaluated in the semen of macaques infected
561 with SIV. Therefore, we evaluated the presence of Fc γ RIIIa dimeric protein-binding semen Abs
562 in infected macaques as a surrogate of ADCC function and investigated whether it may be
563 associated with local viral shedding. We detected Abs that engage the Fc γ RIIIa in the blood of
564 all of the studied macaques. The responses were weaker in the semen, but detectable in four of
565 six animals. Although we did not find a correlation between the presence of Abs that bind the
566 dimeric Fc γ RIIIa and BVL, there was a positive correlation in the semen. Indeed, the two
567 macaques with the highest Fc γ R-binding titers were those with the highest SVL at five months
568 post-infection. These results need to be confirmed in a larger cohort. Although our results
569 suggest that the ADCC response does not protect against viral shedding nor disease progression,
570 they do suggest that Abs with a potential to mediate ADCC could modulate semen infectivity
571 and viral transmission. As reported [38], the presence of ADCC Abs in semen may, at least in
572 part, explain the relatively low rate of sexual transmission during sexual intercourse.

573 The limited amount of available seminal plasma did not allow us to investigate other Fc-
574 mediated antibody functions, such as antibody-mediated viral inhibition (ADVI) and antibody-
575 mediated phagocytosis (ADP), which may also contribute to protection from HIV infection.
576 Future studies should investigate these properties, as ADCVI has been observed with mucosal
577 anti-HIV-1 IgG in the genital tract of HIV-infected women [88] and because it can explain, at
578 least in part, the protective effect of a vaccine against the acquisition of neutralization-resistant
579 SIV challenge in Rhesus macaques [89]. Furthermore, studies investigating the contribution of
580 the biophysical properties of ADCC Abs present in semen, such as epitope avidity, epitope
581 specificity, and IgG subclass distribution, will be of interest, as these functions have been shown
582 to be associated with the control of disease and protection in vaccine studies [90,91].

583 Overall, this comprehensive study found that semen from SIV infected macaques contained
584 high levels of pro-inflammatory cytokines and chemokines and, in contrast to the blood, was
585 enriched for activated CD8⁺ memory T cells. Thus, responses observed in the periphery do not
586 reflect the local immune response and inflammatory features of the MGT response, which could
587 enhance transmission via effects on the functional status of either the seminal leukocytes or the
588 recipient mucosa. High seminal viremia was associated with mucosal IgG responses with
589 potential ADCC function. Identifying and optimizing the protective efficacy of immune
590 responses in the MGT may be key to achieving vaccine-induced immunity to sexually
591 transmitted HIV-1 infection.

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601

602 **Author Contributions Statement**

603 Study conception and design: RL and MC. Acquisition of data: KS, SS, CG, and BD. Analysis
604 and interpretation of data: KS, SS, CG, NB and MC. Drafting of the manuscript: KS and MC.
605 Critical revisions: KS, SK, BW, MH, RL and MC.

606

607 **Conflict of Interest Statement**

608 The authors declare no competing interests.

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In review

925 **Figure legends**

926 **Fig. 1. Cytokines and chemokines affected by SIV infection in macaques**

927 (A) Spearman correlation between SIV viral load in blood and semen. (B) Mean and standard
928 error of the mean (SEM) of 28 cytokine and chemokine levels in the seminal plasma of 12
929 uninfected macaques. (C) Mean and SEM of five pro-inflammatory molecules and the
930 immunoregulatory cytokine IL-13 in SIV⁻ macaques (n = 12, green dots) and SIV⁺ macaques
931 at the primary stage (10 or 14 dpi, n = 10, blue dots), and the chronic stage (n = 16, purple dots).
932 (D-E) Longitudinal follow-up of seminal plasma levels of cytokines and chemokines and BVL
933 (red) and SVL (blue) (both are represented by dotted lines) in SIV-infected macaques (n = 10
934 at baseline and at 14 dpi, seven at 28, 35, and 87 dpi, and six at 148 and 171 dpi). The mean
935 and SEM are shown for each time point. (F) Mean and SEM of IP-10 concentrations in SIV⁻
936 macaques (n = 19) and SIV⁺ macaques at 10 (n = 6), 28 (n = 6), 29 (n=4), 35 dpi (n = 7), 160
937 (n=4) and 315 (n=5). Significant differences between groups are shown (Mann-Whitney test,
938 *p < 0.05, **p < 0.01, and ***p < 0.001).

939 **Fig. 2. Dynamics and phenotype of semen CD8⁺ T cells and comparison with blood**

940 (A, B) Percentage of CD4⁺ and CD8⁺ T cells of total CD45⁺ cells in semen (A) and peripheral
941 blood (B) in uninfected (n = 12) and SIV⁺ macaques at 14 dpi (n = 6) and at the chronic stage
942 (> 1 year pi, n = 12 or 8). The mean and SEM is shown. (C) Percentage of T_{nv} (orchid), T_{cm}
943 (green) and T_{em} (lavender) CD8⁺ T cells in blood (round dots) and semen (squared dots) of 12
944 uninfected macaques. (D-E) Percentage of each CD8⁺ T cell subset in the semen (D) and blood
945 (E) of SIV⁻ (open symbols) and chronic SIV⁺ macaques (filled symbols). (F) CD45RA
946 expression of semen CD8⁺ T cells: percentage of CD45RA⁺CD8⁺ T cells in the blood (red
947 circles) and semen (blue squares) of SIV⁻ (open symbols, n = 3) and SIV⁺ (filled symbols, n =
948 5) macaques is shown. (G) CCR5 and CXCR3 expression on semen CD8⁺ T cells. Percentage
949 of CCR5⁺ (green squares), CXCR3⁺ (rose squares) and CCR5⁺ CXCR3⁺ (blue squares) cells in
950 SIV⁻ (open squares, n = 6) and SIV⁺ (filled squares, n = 6) macaques. (H) LFA-1 integrin
951 (CD11a⁺CD18⁺) expression on semen CD8⁺ T cells. % of LFA-1⁺ (orange squares) and
952 CD11a⁺CD18⁻ cells (violet squares) in SIV⁻ (open squares, n = 8) and SIV⁺ (filled squares, n=
953 8) macaques. The mean and SEM are shown. * indicates significant differences between groups
954 (Mann-Whitney test, *p < 0.05 and **p < 0.01).

955 **Fig. 3. Activation profile of CD8⁺ T cells in semen and blood**

956 (A) Comparison of CD69 expression on CD8⁺ T cells (T_{cm}: dark pink symbols, T_{em}: lavender
957 symbols) in the blood (circles, n = 12) and semen (squares, n = 12) of uninfected macaques.
958 (B-C) percentage of T_{cm} and T_{em} CD69⁺ CD8⁺ T cells in SIV⁻ (open symbols) and SIV⁺ (at >
959 1 year post-infection, filled symbols) macaques in semen (B) and blood (C). (D) Longitudinal
960 follow-up of the percentage of T_{cm} (dark pink line) and T_{em} (lavender line) CD69⁺ CD8⁺ T
961 cells and of BVL (red dashed line) and SVL (blue dotted line) in eight macaques infected with
962 5,000 AID50 IV. Significant differences from baseline are shown (Wilcoxon matched-pairs
963 signed rank test, *p < 0.05). (E, F) Spearman correlation between the percentage of T_{cm} (E)
964 and T_{em} (F) CD69⁺ CD8⁺ T cells in blood and semen of 15 chronically infected macaques. (G)
965 Comparison of HLA-DR (B) expression on CD8⁺ T cells (T_{cm}: brown symbols, T_{em}: blue
966 symbols) in the blood (circles, n = 12) and semen (squares, n = 12) of uninfected macaques.
967 (H-I) The percentage of T_{cm} (brown symbols) and T_{em} (blue symbols) HLA-DR⁺ CD8⁺ T cells
968 in SIV⁻ (open symbols) and SIV⁺ (filled symbols) macaques at > 1 year post-infection in semen
969 (H) and blood (I). The mean and SEM are shown. * indicates significant differences between
970 groups (Mann-Whitney test, *p < 0.05 and **p < 0.01).

971 **Fig. 4. SIV-specific CD8⁺ T cells response in semen of SIVmac251-infected macaques.**

972 Intracellular staining for Th1 cytokines in semen CD8⁺ T cells in response to a 5-h stimulation
973 with SIV *gag* peptide or without stimulation (mock). (A and D) IFN- γ and MIP- β expression
974 in seminal plasma (A) and blood (D) CD8⁺ T cells of all studied animals (n = 7), with (lower
975 panels) or without (upper panels) stimulation with *gag*. (B and E) Polyfunctionality of semen
976 (B) and blood (E) CD8⁺ T cells in seven chronically infected macaques. IL-2 was not included
977 because it was not produced in sufficient amounts. Each color represents one animal and the
978 mean is shown. (C and F) Pie charts showing the polyfunctionality of semen CD8⁺ T cells in
979 seminal plasma (C) and blood (F) of chronically-infected macaques with SIV-specific CD8 T
980 cells (#30690, #31044, and #BC461) at steady state (128 dpi, upper panel) and the late chronic
981 stage (413 dpi, lower panel).

982 **Fig. 5. Viral loads, anti-SIV antibody titers and neutralizing antibody response in semen**
983 **and blood of SIVmac251 infected macaques.**

984 Longitudinal follow-up of viral loads, Ab targeting SIV/HIV-2, and neutralizing antibody
985 response in six infected macaques. Each color represents a different animal. (A) Blood and
986 seminal plasma viral RNA loads (BVL: solid lines, SVL: dotted lines). (B) Spearman
987 correlation between semen SIV-specific Ab titers and SVL. (C) Blood (left panel) and seminal
988 plasma (right panel) titers of SIV-specific Ab for each macaque (the 2 titer values are connected
989 by a line) at baseline and 51 and 148 dpi. (D) Neutralizing antibody response in the blood (left
990 panel) and seminal plasma (right panel) of six infected macaques at 148 dpi. The dotted line
991 represents 50% of viral inhibition. The mean and SEM are shown.

992 **Fig. 6. SIV-specific dimeric rsFc γ RIIIa binding antibodies in paired blood and semen**
993 **samples of SIVmac251 infected macaques.**

994 (A) Fc γ RIIIa-V158 binding Ab response in blood (left panel) and seminal plasma (right panel)
995 to SIVmac251 gp130 protein. Results are presented as optical density (OD) at 450 nm. The
996 mean and SEM are shown, and each color represents a different animal. The responses at 51
997 and 148 dpi were compared using the paired t test. *p < 0.05 was considered significant. (B)
998 Spearman correlation between SIV-specific Ab titers and the Fc γ RIIIa -V158 binding Ab
999 response in blood (left panel) and seminal plasma (right panel). (C) Spearman correlation
1000 between Fc γ RIIIa -V158 binding Ab response and SVL.

1001 **Tables**

1002 **Table 1. Seminal levels of cytokines and chemokines affected by SIV infection.**

1003 Levels of cytokines/chemokines in the seminal plasma of uninfected macaques (n = 12) and
 1004 during primary (10-14 dpi, n = 10) and chronic (> 1-year pi, n = 16) infection. The correlation
 1005 with blood viral load (BVL) and seminal viral load (SVL) is shown.

Molecules	No infection (Mean (pg/ml) ± SD)	Primary infection (Mean (pg/ml) ± SD)	Chronic infection (Mean (pg/ml) ± SD)	Correlation with BVL		Correlation with SVL	
				P value	r value	P value	r value
IL-1β	4±4	13±20	1±2	0·009	0·6225	0·0001	0·7117
IL-4	39±35	57±39	7±16	< 0·0001	0·7312	0·0003	0·6923
IL-8	2066±2392	16271±25874	806±1097	< 0·0001	0·7106	< 0·0001	0·7494
IL-13	10±6	19±11	5±9	0·0156	0·4783	0·0031	0·5889
IL-15	255±173	1073±7467	131±1450	< 0·0001	0·7514	0·0061	0·5541
IL-18	78±76	185±114	31±50	< 0·0001	0·7157	< 0·0001	0·8214
IFN_γ	14±8	27±14	18±19	0·367	0·1884	0·7802	0·0616
RANTES	158±147	863±640	164±303	0·0006	0·6744	0·0022	0·6297
TGF-β1	7571±7924	19544±23463	2144±4403	0·0036	0·6961	0·0083	0·6924
TGF-β2	43017±39647	40763±32440	6768±8162	0·0129	0·6557	0·0423	0·5562
TGF-β3	828±813	1352±964	291±154	0·0055	0·7129	0·0332	0·5797

1006

Figure 1

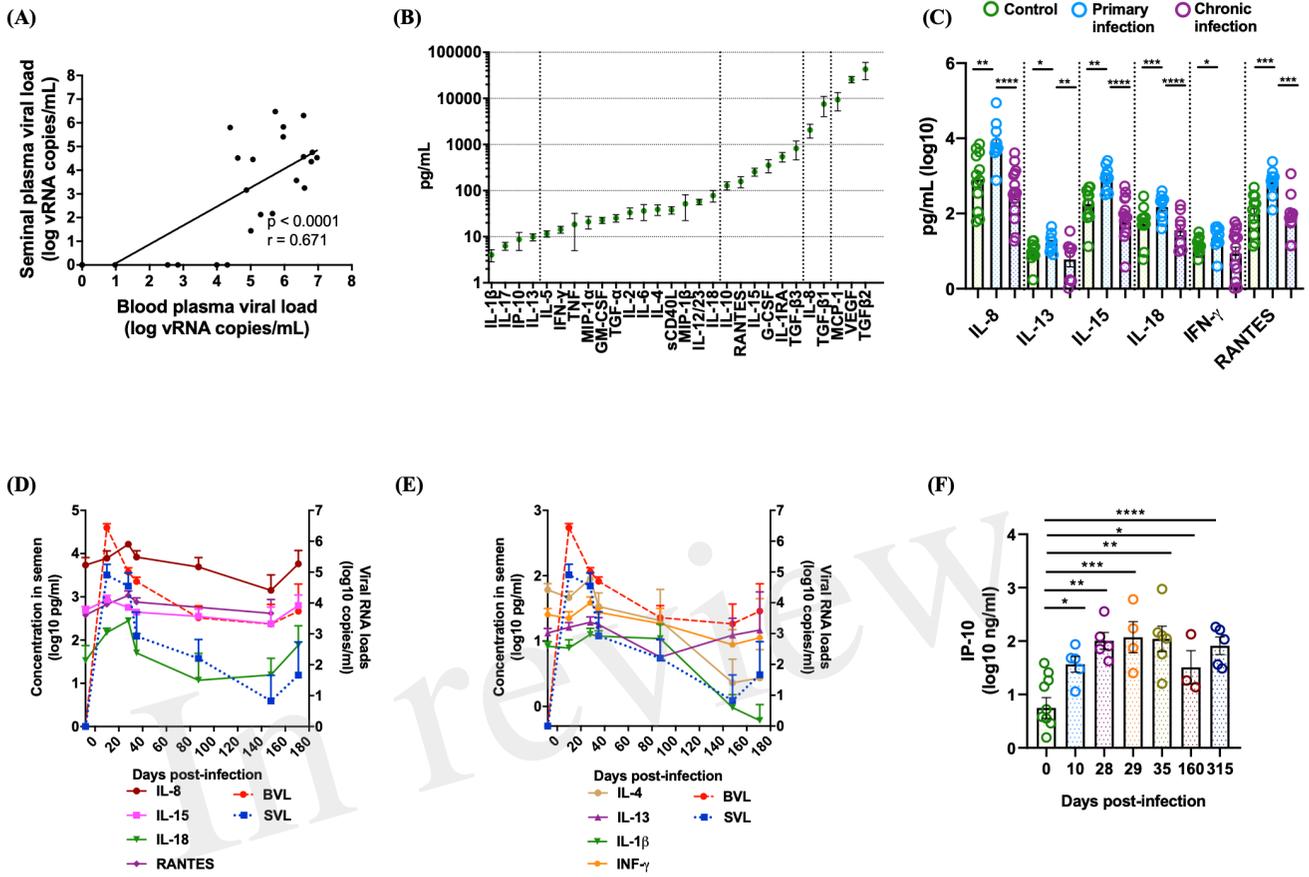


Figure 2

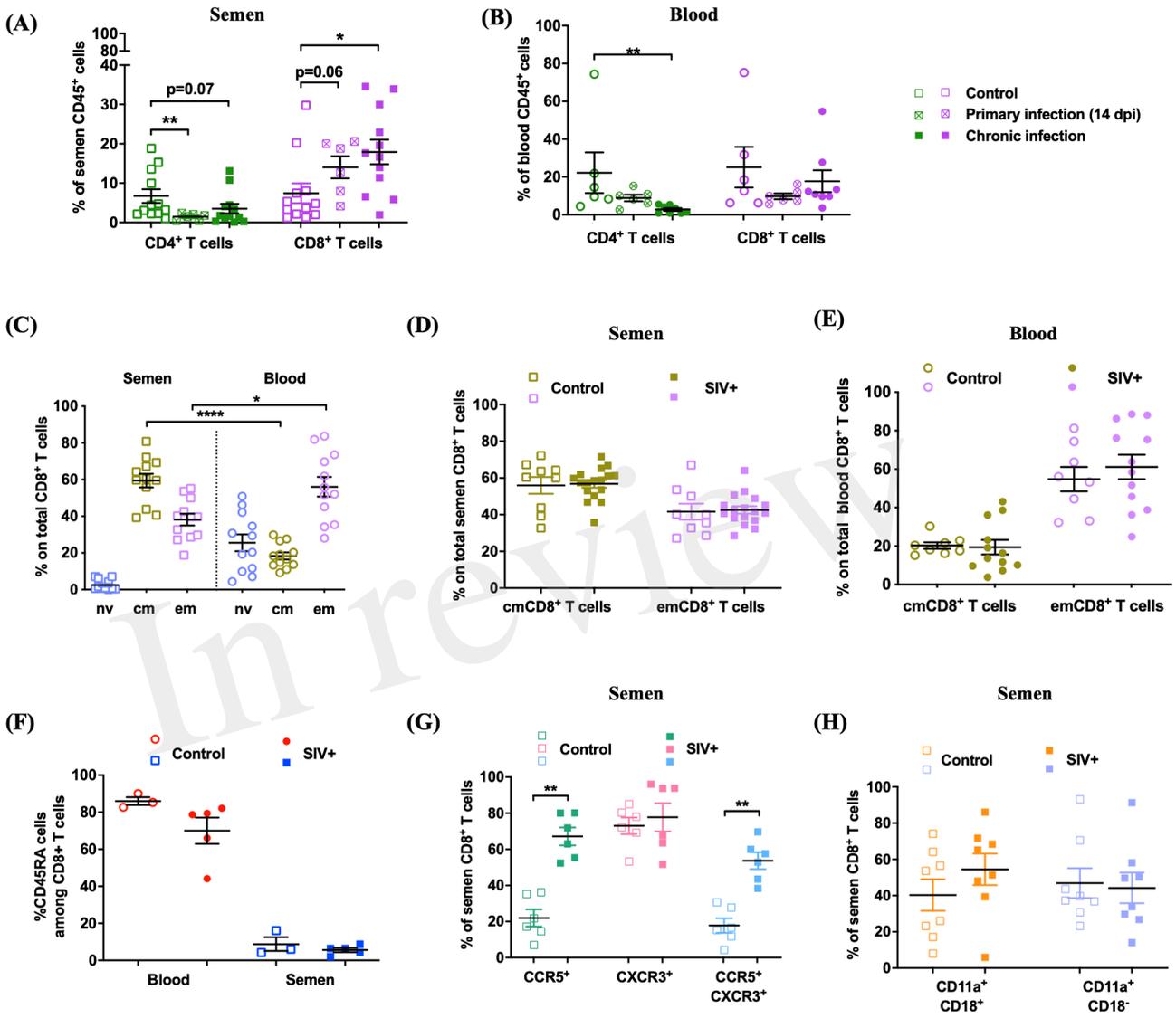


Figure 3

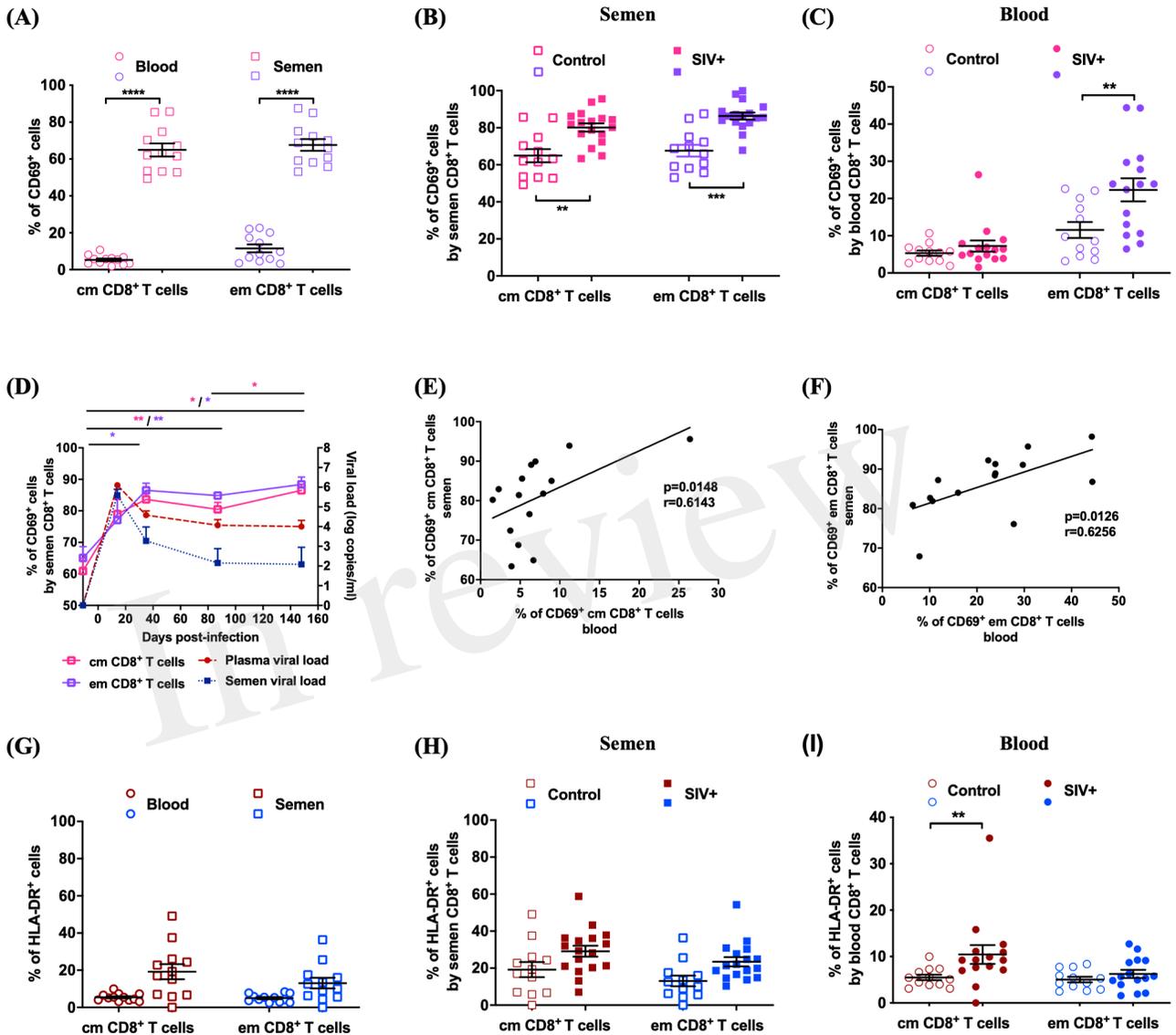


Figure 4.TIFF

Figure 4.

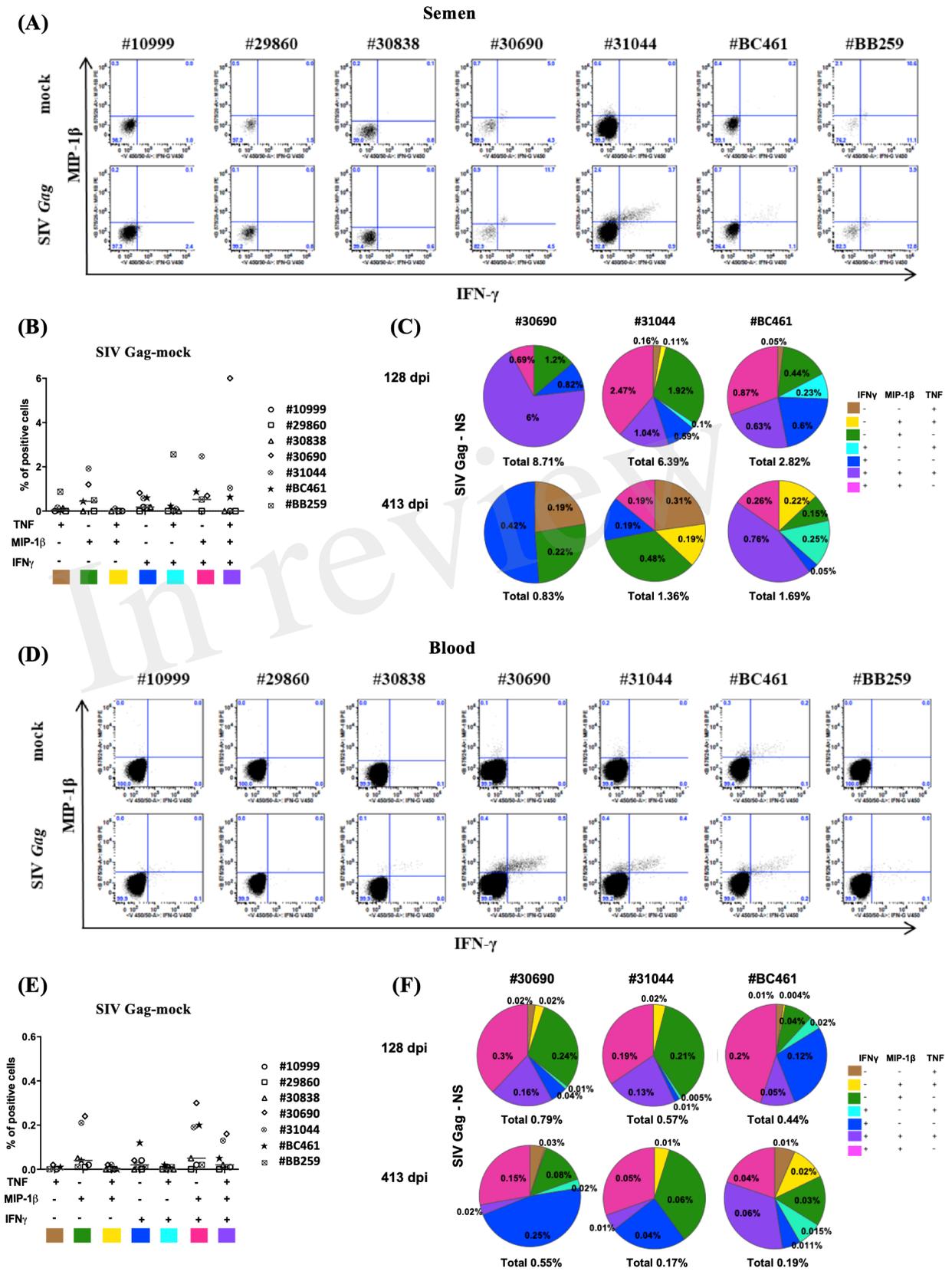


Figure 5

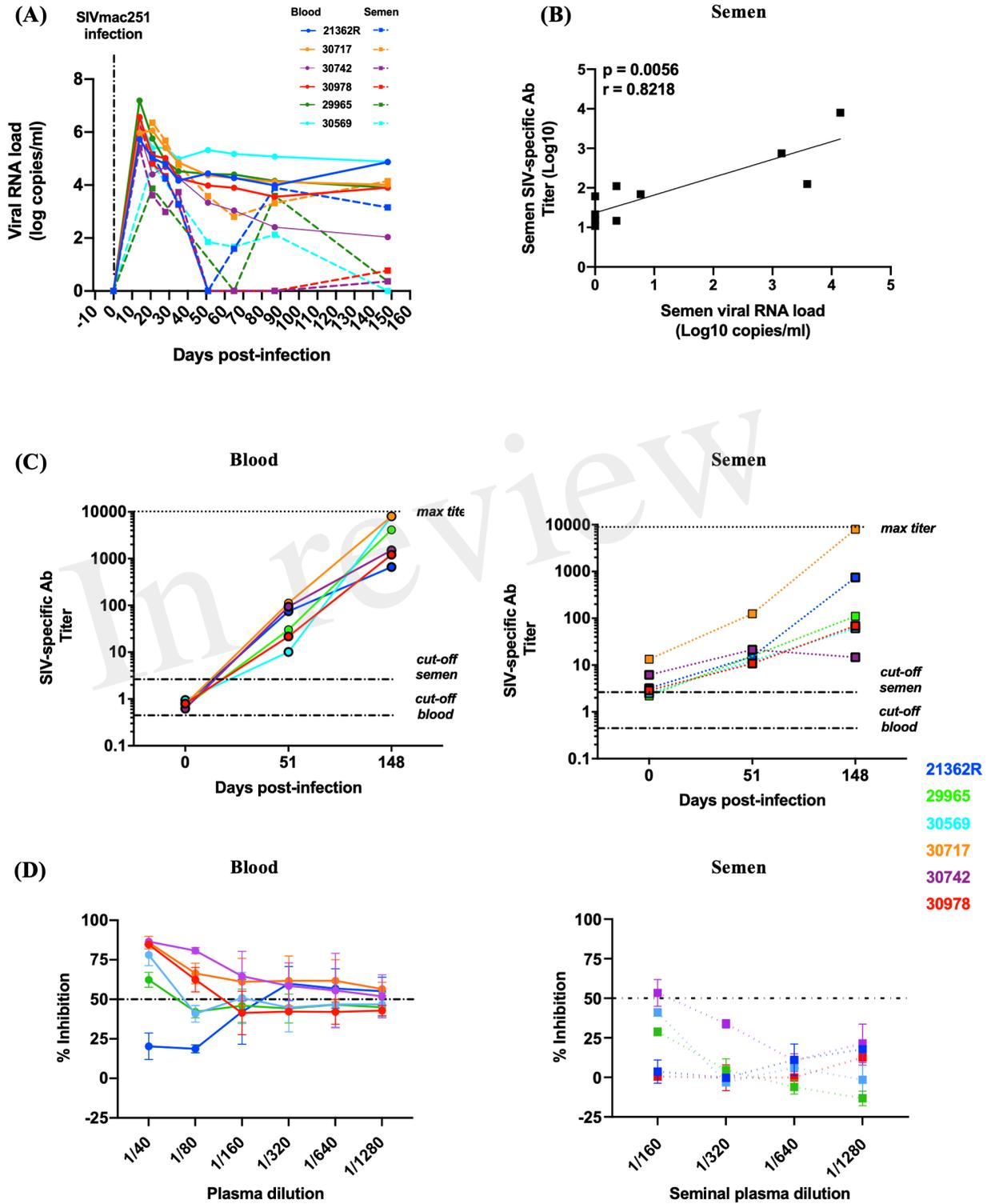
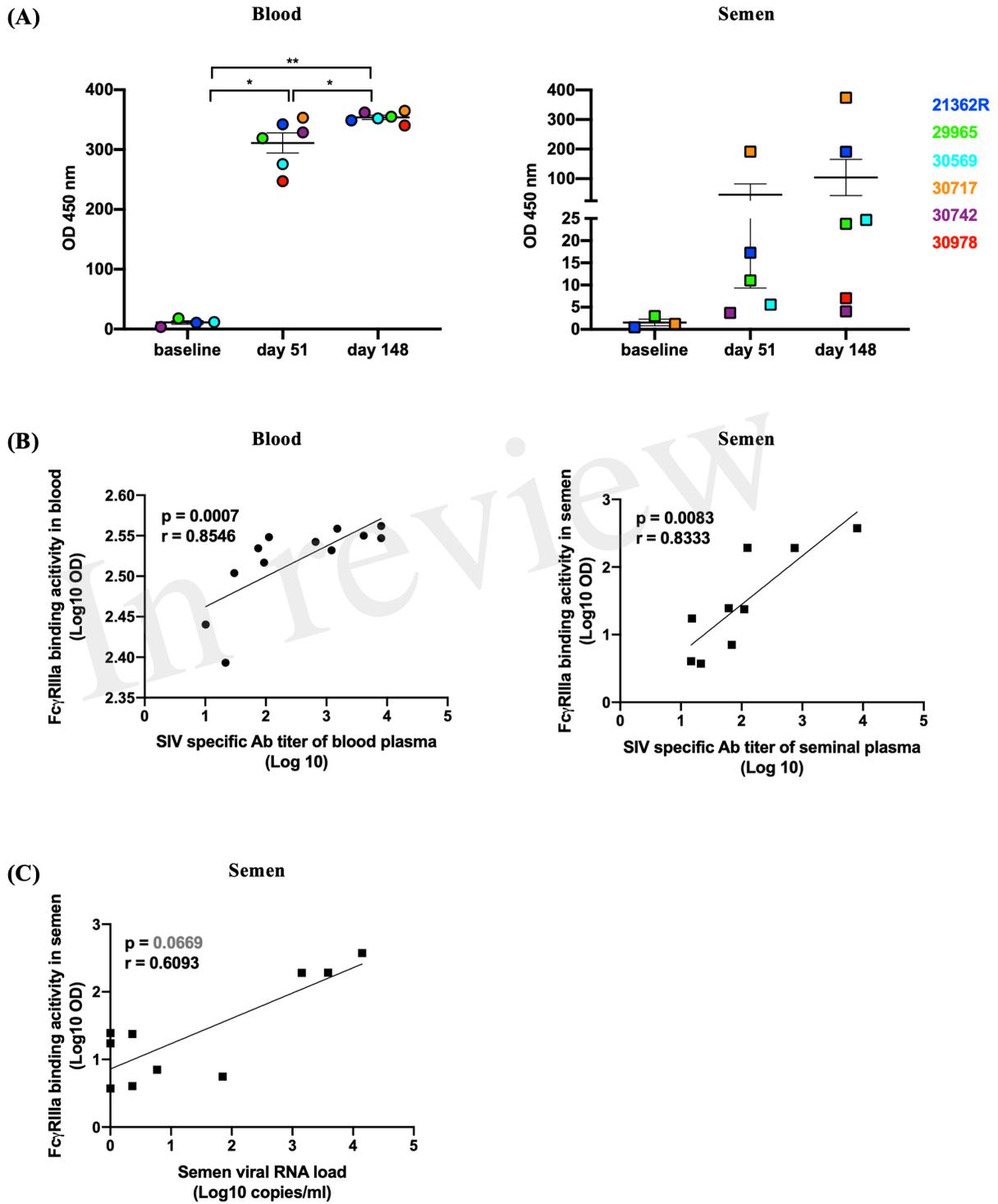


Figure 6



4.2 Second article (In preparation): SHIV transmission by semen leukocytes is efficiently inhibited by broadly neutralizing antibodies

4.2.1 Summary

In the second article, we assessed the efficacy of bNAbs against SHIV_{162P3} cell-to-cell transmission in comparison with cell-free infection. We developed *in vitro* cell-to-cell assays using either TZM-bl or human PBMC as target cells. The donor cells used in the assay were semen leukocytes or splenocytes collected from SHIV_{162P3}-infected cynomolgus macaque at acute phase of infection. The panel of bNAbs, including 1st generation and 2nd generation Abs, was selected to target different epitopes of HIV envelope-V1/V2 loop, V3 loop, CD4 binding site, MPER of gp41 and bridging region of gp120-gp41. The ability of bNAbs to protect from SHIV_{162P3} cell-to-cell transfer by splenocytes was tested *in vitro* in a TZM-bl assay and PBMC assay. To confirm the data, neutralizing activity was assessed using CD45⁺ semen leukocytes.

Inhibition of cell-free infection was achieved with 1 log lower inhibitory concentration (IC)₅₀ values compared to cell-associated transmission, whatever bNAb used. Both types of donor cells in the assay displayed their infectiousness which could be inhibited by bNAb candidates. A combination of 1st generation bNAbs 2F5+2G12+4E10 inhibited cell-to-cell transmission with equal efficiency when both semen leukocytes and splenocytes were used as donor cells in a TZM-bl assay. Protection was highly reduced when the same bNAbs were used in double combination or individually. Interestingly, 2nd generation bNAbs (10-1074, PGT128, PGT151 and N6) even when used individually, revealed to be as efficient against cell-to-cell transmission as the triple combination of the 1st generation bNAbs. Moreover, the anti-V3 bNAbs 10-1074 was able to inhibit cell-to-cell transmission mediated by semen leukocytes and splenocytes with the highest efficiency.

In conclusion, we demonstrated that a subset of bNAbs could efficiently prevent SHIV_{162P3} cell-to-cell transmission *in vitro*. On the base on these results, 10-1074 was selected to evaluate its potential to inhibit *in vivo* SHIV_{162P3} Cell-to-cell transmission following intravaginal challenge of cynomolgus macaques.

4.2.2 Manuscript

1 **SHIV162P3 transmission by semen leukocytes is efficiently**
2 **inhibited by broadly neutralizing antibodies**

3

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21 **Running title:** bNAbs inhibit semen cell-to-cell transmission

22 **Key words:** cell-to-cell transmission, semen cells, bNAbs

23 **Abstract**

24 HIV-1 sexual transmission occurs mostly through contaminated semen, which contains
25 both free virions and infected leukocytes. The importance of transmission initiated by infected
26 cells have been shown by several *in vitro* and *in vivo* studies and a reduced capability of broadly
27 neutralizing antibodies (bNAbs) against cell-to-cell transmission has been reported. At present,
28 there is no indication that bNAbs may prevent transmission mediated by semen leukocytes due
29 to the limitation of available experimental models.

30 We developed *in vitro* cell-to-cell assays using as donor cells either splenocytes or
31 semen leukocytes collected from SHIV_{162P3}-infected cynomolgus macaques at acute phase of
32 infection. bNAbs targeting different epitopes of the HIV-1 envelope were used either alone or
33 in combination to assess their inhibitory potential against both cell-free and cell-to-cell
34 transmission.

35 At variance with 1st generation bNAbs , newer generation of bNAbs, including 10-1074,
36 PGT128 and PGT151, even when used individually, were highly effective against cell-
37 associated transmission mediated by *in vivo* infected spleen cells. Interestingly, bNAbs potency
38 was maintained when transmission was mediated by infected semen cells. These results support
39 the use of bNAbs in preventative or therapeutic studies aiming to block transmission events
40 mediated not only by free viral particles but also by infected cells. Antibodies targeting the V3
41 loop and the BR of the envelope were the most potent to prevent SHIV162P3 cell-to-cell
42 transmission. Our experimental system may be used to predict *in vivo* efficacy of bNAbs.

43

44

45 **Introduction**

46 HIV-1 infection continues to be one of the major public health issue, with semen being
47 responsible of more than 60% of new transmission events (Joint United Nations Programme on
48 HIV/AIDS (UNAIDS), 2018; UNAIDS, 2018). The virus is present in the semen not only as
49 cell free virions but also as infected lymphocytes (Arien, Jespers and Vanham, 2011; Moir,
50 Chun and Fauci, 2011; Gonzalez *et al.*, 2019). Which is the form of virus that initiate infection
51 *in vivo* is unclear. Various *in vitro* and *ex vivo* studies demonstrated that cell-associated virus
52 (CAV) is 10 to 100 fold more potent to transmit infection than cell-free virus (Sagar, 2010;
53 Arien, Jespers and Vanham, 2011; Kolodkin-Gal *et al.*, 2013; Ronen, Sharma and Overbaugh,
54 2015). In addition, we and others have shown that systemic infection can be initiated *in vivo* in
55 macaques following either intravaginal, intrarectal or intravenous inoculation of SIV infected
56 cells (Sallé *et al.*, 2010; Kolodkin-Gal *et al.*, 2013; Parsons *et al.*, 2017). Indeed, semen
57 leucocytes are productively infected during all stages of SIVmac infection in cynomolgus
58 macaques (Bernard-Stoecklin *et al.*, 2013), similarly to what is known in HIV-1 infected people
59 (Quayle *et al.*, 1997; Zhang *et al.*, 1998). Lastly, several clinical studies point towards a role
60 for infected cells during sexual HIV-1 transmission.

61 An increasing number of studies reported that broadly neutralizing antibodies (bNAbs)
62 efficiently prevented *in vivo* infection by cell-free HIV/SHIV (Moldt *et al.*, 2012, 2016; Pegu
63 *et al.*, 2014; Rudicell *et al.*, 2014; Shingai *et al.*, 2014; Saunders *et al.*, 2015; Gautam *et al.*,
64 2016). However, bNAb mediated inhibition of CAV transmission has been largely overlooked
65 and the few *in vitro* studies performed up to date exhibited controversial results, possibly due
66 to the different experimental systems used (Martin *et al.*, 2010; Abela *et al.*, 2012; Malbec *et*
67 *al.*, 2013; Schiffner, Sattentau and Duncan, 2013; Duncan *et al.*, 2014; McCoy *et al.*, 2014;
68 Gombos *et al.*, 2015; Reh *et al.*, 2015; Li *et al.*, 2017). Despite of this, there is a large consensus
69 in attesting that the majority of bNAbs lost potency *in vitro* against cell-to-cell transmission
70 compared to cell-free viral infection (Abela *et al.*, 2012; Malbec *et al.*, 2013; Gombos *et al.*,
71 2015; Reh *et al.*, 2015). More importantly, studies performed so far never made use of *in vivo*
72 infected cells and whether bNAbs prevented CAV transmission mediated by semen leucocytes
73 has never been addressed.

74 At present, there is little evidence regarding which *in vitro* assay could accurately predict
75 the capacity of bNAbs to inhibit cell-to-cell viral spread *in vivo*. In this study, we assessed the
76 efficacy of a panel of bNAb to reduce SHIV_{162P3} cell-to-cell transmission. The source of cell-
77 associated virus used was semen leukocytes or splenocytes collected from SHIV_{162P3}-infected

78 cynomolgus macaques. We demonstrated that bNAbs directed against the V3 loop and the
79 bringing region (BR) of the envelope spike are highly effective against cell-associated
80 transmission mediated by *in vivo* infected spleen cells. Interestingly, bNAbs potency was
81 maintained when transmission was mediated by infected semen cells.

82 Our findings are of high relevance to planned studies of preventative or therapeutic use
83 of bNAbs, particularly those directed to the V3 loop and the BR.

84

85

86 **Materials and methods**

87

88 **Ethics statement**

89 The study we described used nonhuman primate models of HIV/AIDS in accordance
90 with European Union guidelines for animal care (European Union Directive 63/2010), and
91 protocols were approved by the Ethical Committee “Ile-De-France SUD”, as requested by our
92 institution (CEA).

93

94 **Animals, infection and sample collection**

95 Adult cynomolgus macaques (*Macaca fascicularis*) weighing 5 to 11 kg, were imported
96 from Mauritius and housed at CEA/IDMIT infrastructure facilities according to European
97 guidelines for animal care (Journal Officiel des Communautés Européennes, L 358, December
98 18, 1986 and new directive 63/2010). Four male cynomolgus macaques (#AX450, #BA865F,
99 #AX454 and #CI901) were infected by intravenous inoculation with a single dose of 1,000 50%
100 animal infectious dose (AID50) of SHIV_{162P3}. #MF1414, #1103075 and #CA149F were
101 infected by intrarectal inoculation with a single dose of 3 AID50 of SHIV_{162P3}.

102 Semen and blood were collected from sedated animals following 5 mg/kg intra-
103 muscular injection of Zoletil®100 (Virbac, Carros, France). Blood samples were taken in BD
104 Vacutainer® Plus Plastic K3EDTA tubes (for plasma viral load quantification) and in BD
105 Vacutainer® Plus Plastic SST tubes (for serum preparation) (BD Biosciences, Le Pont de Claix,
106 France).

107

108 **Semen collection**

109 Semen was collected at day 12, 13 and 14 post-infection from macaques #1103075
110 #CA147F and #MF1414, respectively. The protocol of semen collection was previously
111 described (Bernard-Stoecklin *et al.*, 2013). Briefly, animals were sedated by a 5 mg/kg
112 intramuscular injection of chlorhydrate Tiletamine (50 mg) combined with chlorhydrate
113 Zolazepan (50 mg) (Virbac, Carros, France) during semen collection. Ejaculation was
114 performed by intrarectal electrostimulation of nervous centers near the prostate, with a probe
115 (12.7 mm diameter) lubricated with conductor gel, and an AC-1 electro-ejaculator (Beltron
116 Instrument, Longmont, USA). Sequential stimulations were performed, with a pattern of 6
117 cycles, each cycle consisting of nine two-second stimulations followed by a tenth stimulation
118 lasting 10 seconds. The voltage was increased every two cycles (1–3 V for the first two cycles,

119 2–4 V volts for the third and fourth cycles and 6–8 V for the last two cycles). If a complete
120 ejaculate had not been obtained after six cycles of stimulation, a 7th cycle of stimulation at 7–
121 10 V was performed. The complete ejaculate was immediately diluted in 1.2 ml of phosphate-
122 buffered saline (PBS).

123

124 **Cells, antibodies and viruses**

125 TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent
126 Program (NIH ARRRP) and cultivated in DMEM containing 10% heat inactivated FCS and
127 antibiotics. Human PBMC from healthy donors were prepared from buffy coat and then
128 cultivated in RPMI containing 10%FCS, 1% PSN, 5µg/ml PHA (Sigma-Aldrich) and 5 units/ml
129 IL-2 (Roche) for 3-4 days before using in the experiment. Buffy coat from healthy human
130 donors was purchased from Établissement français du sang (EFS). PBMCs were isolated and
131 collected with Lympholyte cell separation media (Cedarlane) by centrifugation, aliquoted and
132 frozen at -80°C to be used in neutralization assay. bNAbs 2G12, 2F5, 4E10 and b12, and 5F3
133 (anti-gp41non-neutralizing antibody) were obtained from Polymun Scientific, GmbH
134 (Klosterneuburg, Austria) and whereas bNAbs 10-1074, PGT128, PGT151, N6, 3BNC117,
135 10E8, PG16 and PGDM1400 were provided by Dr. H. MOUQUET, Pasteur Institute, Paris,
136 France. For *in vitro* studies of Ab inhibitory activities, the plasmid to produce the infectious
137 molecular clone (IMC) of SHIV_{162P3} (pNL-LucR.T2A-SHIV-SF162P3.5.ecto) virus was kindly
138 provided by Dr. C. Ochsenbauer, University of Alabama at Birmingham.

139

140 **Semen cells preparation**

141 Seminal cells were separated from seminal plasma immediately after collection by
142 spinning 15 minutes at 775 g, then resuspended with 1 ml of cold PBS supplemented with 10%
143 FC and 2mM EDTA and kept in ice for 1 hour maximum. Cells were then spun 10 minutes at
144 1,500g, filtered through a 70 µM sieve and washed with 5 ml of cold PBS supplemented with
145 10% FC and 2mM EDTA.

146 CD45⁺ semen cells from animals #1103075 #CA147F and #MF1414 were enriched by
147 CD45 magnetic bead and cell sorting by BD FACS Aria Flow Cytometer. Total semen cells
148 were incubated for 15 minutes at 4°C with 20 µl of anti-CD45 magnetic beads (Miltenyi Biotec)
149 and washed once with 2 ml of cold PBS supplemented with 0.5% BSA and 2 mM EDTA
150 (sorting buffer). The CD45⁺ cell fraction was then enriched by magnetic bead sorting, with LS
151 columns (Miltenyi Biotec), used according to the manufacturer's instructions. Cells were eluted
152 in 4 ml of sorting buffer. Following the magnetic bead-based enrichment process, cells were

153 stained, using amine-reactive blue dye (Life Technologies) to identify the dead cells, and anti-
154 CD45 (clone D058-1283, BD Pharmingen). Cells were washed twice and stored at 4°C in PBS/
155 10% FCS. CD45⁺ cells were then sorted by simultaneous four-way sorting on a FACS Aria
156 flow cytometer (BD Biosciences). The enriched cell fraction was used in the neutralization
157 assay.

158

159 **Splenocytes preparation**

160 Spleen cells were mechanically dissociated and isolated using density gradient
161 separation with Lympholyte cell separation media (Cedarlane) from four animals (#AX450,
162 #BA865F, #AX454 and #CI901) euthanized at peak of viremia (between day 10 and 14 post-
163 infection). Aliquots of cells were frozen in 10% of dimethyl sulfoxide (DMSO).

164

165 **Phenotypic characterization of splenocytes and semen leukocytes**

166 Staining was performed on either thawed splenocytes or fresh semen. Amine-reactive
167 blue dye (Live/dead Fixable, Life Technologies) was used to assess cell viability and to exclude
168 dead cell from the analysis. Cells were stained with monoclonal antibodies by incubation for
169 30 minutes at 4°C, washed in PBS/10% FCS and fixed in commercial fixation solution
170 (CellFIX, BD Biosciences). List of antibodies used for splenocytes and semen leukocytes is
171 showed in **Supplementary Table 1** and **Supplementary Table 2**, respectively. Data were
172 acquired on a FORTESSA instrument (BD Biosciences) using Diva software (BD Biosciences)
173 and analyzed with Flowjo v10 (BD Biosciences).

174

175 **Cell-free virus titration and neutralization in the TZM-bl assay**

176 Viral titrations were performed in TZM-bl cells as previously described (Montefiori,
177 2009). The Tissue Culture Infectious Dose (TCID) 50 was defined as the reciprocal of the viral
178 dilution resulting in 50% positive wells (Reed-Muench calculation). A cut-off value of 2.5 times
179 background relative luminescence units (RLU) was used in the TCID and neutralization assays.
180 A standard inoculum corresponding to a virus dilution that yields approximately 300,000-
181 500,000 RLU equivalents (+/- 15,000 RLU) was chosen for the neutralization assay to minimize
182 virus-induced cytopathic effects while maintaining an ability to measure a 2-log reduction in
183 virus infectivity. NAbs were prepared by serial four steps of 3-fold dilution, starting from a
184 concentration of 8.32 µg/ml of a pool of 3 NAbs (2G12+2F5+4E10) and from 10 µg/ml for the
185 singles NAbs 2F5, 2G12 and b12. After 1 hour of Ab incubation with the viral supernatant
186 (IMCs SHIV_{162P3}), 10⁴ TZM-bl cells were added to each well and plates were incubated for 48

187 hours before luciferase activity was measured. Cell-free virus infections were carried out in
188 culture medium containing 15 µg/ml of the polycation DEAE dextran (diethylaminoethyl;
189 Amersham Biosciences, Fairfield, Connecticut, USA). Neutralization titers are the Abs
190 concentration at which RLU were reduced by 50% compared to RLU in virus control wells
191 after subtraction of the background RLUs in the control wells with only cells (IC₅₀). By
192 analogy, the IC₇₅ and IC₉₀ were defined as the concentration of Abs able to decrease the
193 percentage of infected cells by 75% and 90%, respectively. The IC₅₀, IC₇₅, and IC₉₀ were
194 calculated using a linear interpolation method (Heyndrickx *et al.*, 2012). For calculating the
195 mean, a value of greater than the highest Ab concentration used was recorded if 50% inhibition
196 was not achieved. In contrast, if 50% inhibition was not achieved at the lowest Ab concentration
197 used, a two-fold lower concentration than that value was recorded.

198

199 **Assessment of cell-cell transmission and neutralization in the TZM-bl assay**

200 To assess cell-cell transmission and inhibition thereafter, splenocytes or CD45⁺ semen
201 leukocytes were co-cultured with TZM-bl, in presence/absence of NAbs. A titration of infected
202 splenocytes (input 10,000 – 200,000 cells) was performed in order to ensuring the infection of
203 target cells comparable with the free virus infection corresponding to 150,000-200,000 RLU.
204 Cells were pre-incubated with serial 2-fold dilution of bNAbs starting from a concentration of
205 132 µg/ml for 2F5, 4E10 and 2G12 and of 50 µg/ml for b12 for 1 hour at 37 °C before co-
206 culturing with 10,000 TZM-bl per well. 40,000 sorted semen cells were co-cultured with 10,000
207 TZM-bl in presence/absence of 132 µg/ml of a pool of 2F5+2G12+4E10.

208 The co-culture condition in 96 flatted well plate was performed in DMEM medium
209 containing 10% heat inactivated FCS and antibiotics for 48 hours at 37 °C in absence of DEAE
210 dextran. To estimate the inhibition activity, cells were lysed and luciferase reporter gene
211 production in TZM-bl measured upon addition of firefly luciferase substrate (Promega,
212 Madison Wisconsin, USA). IC₅₀, IC₇₅ and IC₉₀ were calculated as previously described.

213

214 **Cell-free viral titration and neutralization in the PBMC assay**

215 Analogously to what described for the TZM-bl assay, the TCID₅₀ was firstly defined.
216 Then, six steps of 2-fold dilution of NAbs, starting from 10 µg/ml, was incubated with 20 and
217 40 TCID₅₀ of viral supernatant (IMCs SHIV162P3) for 1 hour. Thereafter, 100,000 PHA-IL2
218 stimulated PBMCs from two donors were added to each well in 96 round bottom well plate
219 using RPMI medium containing 10% heat inactivated FCS, antibiotics and 20 units/ml IL2 at
220 37 °C. At days 1 and 3 cells were washed and at day 7 the supernatant from each well was

221 collected to detect SIVp27 Ag by ELISA, using the RETRO-TEK SIV p27 Antigen ELISA
222 (Helvetica Health Care, Geneva, Switzerland) according to manufacturer's recommendations.
223 IC50, IC75 and IC90 were calculated as mentioned for the TZM-bl assay.

224

225 **Assessment of cell-cell transmission and neutralization in the PBMC assay**

226 The protocol has been adapted from the cell-free neutralization assays. SHIV_{162P3}
227 infected splenocytes or CD45⁺ semen leucocytes were used as donor cells. A titration of
228 splenocytes (input 40,000 – 160,000 cells) was performed for each stock of splenocytes to
229 define the number of cells allowing efficient cell-cell transmission. bNAbs were prepared and
230 used as previously in the TZM-bl assay at starting concentration of 132 µg/ml and 15 µg/ml for
231 1st and 2nd generation bNAbs, respectively. 40,000 semen CD45⁺ cells were used and four steps
232 of 3-fold dilution starting from 5 µg/ml of 10-1074 bNAbs were prepared. Cells were incubated
233 1 hour with the Ab before co-culturing with 120,000 PHA-IL2 activated PBMC from 2 donors.

234 The co-culture in 96 round bottom well plate was performed in RPMI medium
235 containing 10% heat inactivated FCS, antibiotics and 20 units/ml IL2 at 37 °C. After 7 days of
236 culture, the supernatant was collected to detect viral replication by either RT-qPCR (Superscript
237 III platinum one-step qPCR system) or p27-Ag detection (RETRO-TEK SIV p27 Antigen
238 ELISA, Helvetica Health Care, Geneva, Switzerland) according to manufacturer's
239 recommendations. Cells were instead collected and stained for p27 Ag. IC50, IC75 and IC90
240 were calculated as mention in the TZM-bl assay.

241

242 **Detection of SHIV infected cell by intracellular p27 Ag staining**

243 Staining was performed after 7 days of splenocytes-PBMC co-culture. Dead cells were
244 excluded from the analysis using the amine-reactive blue dye (Live/dead Fixable, Life
245 Technologies). Anti-CD45 antibody (clone D058-1283, BD Pharmingen), that do not cross-
246 react with human cells, and anti-CD3 antibody (clone UCHT1, BD Horizon), that do not cross-
247 react with macaque cells, were used in order to discriminate human PBMC from macaque
248 splenocytes. To estimate the transmission and inhibition activity, PE conjugated anti-p24
249 antibody (clone KC57 displaying cross-reactivity with the p-27 antigen) was used. Cells were
250 incubated with monoclonal antibodies for 15 minutes at 4°C, then washed in PBS/10% FCS,
251 permeabilized with Fixation/Permeabilization solution (BD bioscience) for 20 minutes at 4°C
252 and finally washed twice with Perm/Wash (BD Biosciences). The cells were next stained with
253 KC57 antibody for 15 minutes at room temperature. Samples were washed twice with
254 Perm/Wash and fixed (CellFIX, BD Biosciences). Data were acquired on a FORTESSA

255 instrument (BD Biosciences) using Diva software (BD Biosciences) and analyzed with Flowjo
256 v10 software (BD Biosciences).

257

258 **Quantification of viral load in plasma, seminal plasma and culture supernatants**

259 Blood plasma was isolated from EDTA-treated blood samples by centrifugation for 10
260 minutes at 1,500g and stored frozen at -80 °C. Viral RNA (vRNA) was prepared from 200
261 µL of cell-free plasma or culture supernatants, using Nucleospin 96 virus core kit (Machery-
262 Nagel). Retro-transcription and cDNA amplification and quantification were performed in
263 duplicate by RT-qPCR using the Superscript III Platinum one-step quantitative RT-PCR system
264 (Invitrogen, Carlsbad, USA). RT-PCR was performed as previously described (Karlsson *et al.*,
265 2007). The quantification limit (QL) was estimated to be 111 copies/ml and the detection limit
266 (DL) 37 copies/ml. Semen viral RNA was prepared from 500 µL seminal plasma using the
267 QIAamp Ultrasens Virus kit (Qiagen, Courtaboeuf, France), according to the manufacturer's
268 instructions. Quantitative RT-PCR was performed under the same conditions as above, with a
269 QL of 37 copies/ml and a DL of 12.3 copies/ml.

270

271 **Viral DNA extraction from splenocytes and semen leucocytes, quantification and** 272 **normalization**

273 DNA was extracted from frozen cells using the NucleoSpin Tissue XS kit (Machery-
274 Nagel) according to the manufacturer's instructions. DNA was analyzed in duplicate by real-
275 time PCR Taqman assay using the Platinum qPCR SuperMix-UDG kit (Thermo Fisher
276 Scientific) with SIV-gag primers and probe, as described elsewhere (Mannioui *et al.*, 2009).
277 The SIVmac251 gag complementary DNA sequence, ligated into pCR4-TOPO (Invitrogen)
278 plasmid and purified with the HiSpeed Maxiprep Kit (Invitrogen), was used as standard dilute
279 in 5,000 cells from uninfected macaques (serial 10-fold dilutions). The detection limit was of
280 30 copies per 10⁶ cells. The reaction, data acquisition, and analysis were performed with the
281 iCycler real-time thermocycler (Bio-Rad).

282 Thus, we verified cell numbers in each unknown sample. DNA from 5,000 cells from
283 uninfected macaques was serially diluted 1 in 10 (up to 10⁴). The glyceraldehyde-3-phosphate
284 dehydrogenase (GAPDH) gene was then amplified simultaneously using a primer set and probe,
285 as described elsewhere (Mannioui *et al.*, 2009). GAPDH-DNA levels in unknown samples were
286 inferred by comparing threshold cycle (Ct) value against a calibration curve. Unknown samples
287 displayed levels of amplifiable complementary DNA or DNA equivalent to the levels obtained

288 from 5,000 cells. Absolute numbers of viral DNA were normalized to 5,000 cells. Results were
289 expressed as SIV DNA copy numbers per 10^6 cells.

290

291 **Data visualization and statistical analysis**

292 All data visualization and statistics analysis were performed using GraphPad Prism
293 version 8.1 software (GraphPad Software, La Jolla, USA). Dose-response inhibition curves
294 were plotted using sigmoid dose-response curves (variable slope). Parametric Welch's t-test
295 was used to compare infectivity of the different input of splenocytes in TZM-bl assay. P values
296 of 0.05 or lower were considered significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p <$
297 0.0001 .

298

299 **Results**

300

301 **Splenocytes from infected macaques are phenotypically similar to semen leucocytes and**
302 **transmit infection to target cells *in vitro***

303 We aimed to develop a robust system allowing direct quantification of cell-cell
304 transmission using *in vivo* infected cells. Isolation of semen cells is highly challenging, and the
305 modest number of recovered cells doesn't allow to perform extensive set-up experiments. In
306 order to define a surrogate model, we investigated whether SHIV_{I62P3} spleen cells resembled *in*
307 *vivo* infected semen cells and could represent a valuable model in cell-to-cell transmission
308 experiments.

309 We initially compared immune cells composition of spleen cells with those of semen
310 cells from three SHIV_{I62P3} infected animals in order to validate the choice of the experimental
311 model (**Table 1 and Supplementary Figure 1**). Splenocytes were composed by 1.7±2.5 % of
312 CD14⁺ monocytes/macrophages, 6.8±1.3 % of CD3⁺CD4⁺CD8⁻ T cells, 7.5±2.2 % of
313 CD3⁺CD4⁻CD8⁺ T cells and 29.1±7.9 % of CD20⁺ cells. Most of the CD4⁺ T cells were
314 CD28⁺CD95⁺ central memory (CM) cells (62.1±8.7%). CD28⁻CD95⁺ effector memory (EM)
315 and CD28⁺CD95⁻ naive cells were present in smaller proportions (13.8±3.2% and 18.2±14.1%,
316 respectively). Proportion of CD4⁺ T cells in semen was similar to those in spleen, representing
317 9.1% ± 2.49% of total lymphocytes. Similarities were also observed in the amount of naïve,
318 CM and EM cells, whereas a lower proportion of monocytes/macrophages and a higher amount
319 of B cells was detected in semen compared to spleen. Interestingly central memory (CM) and
320 effector memory (EM) spleen CD4⁺ T cells were the major infected populations with a DNA
321 viral load 219-fold and 500-fold higher than in monocytes/macrophages (7 x 10⁵, 1.6 x 10⁶ and
322 3.2 x 10⁴ SIV DNA copies per 10⁶ cells in CM, EM and monocytes/macrophages, respectively;
323 data from one donor). This is in agreement with our previous observation that, while both CD4⁺
324 T cells and macrophages were infected in the semen of macaques, lymphocytes constituted the
325 major source of CAV (Bernard-Stoecklin *et al.*, 2013). These results together with the
326 observation that infected splenocytes initiated systemic infection in macaques *in vivo* (Sallé *et*
327 *al.*, 2010; Parsons *et al.*, 2017) prompted us to use splenocytes in cell-to-cell transmission
328 experiments and related bNAb-inhibition.

329 Stocks of splenocytes were prepared from four macaques (#AX450, #AX454, #BA865F and
330 #CI901) intravenously infected with SHIV_{I62P3} and sacrificed between day 10 and day 13 p.i.
331 when the blood viral load (BVL) ranged between 10⁵ and 10⁷ copies/ml (**Figure 1A**). The BVL

332 was mirrored by the pro-viral load detected in the splenocytes, with cells from #AX454
333 containing the highest amount of integrated DNA (4×10^5 , 3.8×10^3 , 3.29×10^3 and 7.91×10^2 viral
334 DNA copies per 10^6 cells for #AX450, #BA865F, #AX454, and #CI901, respectively) (**Figure**
335 **1B**). In order to identify the best experimental conditions to successfully achieve cell-cell
336 transmission, a titration of splenocytes was performed using both TZM-bl (**Figure 1C**) and
337 PBMCs (**Figure 1D**) as target cells. A linear increase in the transfer of cell-cell SHIV_{162P3}
338 infection was observed when the number of spleen cells was progressively increased. #AX450
339 splenocytes were the most efficient to transmit infection in both experimental systems, which
340 possibly reflected the amount of integrated viral DNA. When co-cultured with PBMCs, 40,000
341 splenocytes from #AX450 produced $2.2 \times 10^8 \pm 2.15 \times 10^8$ RNA copies/ml, whereas at least a
342 double amount of cells from the other three donors was needed to reach similar amount of
343 infection. On the base of these results, we decided to use spleen cells from #AX450 to assess
344 bNAbs-mediated inhibition of SHIV cell-to-cell transmission.

345

346 **Inhibitory activity of a mix of bNAbs against cell-free and cell-cell SHIV_{162P3} transmission**

347 In order to compare the potency of NAb in neutralization assay against cell-free versus
348 cell-associated SHIV transmission, we initially determined the Tissue Culture Infectious Dose
349 50 (TCID₅₀) of cell-free SHIV_{162P3} in TZM-bl cells. DEAE-dextran was used to distinguish
350 cell-free from cell-associated virus infection (Abela *et al.*, 2012; Gombos *et al.*, 2015). A
351 standard inoculum corresponding to about 150,000-200,000 RLU equivalents, and an input of
352 10^5 spleen cells which yield a comparable virus infectivity, was chosen for the neutralization
353 assay. This allowed us to minimize virus-induced cytopathic effects while comparing the same
354 input of virus in the two infection models (**data not shown**).

355 Previous work of the group showed the success to inhibit *in vitro* and *in vivo* cell-free
356 transmission of SHIV_{162P3} by the pool of 2F5, 2G12 and 4E10 NAbs (Moog *et al.*, 2014). Thus,
357 the same panel of NAbs was employed to evaluate their inhibitory activity against cell-to-cell
358 SHIV_{162P3} transmission. Coculture experiments were run in presence of increasing amount of a
359 pool of 2F5, 2G12 and 4E10 NAbs until 100% viral infectivity was regained. The NAbs pool
360 inhibited SHIV_{162P3} cell-cell transmission with a mean IC₅₀ value of 78.09 ± 36.85 $\mu\text{g/ml}$ in
361 the TZM-bl assay (**Figure 2A and Table 2**) and a mean IC₉₀ value of 53.45 ± 12.65 $\mu\text{g/ml}$ in
362 the PBMC assay (**Figure 2B and Table 2**). The comparison of the inhibitory activity under the
363 two transmission modes revealed that the Ab mix lost considerable potency when cell-cell
364 transmission occurred (IC decrease of 132 and 189 folds compared to cell-free infection in
365 TZM-bl and PBMC, respectively) (**Table 2**). It is still under discussion whether it is preferable

366 to use combination of bNAbs targeting different sites of vulnerability of the envelope. To
367 compare efficiency of triple versus double Ab pool, we combined the Abs in order to target
368 only the membrane proximal external region (MPER) of the gp41 (2F5+4E10) or both the
369 gp120 and the MPER (2G12 + 4E10 and 2F5 + 2G12). Double Ab combinations were still
370 active although with some variance between the two assays employed. While in the TZM-bl
371 assay Abs targeting both the gp120 and the MPER revealed as effective as using the triple
372 combination of Ab ($IC_{50} = 48.93 \pm 25.65 \mu\text{g/ml}$ for 2G12 + 4E10 and $36.64 \pm 32.30 \mu\text{g/ml}$ for
373 2F5 + 2G12), in the PBMC assay protection from cell-to-cell transmission was lost when using
374 2G12 + 4E10 combination ($IC_{90} > 132 \mu\text{g/ml}$). In general, however, not significant differences
375 were observed, and the triple and double combinations displayed comparable ICs values
376 (**Figure 2 and Table 2**). As expected, individual Abs completely lost efficacy against cell-to-
377 cell transmission. Among the Ab tested, the IgG1b12 was the most active against SHIV_{162P3}
378 transmission when used individually, with an IC_{50} of $3.21 \pm 1.07 \mu\text{g/ml}$ in the TZM-bl assay.
379 However, it's efficiency was considerable reduced when considering 75 and 90 endpoint titers,
380 in both TZM-bl and PBMC assays ($IC_{75} = 8.46 \pm 4.74 \mu\text{g/ml}$ and $11.03 \pm 5.35 \mu\text{g/ml}$ and IC_{90}
381 $= 26.64 \pm 1.95 \mu\text{g/ml}$ and 25.98 ± 13.08 in TZM-bl and PBMC, respectively, **Table 2 and data**
382 **not shown**).

383

384 **Antibodies targeting the V3-loop and the bringing region of the viral envelope are better** 385 **candidates to inhibit SHIV_{162P3} cell-to-cell transmission**

386 The identification of newer generation bNAbs with potencies 10 to 100 folds greater
387 than earlier bNAbs has renewed interest in their potential clinical use. We investigated whether
388 protection from cell-mediated transmission could be improved employing second generation
389 bNAbs. SHIV_{162P3} infection of PBMC was determined in the presence or absence of 8 different
390 monoclonal Abs to evaluate their relative neutralization efficiencies when used alone or in
391 double and triple combinations. Ab used had different targets on the virus or the target cells.
392 PGDM1400 was predicted to be nonneutralizing before any experiment was performed. A
393 maximum Ab concentration of $5 \mu\text{g/ml}$ was used, compared to $132 \mu\text{g/ml}$ used when assaying
394 1st generation bNAbs. Individual Abs were also used against cell-free transmission. The ICs of
395 the bNAbs are shown in **Table 3**. Among individual Abs used, the anti V3 loop 10-1074 and
396 PGT128 and the anti-bridging region (BR) Ab PGT151 had similar inhibitory potency against
397 cell-to-cell transmission, with IC_{90} s of 1.56 ± 1.09 , 1.93 ± 0.9 and $1.71 \pm 1.17 \mu\text{g/ml}$, and
398 IC_{50} s of 0.44 ± 0.19 , 0.33 ± 0.11 and $0.46 \pm 0.13 \mu\text{g/ml}$, respectively (**Figure 3A**). Those titers
399 were > 50 -fold lower compared to the 2F5+4E10+2G12 combination. Anti CD4bs Ab N6 and

400 3BNC117 were instead less effective, with IC₉₀ values of 4.45 ± 4.44 and $> 15 \mu\text{g/ml}$,
401 respectively. 10E8 and PG16, analogously to PGDM1400, were not neutralizing SHIV_{162P3}
402 (**Figure 3A**). Cell-free SHIV transmission was blocked at Ab concentration 11 - 33 folds lower
403 compared to CAV transmission (**Table 3**). We then combined Abs in order to target different
404 epitopes. Seven double combinations (**Figure 3B**) and three triple combinations (**Figure 3C**)
405 were tested. An additive effect of Ab combination was however, not observed. Indeed, IC₉₀
406 values remained in the same order of magnitude as single Abs. 10-1074, which maintained
407 considerable potency when used alone, resulted particularly interesting and was selected to test
408 its potential to block SHIV transmission mediated by semen leucocytes.

409 To further confirm the specific inhibition of cell-to-cell transmission by bNAbs, we
410 differentiated splenocytes from PBMC in the coculture system by flow cytometry and assessed
411 viral transfer to PBMCs by intracellular p27 Ag staining. This strategy allowed us to
412 discriminate CD3⁻CD45⁺ splenocytes and three populations of cells among PBMCs:
413 CD3^{low}CD45⁻, CD3^{high}CD45⁻ and CD3⁻CD45⁺ (**Figure 4A**). Interestingly, both CD3^{low}CD45⁻
414 and CD3^{high}CD45⁻ cells were infected with values ranging from $1.08 \pm 0.05\%$ to $5.41 \pm 1.11\%$ for
415 the CD3^{low}CD45⁻ and from $0.36 \pm 0.03\%$ to $0.39 \pm 0.08\%$ for the CD3^{high}CD45⁻ cells. In presence
416 of 2nd generation bNAbs the % of infected cells was consistently reduced, with greatest potency
417 compared to the 2F5+2G12+4EE10 pool (**Table 4**). Among the different Ab/Ab combinations
418 used, 10-1074 was one of the most potent, showing a reduction of $> 96\%$ of infected p27⁺ cells
419 when used at $5 \mu\text{g/ml}$ (**Figure 4B** and **Table 4**). As expected, no inhibition was observed in
420 presence of the NNAb 5F3 (**Figure 4C**) or PDGM 1400 (**Table 4**).

421

422 **bNAbs inhibit transmission by SHIV_{162P3} infected semen cells *in vitro***

423 We sought to determine whether Abs could still inhibit cell-mediated transmission when
424 CD45⁺ semen leukocytes were used as source of virus. Semen was collected from three
425 macaques (#1103075 #CA147F and #MF1414) at day 12, 13 and 14 p.i, respectively when the
426 blood viral load (BVL) ranged between $1.77\text{E}+06$ and $1.58\text{E}+05$ copies/ml and the seminal
427 viral load (SVL) ranged between $3.03\text{E}+03$ and $2.9\text{E}+05$ copies/ml (**Figure 5A**).

428 A total of 5.0×10^5 , 1.8×10^6 and 3.6×10^5 CD45⁺ leucocytes were isolated from $84 \mu\text{l}$,
429 $315 \mu\text{l}$ and $52 \mu\text{l}$ of semen from #1103075, #CA147F and #MF1414, respectively. Semen
430 CD45⁺ cells transferred infection to PBMC (mean of $1.15 \times 10^8 \pm 1.21 \times 10^8$ RNA copies/ml at
431 day 7 post-coculture) and the anti-V3 bNAbs 10-1074 inhibited transmission of infection up to
432 99% when used at concentration as low as $0.062 \mu\text{g/ml}$ (**Figure 5B**). Thus, 10-1074 Ab strongly

433 inhibited transmission mediated by semen cells, with IC90 value (<0.062 µg/ml) lower to those
434 obtained when splenocytes were used as donor cells (**Table 3**).

435 Our results support the potential use of this Ab *in vivo* against cell-associated SHIV_{162P3}
436 challenge.

437 **Discussion**

438 In this study we have evaluated for the first time the capability of a panel of bNAbs to
439 inhibit cell-to-cell transmission of SHIV_{162P3} in a setting in which *in vivo* infected cells, notably
440 semen cells, were used as source of virus. HIV-1 transmission from infected to uninfected cells
441 is likely playing a predominant role in infecting individuals (Murooka *et al.*, 2012) and several
442 studies have shown that this mode of viral spread may represent a mechanism through which
443 the virus can evade antibody-based immunity (Ganesh *et al.*, 2004; Abela *et al.*, 2012; Malbec
444 *et al.*, 2013; Gombos *et al.*, 2015). Previous studies reported inability of bNAbs to interfere
445 with cell-to-cell transmission but also demonstrated that these observations depend on the cell
446 type and the Ab used. Moreover, those studies primarily focus on *in vitro* infected cells. Due to
447 the diversity between cell lines or even CD4⁺ T cells derived from blood and semen T cells; it
448 is thus of utmost relevance to establish more physiologically relevant *in vitro* systems. The cell-
449 to-cell transmission system used here allowed us to evaluate bNAbs-mediated inhibition in a
450 setting in which both the percentage of infected cells and the proviral level reflect those found
451 *in vivo*.

452 In both human and macaques, infected cells in the semen are mostly represented by T
453 lymphocytes and macrophages (Quayle *et al.*, 1997; Zhang *et al.*, 1998; Bernard-Stoecklin *et*
454 *al.*, 2013). Here we used spleen cells collected at a time of high plasma viremia of an acutely
455 SHIV_{162P3}-infected macaque, as surrogate of semen cells. We demonstrated that the major
456 difference between the two cells types is represented by the percentage of macrophages, which
457 are present in higher amount in the spleen of infected macaques. However, the proportion of
458 CD4⁺ T lymphocytes, both total and subsets of naïve, central memory and infected memory
459 cells, were comparable. While the more relative abundance of macrophages may favor
460 transmission of the R5 tropic SHIV, our results that semen cells more efficiently transmitted
461 infection to TZM-bl indicated that possibly macrophages were not the predominant transmitting
462 cells. This is confirmed by proviral load results we obtained, demonstrating that lymphocytes
463 were the predominant infected cells among splenocytes.

464 Our results also suggested that cells carrying a high proviral content provided proof for
465 high local MOI, as previously reported (Del Portillo *et al.*, 2011). Indeed, cell-to-cell
466 transmission was more effective when the DNA content was above 10⁴ copies per million of
467 cells.

468 The splenocytes/target cells transmission assay allowed us to evaluate whether the mode
469 of HIV transfer has an influence on the potency of neutralizing antibodies. Indeed, it is

470 recognized that only a subset of potent HIV-1 broadly NAb are capable of inhibiting most of
471 the steps implicated in the lymphocyte-to-lymphocyte spread of HIV-1 including the formation
472 of virological synapses and the transfer of viral material from infected cells to the targets
473 (Malbec *et al.*, 2013). Here we shown that many of the second generation bNAbs tested were
474 effective in SHIV_{162P3} cell-to-cell transmission, with potency more than 50 folds higher when
475 used individually compared to the triple combination of first generation 2F5+4E10+2G12.
476 Interestingly, similar results were obtained when comparing the capability of the
477 2F5+2G12+4E10 pool and the individual 10-1074 Ab to prevent cell-cell transmission
478 mediated by seminal leukocytes.

479 Those results confirmed again that PBMC were more susceptible to cell-to-cell
480 transmission compared to TZM-bl. They also indicated that the possibility to predict
481 transmissibility on the base of the SVL is linked to the experimental system used. Indeed, while
482 at the time of semen collection #MF1414 and #CI901 had similar SVL, only semen cells from
483 #MF1414 could transmit infection to target cells.

484 In conclusion, our results confirmed previous observations that cell-to-cell transmission
485 is more difficult to neutralize compared to cell-free virus and indicated that 1st generation
486 bNAbs, when used alone, might not inhibit SHIV cell-to-cell transmission. It was not the case
487 for some of the newer bNAbs, including the anti-V3 glycans 10-1074, which maintained
488 efficacy even when used individually against SHIV transmission mediated by both spleen and
489 semen cells. We believe that the cell-to-cell transmission model described here may provide a
490 more reliable method for predicting the potency of bNAbs *in vivo*.

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501 **Author Contributions Statement**

502 Study conception and design: RLG and MC. Acquisition of data: KS, MT, SH. Management
503 of animal: DD. Analysis and interpretation of data: KS, MT, NDB and MC. Draft of manuscript:
504 KS and MC. Critical revisions: HM, GS, RLG and MC.

505 **Conflict of Interest Statement**

506 The authors declare that they have no competing interests.

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619

620 **Figure legends**

621 **Figure 1. *In vitro* infectiousness of SHIV_{162P3} infected splenocytes from four different**
622 **macaques and related viral content**

623 (A) Follow-up of blood viral RNA load in macaques up to the day of euthanasia. (B) Proviral
624 SIV DNA content of SHIV_{162P3} infected spleen cells. (C) Titration of splenocytes in coculture
625 with TZM-bl cells. Infection was recorded as Relative Light Unit (RLU) at 48 hours post-
626 coculture after subtraction of control well's RLU (TZM-bl alone). (D) Titration of splenocytes
627 in coculture with PBMC. Viral production was detected by RT-qPCR 7 days post-coculture.
628 Each bar represents mean and standard error of the mean (SEM) of four experiments performed
629 in triplicate except for #AX450 titration in the TZM-bl that was performed in triplicate. Each
630 color represents a different animal.

631

632 **Figure 2. Neutralization of SHIV_{162P3} cell-cell transmission by 2F5, 4E10 and 2G12 bNAbs**
633 **used in single, double and triple combination.**

634 (A) TZM-bl neutralization assay. Results after 48 hours of splenocytes/TZM-bl coculture are
635 shown. (B) PBMC neutralization assay. Infection was analyzed by SIV p27 Ag ELISA 7 days
636 post-coculture. Mean and SEM of three to four independent experiments is shown. Dot lines
637 indicate 0, 50 and 90 % of viral inhibition.

638

639 **Figure 3. Neutralization of SHIV_{162P3} cell-cell transmission by a panel of 2nd generation**
640 **bNAbs in a PBMC assay.**

641 bNAbs were used singularly (A) and in combination (B, C). Infection was analyzed by SIV p27
642 Ag ELISA 7 days post-coculture. Mean and SEM of three to four independent experiments is
643 shown. Dot lines indicate 0, 50 and 90 % of viral inhibition.

644

645 **Figure 4. Assessment of target cell infection in the PBMC neutralization assay by**
646 **intracellular p27 staining.**

647 (A) Gating strategy used to discriminate macaques splenocytes from human PBMC. After
648 gating on live cells, macaques splenocytes were identified as CD45⁺CD3⁻ cells (the anti-CD3
649 antibody that do not cross-react with macaque cells) and human PBMC as CD45⁻ cells (the anti-
650 CD45 antibody do not cross-react with human cells). (B) Dose-dependent inhibition of cell-to-
651 cell transmission mediated by 10-1074 used at a starting concentration of 5 µg/ml, in the
652 population of CD3⁺/CD45⁻ cells (upper panel) and CD3⁻/CD45⁻ cells (lower panel) in the

653 presence of serial dilution of bNAbs. Infected cells were identified by intracellular p-27 Ag
654 staining. (C) Dose-dependent inhibition of cell-to-cell transmission mediated by the NNAb 5F3
655 used as negative control at a starting concentration of 132 $\mu\text{g/ml}$, in the population of
656 $\text{CD3}^+/\text{CD45}^-$ cells (upper panel) and $\text{CD3}^-/\text{CD45}^-$ cells (lower panel). Dot plots from one
657 representative experiment are shown.

658

659 **Figure 5. bNAb-mediated inhibition of semen leukocytes SHIV_{162P3} transmission to target**
660 **cells**

661 (A) Follow-up of viral RNA loads in blood (plain line) and in semen (dashed line) of three
662 macaques. Each color represents a different macaque. (B) 10-1074 mediated inhibition of
663 transmission from CD45^+ semen cells to PBMC. Infection was analyzed by RT-qPCR 7 days
664 post-coculture. Each condition was assessed in duplicate. Mean and SEM of three independent
665 experiments is shown.

666

667 **Supplementary figure legends**

668 **Supplementary Figure 1. Gating strategy used to characterize the phenotype of SHIV_{162P3}**
669 **infected cells from #AX450 spleen and #MF1414 semen.**

670 After gating on live cells, among CD45⁺ cells we identified: monocytes/macrophages (CD3⁻
671 CD8⁻) and B lymphocytes (HLADR⁺CD20⁺). T lymphocytes (HLADR⁺CD20⁻) were further
672 classified as CD4⁺ T cells (CD3⁺CD8⁻CD4⁺) and CD8⁺ T cells (CD3⁺CD8⁺CD4⁻).

673

674 **Tables**

675 **Table 1. Comparison of immune cell populations from the spleen and the semen of SHIV_{162P3} infected macaques.**

676

Number of animals	Days post infection	Origin	Monocyte/ macrophage	B lymphocytes	CD8 ⁺ T lymphocytes	CD4 ⁺ T lymphocytes	CD4 ⁺ T lymphocytes		
							Central Memory	Effector Memory	Naive
3	10	Spleen	1.7 ± 2.5 %	29.1 ± 7.9%	7.5 ± 2.2%	6.8 ± 1.3%	62.1 ± 8.7%	13.8 ± 3.2%	18.2 ± 14.1%
3	13-28	Semen	0.8 ± 0.7%	0.7 ± 0.7%	23.4 ± 11.1%	9.1 ± 2.5%	73.2 ± 5.7%	4.2 ± 1.8%	17.9 ± 5.6%

677

678

679 **Table 2. IC values of a panel of 1st generation bNAbs against SHIV_{162P3} transmission.**

680

681 Mean ± standard deviation from three ^(a) or from three to four ^(b) independent experiments is shown. ND=Not Done

Neutralizing antibody	IC 50 (µg/ml) ^a		IC 90 (µg/ml) ^b	
	TZM-bl assay		hPBMC assay	
	Cell-free	Cell-cell	Cell-free	Cell-cell
2F5+2G12+4E10	0.59±0.25	78.09±36.85	0.28±0.23	53.45±12.65
2F5+4E10	ND	>132	ND	95.06±25.23
2G12+4E10	ND	48.93±25.65	ND	>132
2F5+2G12	ND	36.65±32.30	ND	87.64±9.27
2F5	1.6±1.17	>132	1.34±1.16	>132
4E10	9.18±9.41	>132	5.01±2.13	>132
2G12	>10	>132	>10	>132
b12	0.95±0.9	3.21±1.07	0.3±0.2	25.98±13.08
5F3	ND	>132	>10	>132

682

683 **Table 3. IC values of a panel of 2nd generation bNAbs against SHIV_{162P3} transmission.**

Neutralizing antibody	hPBMC assay ^a			
	Cell-free IC 90 (µg/ml)	IC 50 (µg/ml)	IC 75 (µg/ml)	Cell-cell IC 90 (µg/ml)
10-1074+N6+ PGDM1400	ND	0.17±0.13	0.36±0.35	1.10±1.10
10-1074+N6+ PGT151	ND	0.15±0.25	0.53±0.31	0.83±0.47
10-1074+PGT128+ PGT151^b	ND	< 0.062	< 0.062	0.28
10-1074+N6	ND	0.36±0.17	0.70±0.16	1.18±0.10
N6+PGT151	ND	0.61±0.07	1.08±0.77	1.41±0.10
10-1074+PGT151	ND	0.26±0.06	0.45±0.06	0.69±0.30
10-1074+PGT128	ND	0.27±0.12	0.58±0.07	1.11±0.08
N6+PGDM1400	ND	1.73±0.55	>5	>5
10-1074+PGDM1400	ND	0.49±0.07	0.90±0.10	1.34±0.07
PGT128+PGT151^b	ND	< 0.062	0.12	0.29
10-1074	0.14±0.03	0.44±0.19	0.85±0.50	1.56±1.09
PGT128	0.06±0.04	0.33±0.11	0.87±0.42	1.93±0.90
PGT151	0.15±0.08	0.46±0.13	0.95±0.70	1.71±1.17
N6	0.23±0.15	1.01±0.83	2.57±2.56	4.45±4.44
3BNC117	1.02±1.03	8.48±14.40	>15	>15
10E8	1.07±0.69	>15	>15	>15
PG16	>3.3	>15	>15	>15
PGDM1400	>3.3	>15	>15	>15

684 Mean ± standard deviation from three to four (^a) or from one (^b) independent experiments is
685 shown. ND=Not Done

686 **Table 4 Percentage of inhibition of p27+ cells in presence of bNAbs**

687

bNAbs [Ab] (µg/ml)	Percentage of inhibition on infected cells: CD3 ^{low} /CD45 ⁻ ; CD3 ^{high} /CD45 ⁻								bNAbs ⁶⁸⁸ 689 [b12] (µg/ml)
	2F5+2G12 +4E10	2F5+4E10	2G12+ 4E10	2F5+ 2G12	2F5	4E10	2G12	b12	
132	98.9; 94.4	88; 75	54.3; 39.8	97.6; 95.3	77.3; 66.3	47.1; 54.9	neg; 7.5	77.8; 66.6	50
66	88.4; 79.1	49.7; 7.3	3.9; neg	60.2; 39.8	43.3; 39.3	29.3; 48.8	neg; neg	27.5; 10.4	16.67
33	39.6; 23.2	33.5; neg	1.9; neg	12.7; neg	42.5; 27.9	20.1; 28.6	1.7; 24.6	17.4; neg	5.56
16.5	12.8; neg	30; neg	7.2; neg	neg; neg	6.7; 0.9	neg; 12.3	neg; neg	neg; neg	1.85
8.25	neg; neg	18.3; neg	neg; neg	neg; neg	6.7; 4.6	neg; 33.8	5.8; neg	neg; neg	0.62
bNAbs [Ab] (µg/ml)	N6+ 10-1074+ PGDM 1400	N6+ 10-1074+ PGT151	10-1074+ PGT151	10-1074	N6	PGDM 1400	PGT151	5F3	bNAbs [Ab] (µg/ml)
5	99.5; 98.3	99.5; 97.7	99.7; 97.2	97.9; 96.4	97.5; 94.8	neg; neg	95; 94.8	16.3; 30.1	5
1.67	99.5; 98.4	99.5; 97.7	99.7; 97.5	97; 96.3	44.1; 62.3	1.1; 12.5	97.5; 96.6	8.6; 20.3	1.67
0.56	83.4; 86.5	99.3; 98.1	99.3; 98.4	83; 85	30.9; 48.5	0.18; 15.3	89.9; 81.6	1.3; 12.3	0.56
0.19	30.7; 47.9	75.4; 78.5	69.8; 74.5	14.1; 21.8	neg; 17.2	neg; 16.7	20.4; neg	15.2; 32.5	0.19
0.063	10.2; 30.4	28.6; 42.9	11.1; 16.7	neg; neg	neg; neg	1.8; 17.2	15.4; 13.8	neg; 19.3	0.063

690 **Supplementary Tables**691 **Supplementary Table 1: List of monoclonal antibodies used to characterize the phenotype**
692 **of infected splenocytes**

693

Antibody	Clone	Company
CD45	D058-1283	BD Pharmingen
CD3	SP34-2	BD Pharmingen
CD4	L200	BD Pharmingen
CD8	Bw135/80	Miltenyi
CD95	DXk2	BD Pharmingen
CD28	CD28.2	Beckman Coulter
CD69	FN50	BD Pharmingen
HLA-DR	L243 (G46-6)	BD Pharmingen
CD20	2H7	Ebioscience
CD27	MT-271	BD Pharmingen
IgD FITC	Polyclonal	AbDserotec

694

695

696 **Supplementary Table 2: List of monoclonal antibodies used to characterize the phenotype**
 697 **of infected semen cells**

698

Antibody	Clone	Company
CD45	D058-1283	BD Pharmingen
CD3	SP34-2	BD Pharmingen
CD4	L200	BD Pharmingen
CD8	RPA-T8	BD Horizon
CD20	2H7	BD Horizon
HLA-DR	G46-6	BD Pharmingen
CD16	3G8	BD Horizon
NKG2A	Z199	Beckman Coulter
CD123	7G3	BD Pharmingen
CD14	M5E2	BD Pharmingen
CD66	TET2	Miltenyi
CD11c	S-HCL-3	BD Pharmingen
NkP44	2.29	Miltenyi

699

Figure 1.

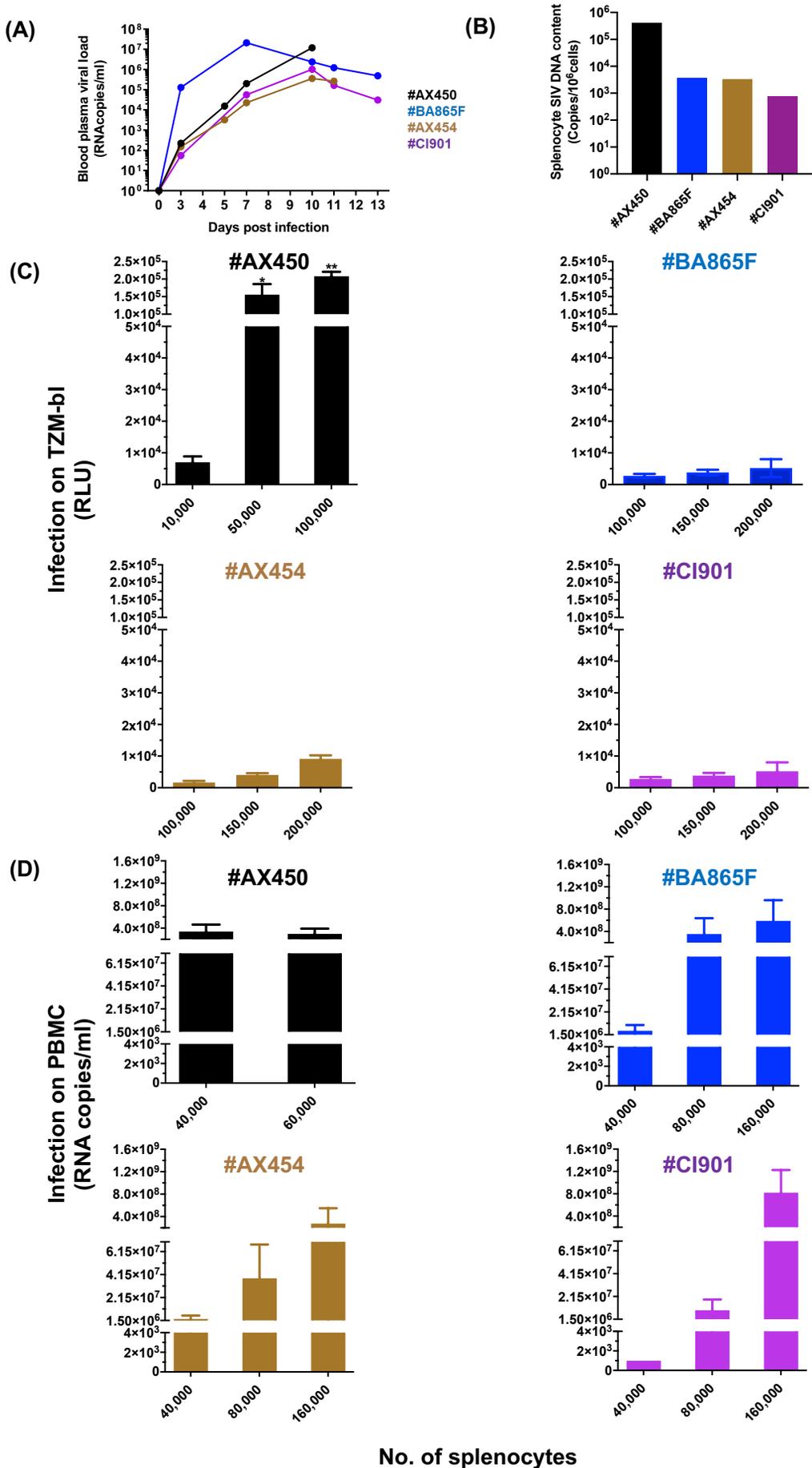


Figure 2.

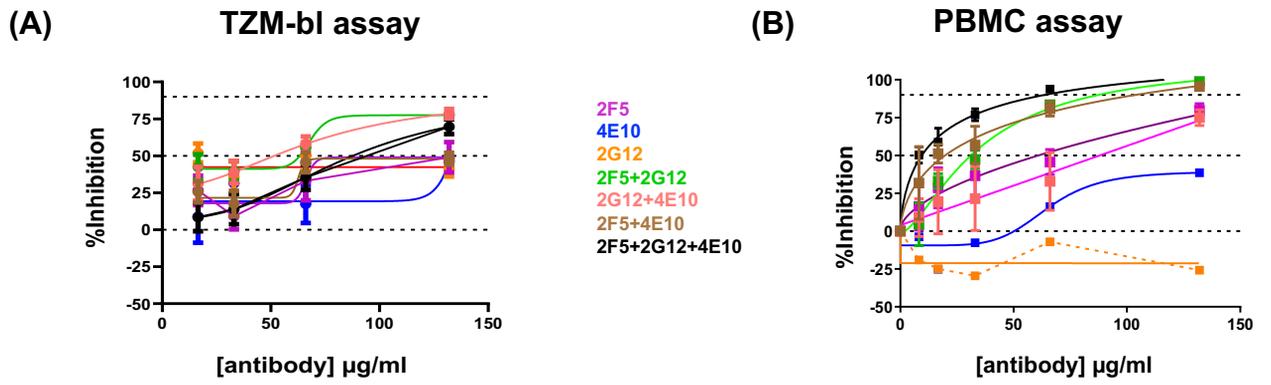


Figure 3.

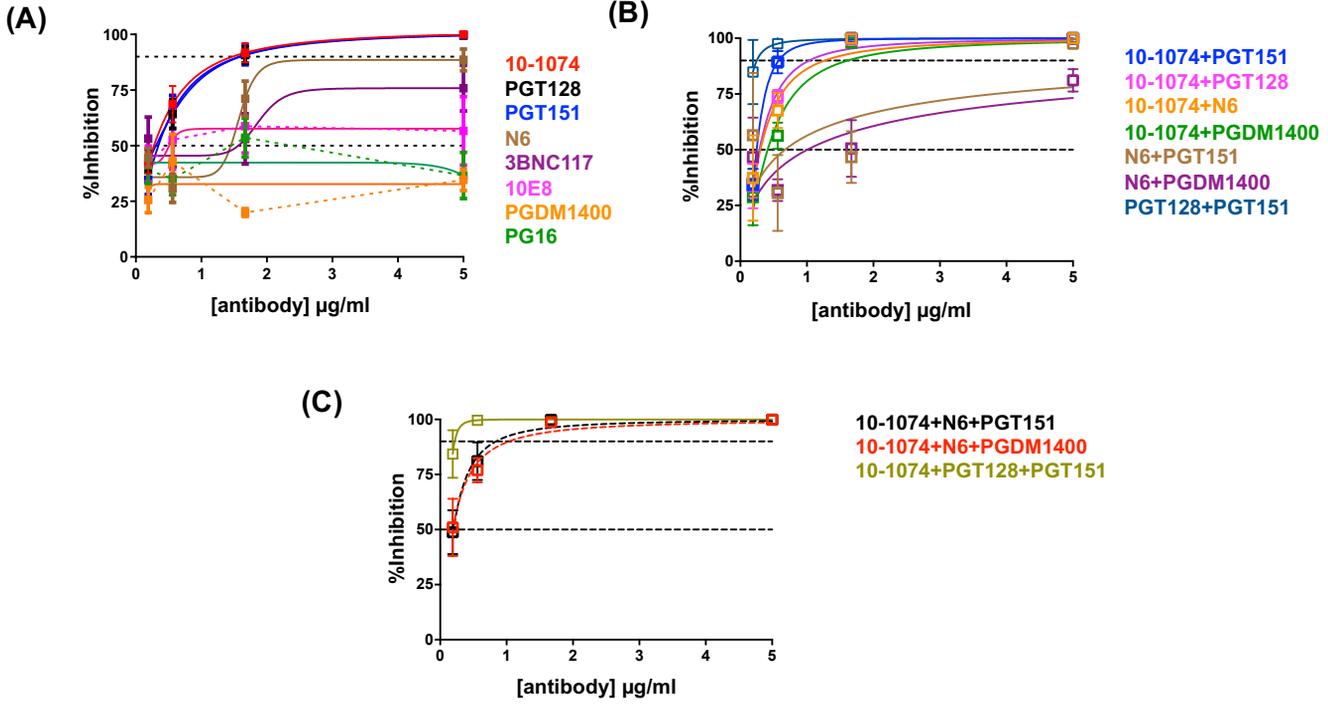
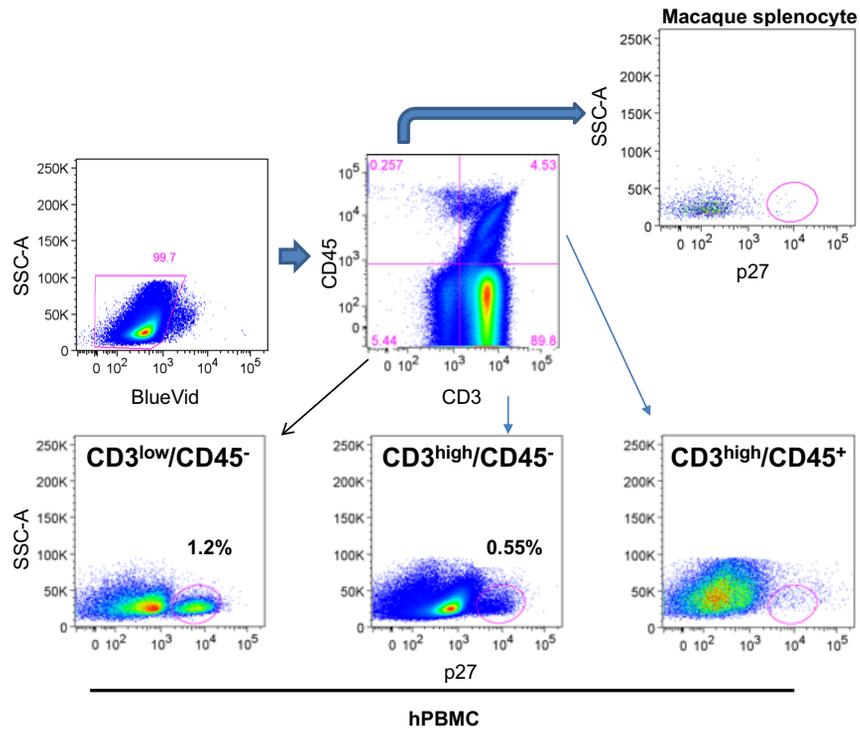
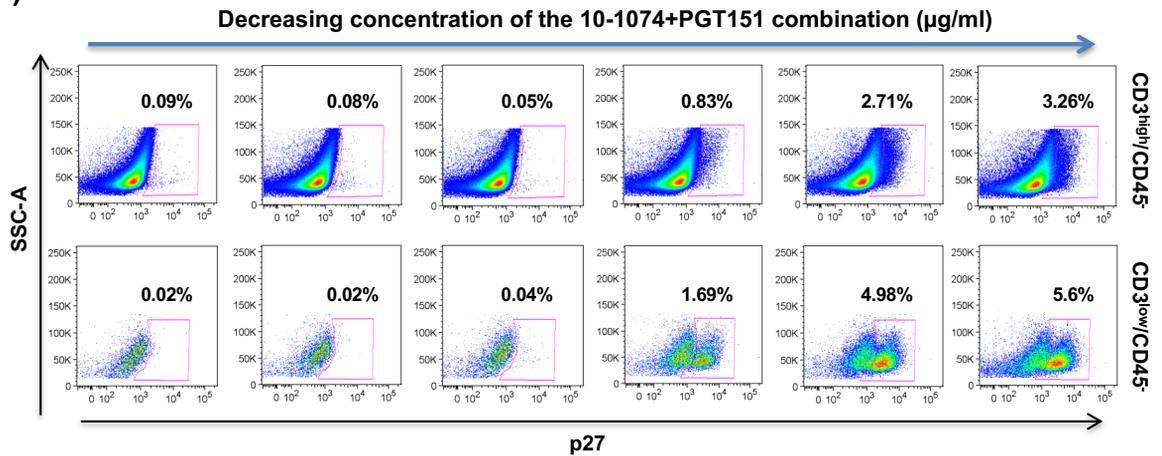


Figure 4.

(A)



(B)



(C)

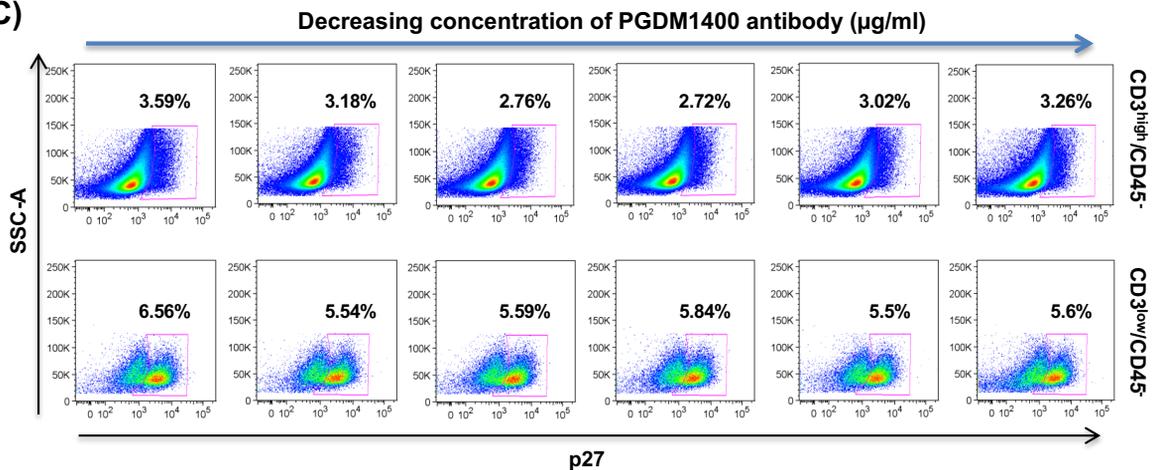
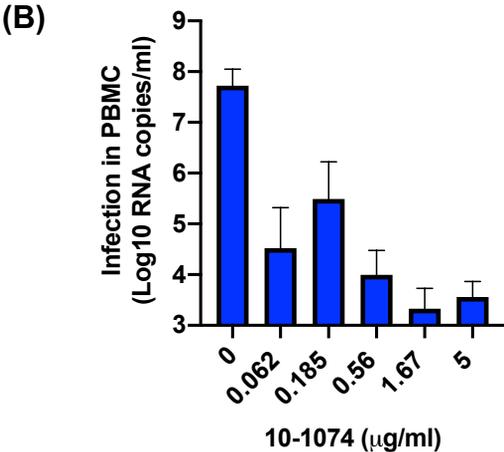
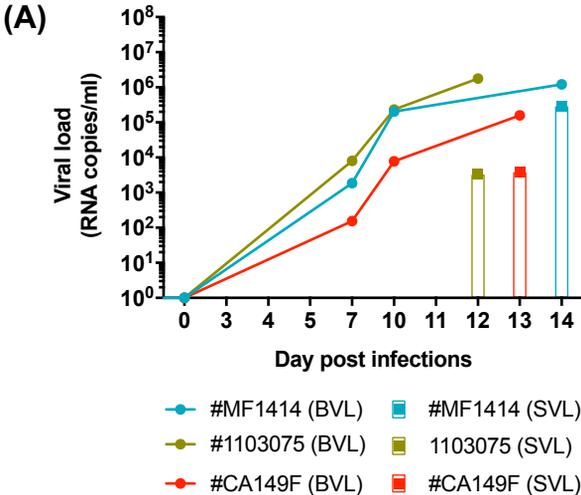


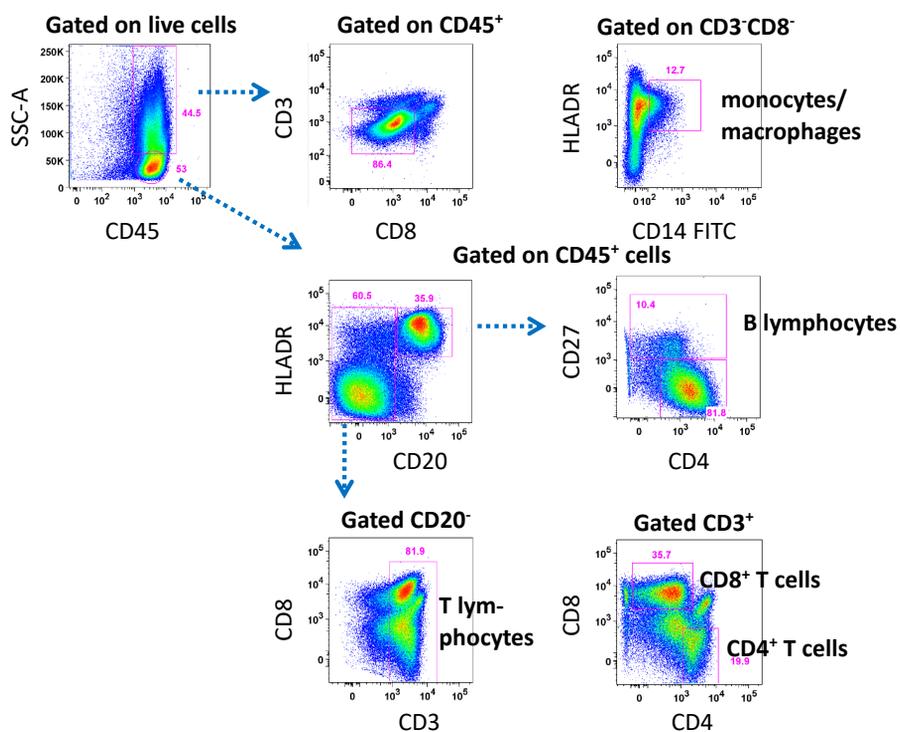
Figure 5.



Supplementary Figure 1.

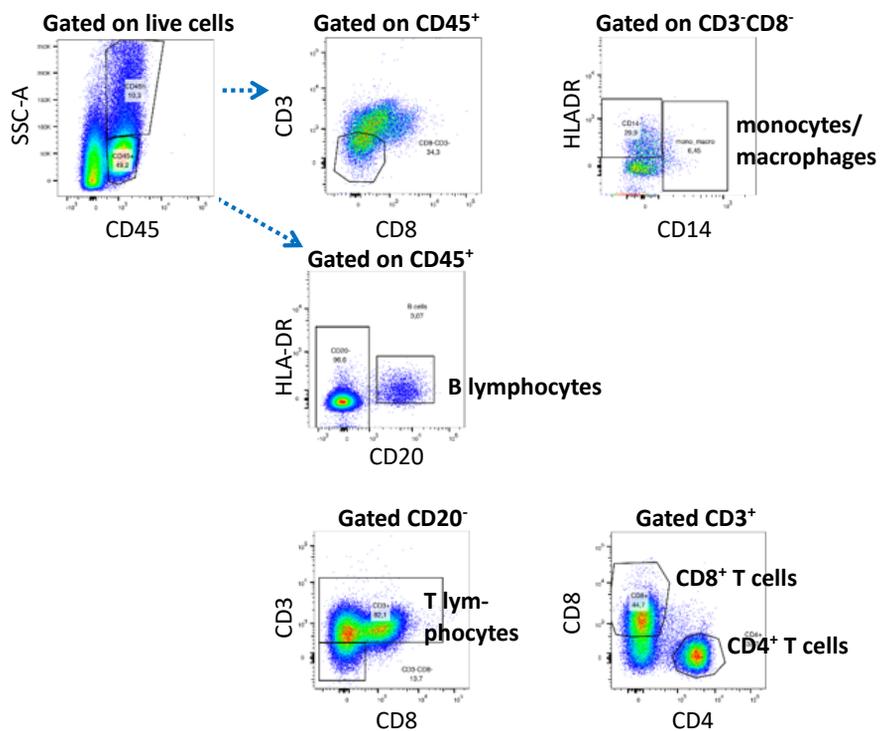
(A)

#AX450 splenocytes



(B)

#MF1414 semen cells



4.3 Complementary results

4.3.1 Establishment of an *in vivo* SHIV model of CAV transmission in cynomolgus macaques for assessment of bNAbs-conferred protection

The project aims to assess the efficacy of the anti-V3 10-1074 bNAb, which was selected through the *in vitro* screening of a panel of 11 bNAbs (see **Chapter 4.2**, second paper), to prevent SHIV_{162P3} CAV transmission in cynomolgus macaques. To achieve this purpose, the first step to accomplish is to validate the infectiousness of SHIV_{162P3} infected splenocytes following vaginal inoculation of animals.

*a) Assessment of the *in vivo* infectivity of SHIV_{162P3} infected spleen cells following vaginal challenge of cynomolgus macaques*

Our group has previously reported that SIVmac251 infected spleen cells efficiently transmit infection after vaginal exposure of cynomolgus macaques. The dose needed to infect 50% of the animals (AID50) was $6.69 \times 10^5 \pm 2.08 \times 10^5$ viral DNA copies [71].

The assessment of anti-HIV-1 bNAb-mediated protection from infection following exposure to infected cells requires viruses expressing the HIV-1 envelope, such as SHIV. Thus, we investigated whether systemic infection could be initiated by inoculating female macaques intravaginally with SHIV_{162P3} infected spleen cells. As source of infected cells, we used the same stock of splenocytes from animal AX450 used in the *in vitro* study of bNAb inhibition of CAV transmission (see **Chapter 4.2**, second article). This challenge stock was generated by harvesting the spleen of a macaque (AX450) intravenously infected with 5000 AID50 of SHIV_{162P3} virus. At day 10 post-infection, when plasma viral RNA load was 1.2×10^7 copies per mL (**Figure 14**) the animal was euthanized and splenocytes were cryopreserved. Spleen cells were phenotypically characterized (**Figure 15**) and the SIV DNA was quantified in spleen cells (4×10^5 copies/million of cells), PBMCs (3×10^5 copies/million of cells) and peripheral LNs (1×10^6 copies/million of cells).

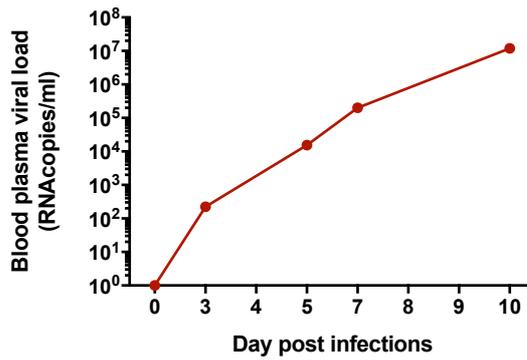


Figure 14: Dynamic of blood plasma viral load of macaque AX450 till day 10 post-infection (corresponding to the time of euthanasia).

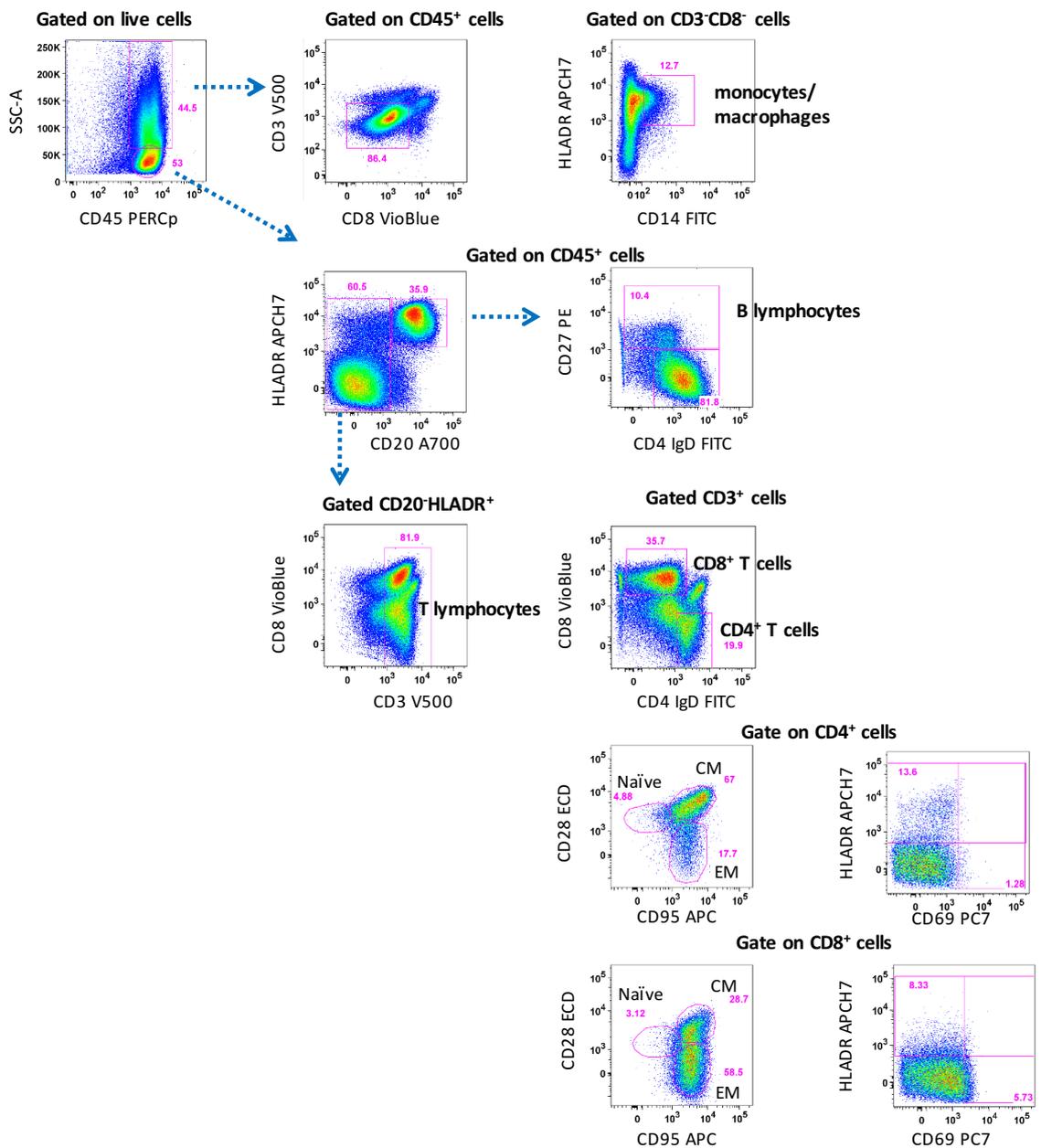


Figure 15: Phenotypic characterization of splenocytes collected from macaque AX450.

Proportions of macrophages and lymphocytes were determined from the live, CD45⁺ cells. CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined for the lymphocyte gate. Naive, central memory, and effector memory T cells were defined as CD28⁺CD95⁻, CD28⁺CD95⁺, and CD28⁻CD95⁺, respectively.

Next, in order to establish the infectiousness of the CAV challenge stock, 10 x 10⁶ live splenocytes, corresponding to a dose of 4X10⁶ viral DNA copies, were thawed, washed twice and resuspended in medium to be administered to two animals (CE468 and CE470; age 2.8 years). Animals were treated with 30 mg/Kg of medroxyprogesterone acetate (Depo-Promone[®]) one month prior the first challenge in order to thin the vagina epithelium and facilitate transmission. Splenocytes administration was continued once a week until detectable viral load was measured. **Table 3** reports the MHC haplotypes of the “donor” (AX450) and the “receivers” (CE468 and CE470) macaques. Haplotypes known to be associated to protection from infection [390,399,400] were avoided.

Table 3: MHC haplotype of the macaques used in the study.

	Macaque	MHC Haplotype
Donor	AX450	H2/H2
Receiver	CE468	H6/rech4H3
	CE470	H3/H2

Both CE470 and CE468 macaques become infected following one and seven inoculations of cells, respectively (**Figure 16**). Indeed, CE470 displayed detectable blood viral RNA load (166.89 copies/mL) one week after the 1st splenocytes inoculation followed by a peak of infection (3.45E+06 copies/mL) at day 14 and then a decrease of viremia. CE468 displayed instead a delayed infection, blood viremia was detectable after the 6th challenge (1.65E+04 RNA copies/mL) and the peak of

infection ($1.0E+06$ RNA copies/mL) was measured one week later. Viral load follow-up was discontinued at day 91 after the first challenge.

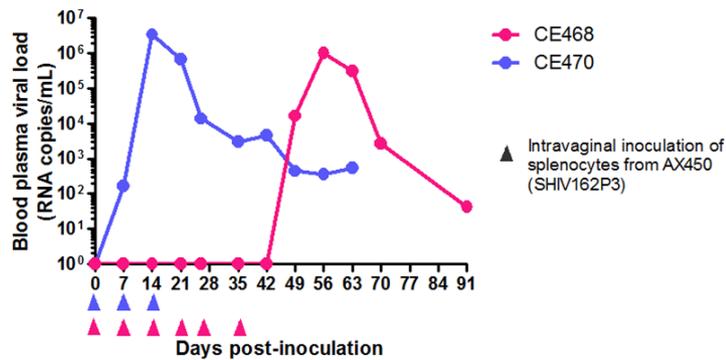


Figure 16: Blood plasma viral load of macaques CE468 and CE470 following intravaginal exposure to SHIV_{162P3} infected splenocytes.

Thus, we provided the proof that systemic infection of macaques can be induced following mucosal vaginal challenge of SHIV-infected cells. However, as most of the stock of AX450 cells was used to perform the *in vitro* study described in **chapter 4.2**, we were in the need to produce a new stock(s) of splenocytes for the assessment of bNAbs-mediated protection of macaques from CAV transmission.

b) Validation of the infectivity of the newly produced stock(s) of SHIV_{162P3} infected splenocytes: in vitro and in vivo studies

i) Constitution of the stocks of cells and determination of their infectivity in vitro

The results of the *in vivo* challenge study described in the previous paragraph shows that a viral inoculum corresponding to 4×10^5 DNA copies/million of cells could transmit infection to female macaques. To reproduce these conditions, we aimed to produce a new stock of splenocytes with a viral DNA content of at least 1×10^5 copies/million of spleen cells. To meet this goal, seven male cynomolgus macaques were infected with 5000 AID50 SHIV_{162P3} virus through the intravenous route. Viremia was measured every 3 days and the animals were euthanized at a time point expected to correspond to the peak of blood viremia, i.e. between day 10 and day 13. More precisely, three animals were sacrificed at day 10 post-infection (p.i.), one at day 11, one at day 12 and two at day 13 p.i. (**Figure 17**).

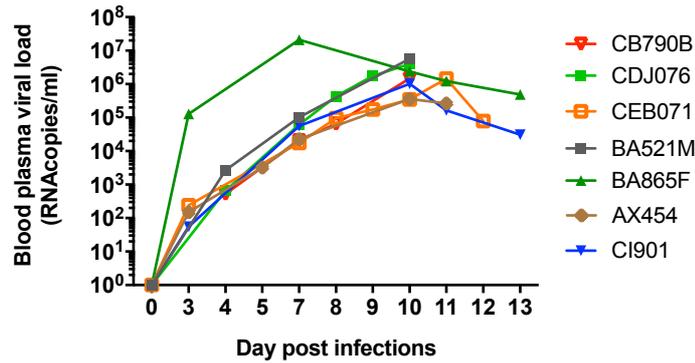


Figure 17: Kinetics of blood plasma viral load of seven macaque intravenously infected with 5000 AID50 SHIV_{162P3} virus.

The macaques BA865F was the only one that reached a viremia level comparable to macaque AX450 (2.1E+07 and 1.2E+07 copies/mL, respectively). However, the peak of viral load was detected unusually early, at day 7 p.i. and we “missed” it because the animal was euthanized at day 13 p.i., when the viral load was already declined. Two out of seven macaques (CEB071 and CI901) exhibited the lowest viral load, corresponding to 7.91x10⁴ and 3.1x10⁴ viral RNA copies/mL, respectively. The remaining macaques had a blood viral RNA content of 5.72x10⁶, 4.0x10⁶, 1.48x10⁶, 4.93x10⁵, 2.65x10⁵ and for BA521M, CDJ067, CB790B, BA865F and AX454 respectively. Analogously to the previous study, at euthanasia we collected the spleen and cells were frozen in FCS supplemented with 10% DMSO. Viral DNA content of the different stock of spleen cells is reported in **Table 4**. Despite our effort, all the stock of splenocytes had SIV DNA contents below 1X10⁵ copies/million of cells. The highest viral DNA was found in 3 stocks (CB790B, CDJ067 and CEB071) with values of 8.30X10⁴, 4.74X10⁴ and 1.15X10⁴ copies/million of cells for macaque CB790B, CDJ067 and CEB071, respectively.

Table 4: SIV DNA contents of SHIV_{162P3}-infected spleen cells from seven macaques.

Macaque	Splenocytes SIV DNA
code	(copies/10⁶ cells)
CB790B	8.30X10 ⁴
CDJ067	4.74X10 ⁴
CEB071	1.15X10 ⁴
BA521M	8.5X10 ³
BA865F	3.80X10 ³
AX454	3.29X10 ³
CI901	7.91X10 ²

Next, we tested the infectivity of each stock of spleen cells using the *in vitro* cell-to-cell transmission assays we developed, which make use of either TZM-bl or PBMC as target cells. Methodological details of the assays have been described in the **Chapter 4.2**, second paper of this thesis. Briefly, serial dilutions of splenocytes, ranging between 100,000 to 200,000 cells for the TZM-bl assay and between 40,000 to 160,000 cells for the PBMC assay, were used. Splenocytes were incubated with a fix amount of 10,000 TZM-bl or 120,000 PBMCs (activated with PHA and IL-2). The read-out of the infection was the luciferase production after 48h for the TZM-bl assay and viral load quantification by RT-qPCR after 7 days of culture for the PBMC assay. In general, the PBMC assay revealed to be more sensitive to detect cell-to-cell transmission compared to the TZM-bl assay (**Figure 18**). In agreement with the SIV DNA content of the cells, spleen cells from macaques CB790B, CDJ067 and CEB071 were the most efficient to transmit infection *in vitro* to both TZM-bl and PBMC target cells. Thus, these three stocks of splenocytes were selected as CAV challenge stocks.

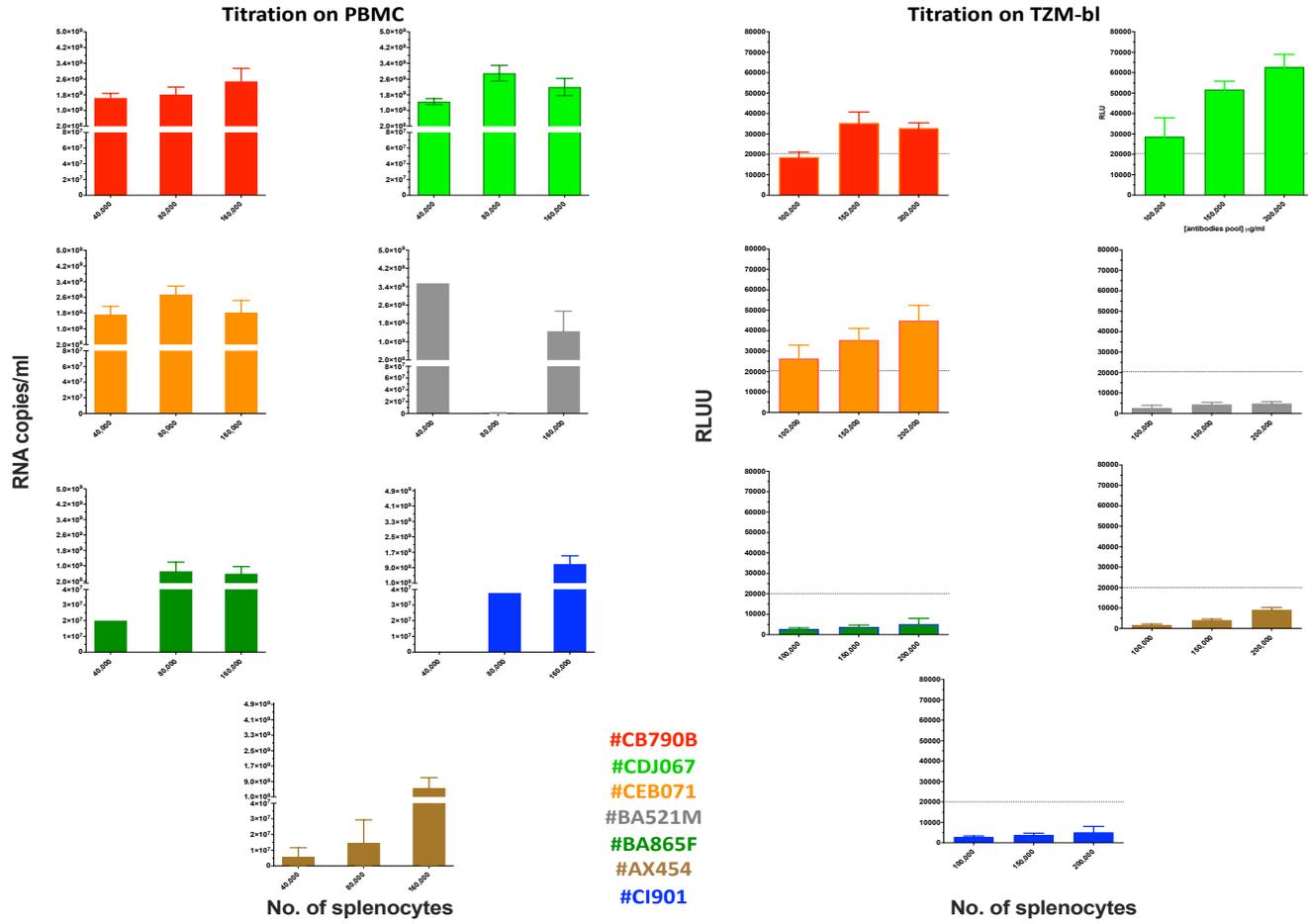


Figure 18: Splenocytes titration on PBMC (left panel) and on TZM-bl (right panel).

The dotted line represents the cut-off of infection.

ii) Phenotypic characterization of CAV challenge stocks of SHIV_{162P3}

infected splenocytes

We analyzed the proportion of different cell types on splenocytes from macaques CB790B and CDJ067. Phenotype characterization was performed using cells left over of the time of the *in vivo* challenge described in section iv) of this chapter. No left over of cells remained from macaque CEB071, thus they were not analyzed. Two-different antibody panels were used: panel 1, targeting lymphocyte populations and T cell activation markers and panel 2, targeting NK cells, monocytes and neutrophils. The list of the antibodies used is provided in **Table 5**.

Table 5: List of the antibodies used for splenocyte characterization.

Panel 1			Panel 2		
Antibody	Fluorochrome	Clone	Antibody	Fluorochrome	Clone
CD45	PerCp	D058-1283	CD45	PerCp	D058-1283
CD3	V500	SP34-2	CD3	V500	SP34-2
CD4	V450	L200	CD8	BV650	RPA-T8
CD8	BV650	RPA-T8	CD20	BV711	2H7
CD20	PE-CF594	2H7	CD16	PE-CF594	3G8
CD27	PE	MT-271	NKG2A	PE	Z199
CD45RA	PC7	L48	HLA-DR	APC-H7	G46-6
HLA-DR	APC-H7	G46-6	CD11c	APC	S-HCL-3
CD69	Alexa-700	FN50	CD14	Alexa-700	M5E2
CD21	BV711	B-Ly4	CD69	V450	FN50
CD28	FITC	CD28,2	CD66	FITC	TET2
CD95	APC	DX2	CD123	PC7	7G3
			NKp44	Pure	2.29

Similar proportion of cell types were observed in the two stock of splenocytes (**Table 6** and **Figure 19-20**). The major cells were T lymphocytes (32.38% and 27.58% for macaque CDJ067 and CB790B respectively) and B lymphocytes (35.48% and 32.7% for macaque CDJ067 and CB790B respectively). Regarding T lymphocyte subsets, we observed the highest proportion of central memory T cells for macaque CDJ067, whereas for macaque CB790B CD4⁺ T cells mostly had an effector memory phenotype and CD8⁺ T cells were mostly naïve cells. Neutrophil (5.12% and 6.66% for macaque CDJ067 and CB790B respectively) and NK cells (5.24% and 4.53% for macaque CDJ067 and CB790B respectively) were also present. Very small proportion

of macrophages (0.0024 % and 0.053% for macaque CDJ067 and CB790B respectively), of mDC (0.174% and 0.14% for macaque CDJ067 and CB790B respectively) and of DC (0.19% and 0.069% for macaque CDJ067 and CB790B respectively) were observed.

In the future we plan to determine the SIV DNA content of each cellular population to define the main source of CAV.

Table 6: Proportion of leukocytes presence in splenocytes from macaque CDJ067 and CB790B.

Percentage, among CD45⁺ cells, of CD4⁺ and CD8⁺ T lymphocyte subsets, B cells, NK cells, monocytes/macrophages, dendritic cells and neutrophils of the stock of spleen cells from macaques CDJ067 and CB709B.

	Macaque CDJ067	Macaque CB790B
Total T lymphocytes	32.38 %	27.58 %
CD4⁺ T cells	7.17 %	8.04 %
- Central memory	50.9%	13.2%
- Effector memory	10.6%	68.6%
- Naïve	35.6%	14.2%
CD8⁺ T cells	4.75 %	8.35 %
- Central memory	46.5%	11.9%
- Effector memory	11.1%	30.1%
- Naïve	39.4%	50.2%
B cells	35.48 %	32.7 %
NK cells	5.24 %	4.53 %
Monocytes/Macrophages	0.025 %	0.05 %
mDC (CD11c⁺/CD123⁻)	0.14 %	0.14 %
pDC (CD11c⁻/CD123⁺)	0.19 %	0.07 %
Neutrophils	5.12 %	6.66 %

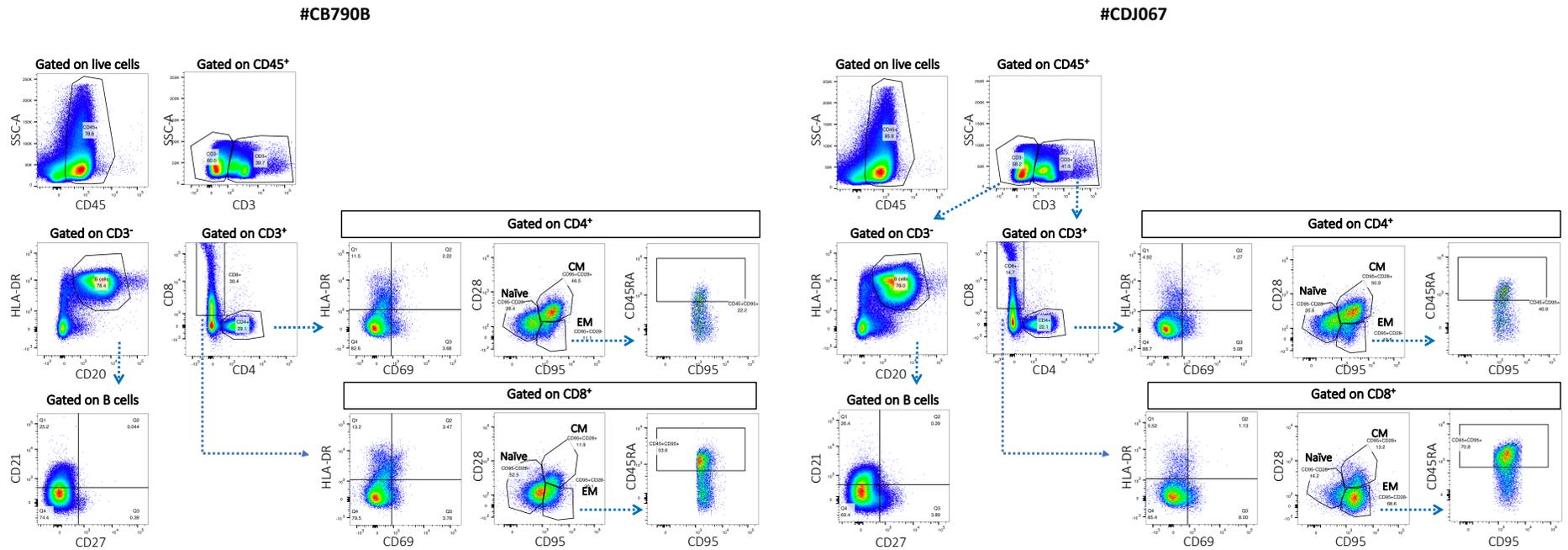


Figure 19: Gating strategy used to phenotype T and B lymphocytes among SHIV_{162P3} infected splenocytes from macaque CB790B (left panel) and CDJ067 macaque (right panel).

Proportions of lymphocytes were determined from the live, CD45⁺ cells. CD3⁻ HLA-DR⁺CD20⁺ cells were categorized as B lymphocytes. CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined for T lymphocyte gate. Naive, central memory, and effector memory T cells were defined as CD28⁺CD95⁻, CD28⁺CD95⁺, and CD28⁻CD95⁺, respectively. Effector memory T cells were further characterized for CD45RA expression.

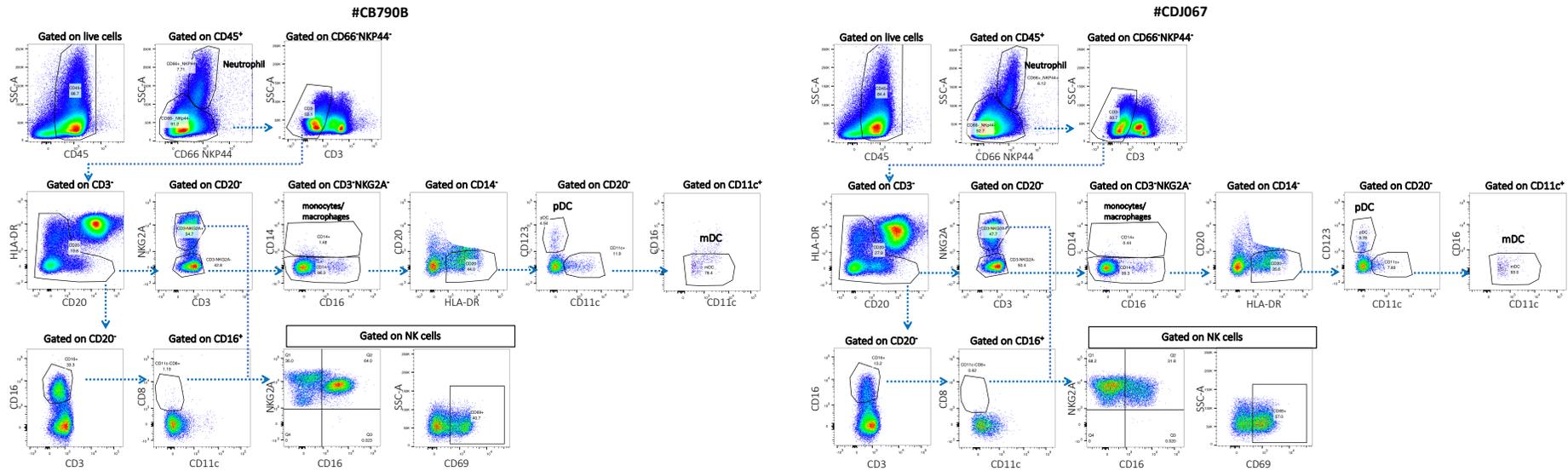


Figure 20: Gating strategy used to phenotype NK cells, monocytes, macrophages, DCs and neutrophils among SHIV_{162P3} infected splenocytes from macaque CB790B (left panel) and CDJ067 macaque (right panel).

Proportions of leukocytes were determined from the live, CD45⁺ cells. CD66⁺NKP44⁺ cells were categorized as neutrophils. CD3⁺NKG2A⁺ cells were classified as monocytes/macrophages. CD3⁺CD14⁻CD20⁻ were determined for pDC and mDC as CD11c⁻CD123⁺ and CD11c⁺CD123⁻CD16⁻, respectively. CD3⁺NKG2A⁺ or CD3⁺CD20⁻CD16⁺CD8⁺CD11c⁻ cells were categorized as NK cells.

iii) *In vitro* neutralizing activity of the bNAbs 10-1074 against SHIV_{162P3}

CAV transmission

We next determined the ability of 10-1074 bNAbs to inhibit infection of PBMCs when splenocytes from CB790B, CDJ067 and CEB071 were used as source of virus. 40,000 spleen cells, either from one single stock or mixing the splenocytes of two stocks (at 1:1 ratio) were co-cultured with 120,000 PBMCs (activated with IL-2 and PHA) in presence/absence of the Ab. The infection and neutralizing activity were measured by RT-qPCR 7 days post-coculture.

Of note, whatever it was the stock of cells used, 10-1074 inhibited CAV transmission with similar efficacy, as evidenced by the IC values reported in **Table 7**. We expect that the *in vitro* results might be predictive of the *in vivo* potency of 10-1074.

Table 7: Inhibitory concentration (IC) 50, 75 and 90 of 10-1074 in a PBMC-based neutralization assay using three stocks of spleen cells.

*Results with AX450 splenocytes are derived from previous experiments and reported here as comparison.

Splenocytes	10-1074 bNAbs ($\mu\text{g/ml}$)		
	IC50	IC75	IC90
CB790B+CDJ067	0.42	0.54	1.03
CB790B+CEB071	< 0.062	0.3	0.43
CB790B	0.18 \pm 0.04	0.26 \pm 0.12	0.32 \pm 0.2
CDJ067	0.2	0.59	1.1
CEB071	1.07	1.33	1.52
AX450*	0.40 \pm 0.23	0.85 \pm 0.49	1.61 \pm 1.02

iv) *In vivo* challenge study

We performed a pilot study to confirm *in vivo* the infectivity of the stocks. Two juvenile female cynomolgus macaques (*Macaca fascicularis*) (animal codes; CFF007 and CFC037) with an age of 2.6 years and 2.8 years, respectively, imported from Mauritius, were involved in the study. The females were treated with 30 mg of medroxyprogesterone acetate (Depo-Promone[®]) one month prior the first challenge.

The *in vivo* challenge study described in **section 4.3.1 a** showed that the animal with an MHC haplotype that did not match the one of the donor cells, get infected after one inoculation of cells. On the contrary the second macaque, who get infected after seven inoculations, had an MHC haplotype that partially matched the one of the “donor” (see **Table 3**). This observation prompted us to select animals with an MHC haplotype that did not match those of the stock of spleen cell used for the challenge (**Table 8**).

Table 8: MHC haplotype of the “donor” and the “receiver” macaques for pilot study.

	Macaques	MHC Haplotype
Donor	CEB071	recH3H1/H7
	CDJ067	recH3H5/H7
	CB790B	H3/H1
Receiver	CFF007	H6/recH4H7
	CFC037	H2/H5

We planned to challenge the two females intravaginal every week for up to 7 weeks (8 challenges maximum in total). Challenges will discontinue when the animal will have a measurable viremia (>100 viral RNA copies per milliliter of blood plasma by RT-qPCR) for two consecutive time points. During the study, blood and vaginal secretion were collected to measure viral load and anti-SHIV antibody response. The study plan is depicted in **Figure 21**.

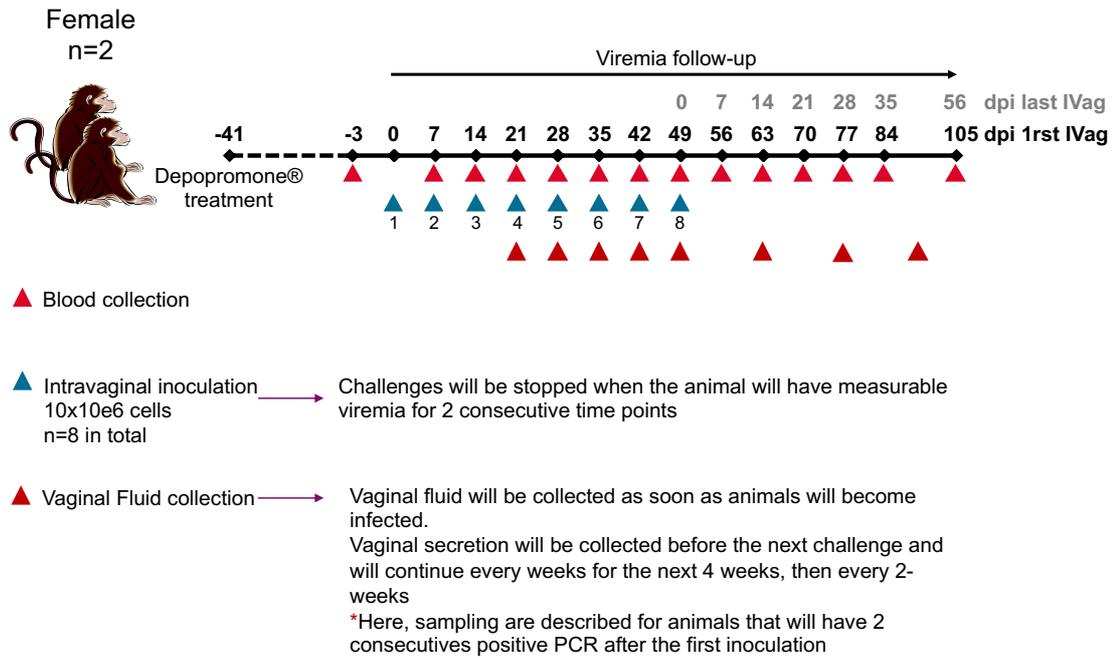


Figure 21: Study plan to assay in vivo infectivity of SHIV_{162P3} infected splenocytes after vaginal challenge of two cynomolgus macaques.

We also decided to mix, at each challenge, splenocytes from two donors at a ratio dilution 1:1 in order to induce an allogenic reaction and thus, facilitate the infection. Moreover, this choice was justified by a practical reason, as we made an estimation and the cells from one stock would not be sufficient to perform eight challenges of 12 macaques in the future study we intend to perform (6 animals in the control group and six animals treated with the antibody containing gel). Thus, receivers were challenged with 1×10^7 live splenocytes (mixed from two out of three stocks) thawed 1 hour before inoculation, washed three times and resuspended in RPMI medium. **Table 9** shows the splenocytes combination we decided to use for each challenge. The inoculation dose for each challenge was $6.16\text{E}+05$ viral DNA when combining cells from CB790B and CDJ067 and $4.73\text{E}+05$ viral DNA when combining cells from CB790B and CEB071.

Table 9: Splenocytes preparation for the intravaginal challenge.

Splenocyte preparation for each challenge (5x10 ⁶ living cells/stock)							
1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th
Week 0 pi	Week 1 pi	Week 2 pi	Week 3 pi	Week 4 pi	Week 5 pi	Week 6 pi	Week 7 pi
CEB071	-	CEB071	-	CEB071	-	CEB071	-
-	CDJ067	-	CDJ067	-	CDJ067	-	CDJ067
CB790B	CB790B	CB790B	CB790B	CB790B	CB790B	CB790B	CB790B

The results of the challenge are shown in **Figure 22**: CFC037 and CFF007 displayed detectable viremia 1 and 2 weeks after the first inoculation, respectively. CFC037 demonstrated a rapid infection with a detectable blood viral RNA load (200 copies/mL) at one week after the 1st inoculation, followed by a peak of infection at day 14 and then a decrease and a stabilization until the end of study (84 days post first inoculation). A similar kinetics was also observed in macaque CFF007 but the animal had a detectable viremia (10 copies/mL) after the second challenge and the peak of infection was reached after three challenges. Interestingly, the peak of viremia was comparable in both macaques, with values of 2.80E+06 and 2.82E+06 RNA copies/mL for CFC037 and CFF007, respectively. In summary, CFC037 received three inoculations and CFF007 received four inoculations.

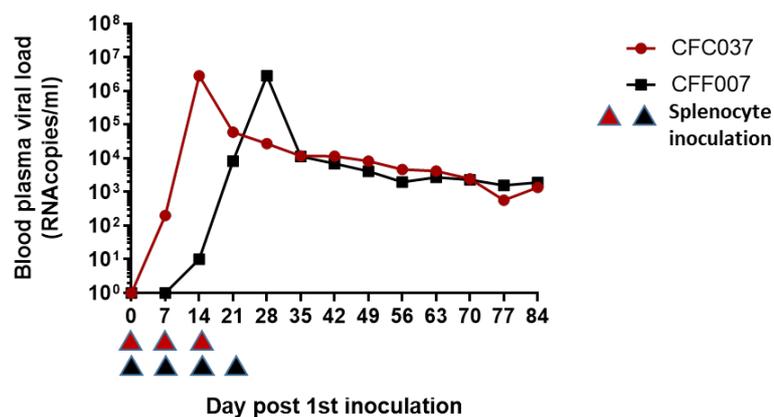


Figure 22: Blood plasma viral load of macaque CFC037 and CFF007 following intravaginal exposure with SHIV_{162P3} infected splenocytes.

Finally, to confirm that infection of macaques was initiated by infected cells and not by free virus present in the inoculum, we collected the supernatant of the

last wash of the cells stock CB790B + CEB971 and CB790B + CDJ067 before inoculation of cells to macaques and we measured the residual viral RNA by RT-qPCR. We detected residual amount of viral RNA in the last wash supernatants at an average of $2.07 \times 10^4 \pm 1.27 \times 10^4$ copies/mL for the combination CB790B + CEB971 and an average of $6.51 \times 10^4 \pm 2.16 \times 10^4$ copies/mL for the combination of CB790B + CDJ067. Comparing our data with the previous publication by Sallé *et al* (2013), the last wash supernatant of SIV infected cells of K833 stock, containing 3.51×10^4 viral RNA copies/mL, did not initiate the infection in macaques following vagina inoculation [71]. As the last wash supernatants of our study have comparable residual viral RNA content to the supernatant from the splenocytes K833, we expect that free viral particles contaminating the inoculum were not responsible of the infection of macaques we observed. We will confirm *in vivo* that the last wash supernatant is not transmitting infection.

In conclusion, we have successfully established an *in vivo* model of SHIV CAV transmission that will be further exploited to assess protection conferred by a gel containing the anti-V3 bNAb 10-1074.

Chapter 5 Validation of the experimental approach

5.1 Characteristics of macaque semen and comparison with human semen and related semen parameters

Using electroejaculation method, the volume of cynomolgus macaque semen we could collect ranked from 100 to 500 μL , which is similar to what has been described in rhesus macaques [397]. On the other hand, the volume of a human ejaculation ranked between 2 to 5 mL [11]. The pH of cynomolgus macaque semen was slightly alkaline, ranking from 7 to 9, similarly to human semen which pH rank between 7.2-7.8 [106].

The major leukocytes in human semen are polymorphonuclear cells, macrophages and T cells [72,130]. We previously showed that those populations are also found in cynomolgus macaque semen, although with differences in the relative proportions of each population [73] (**Table 10**). The cause of this discrepancy may be due to the different techniques used. Whereas the technique usually used to quantify leukocytes populations in human semen is numeration from semen cells smears, we used multi-parameters flow cytometry to categorize the semen cell population in cynomolgus macaques. Although variability between human and cynomolgus macaque semen may really exist, cynomolgus macaque semen shared many similarities with human semen. Notably, the major HIV-1/SIV target cells (CD4^+ T cells and macrophages) that are found in human semen are also present in macaque semen [73]. They remain present at all stages of infection, despite a profound depletion of CD4^+ T cells. Analogously to what reported in HIV⁺ patients, the total leukocytes count correlated with semen viral shedding [164]. Moreover, during acute infection 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. Thus, the use of semen cells could be considered as a pertinent model for studying cell-associated virus in HIV-1 mucosal transmission.

Table 10: Comparison of semen cell populations between human and macaque.

Population	Human semen ^a	Cynomolgus semen ^b
Polymorphonuclear	60-70%	26%±6.7%
Macrophages	20-30%	22.22%±5.06%
Lymphocytes	4-5%	15.95%±4.29%

a. Anderson *et al.*, 2010 and Wolff & Anderson, 1988 [72,130]; b. Bernard-Stoecklin *et al.*, 2013 [73].

5.2 Characteristics of infected splenocytes and comparison with infected semen cells

The collection of semen cells to perform *in vitro* cell-to-cell transmission assays and related experiments of bNAbs inhibition is challenging, due to the high number of cells requested. Moreover, semen should be collected from animals at the acute phase of infection, i.e. between day 10 and day 14 post-infection, otherwise the amount of integrated viral DNA is not enough to induce infection, not *in vitro* neither *in vivo* (as showed by our results). For all those reasons, the screening of bNAbs with the potential to inhibit SHIV_{162P3} cell-to-cell transmission was performed using splenocytes as surrogate of semen cells. In the majority of reported cases, infected cells in the human semen mostly consist of T lymphocytes (75% of patients) and macrophages (38% of patients) [122]. We confirmed that in macaques infected with SIVmac251 strain, T lymphocytes and, to a lesser extent, macrophages also represent the predominant infected cells in the male genital tract, which may seed the semen [153]. When comparing semen cells and splenocytes from SHIV_{162P3} infected macaques, a lower number of macrophages and B cells was found in the semen compared to spleen cells, however no major differences were observed in the lymphocyte's population (reported in **chapter 4.2**). Moreover, the infectiousness of splenocytes was demonstrated following intravaginal inoculation using either SIVmac251 infected splenocytes [71] or SHIV_{162P3} infected splenocytes (unpublished results reported in **chapter 4.3**). Taken together, we consider that infected splenocytes shares enough similarities with macaque and human semen and

represent a valuable tool for the development of CAV mucosal transmission models and related inhibition by bNAbs.

5.2 Target cells used in *in vitro* assays of cell-to-cell transmission

TZM-bl and PBMC-based neutralization assays have been widely used by the scientific community [274,401].

TZM-bl is a cell line, containing a tat-inducible luciferase reporter gene. TZM-bl based neutralization assay is a standardized, reproducible and rapid method, which is suitable for screening tests [274]. However, the cells express 100 times higher level of CCR5 coreceptor than activated or non-activated human PBMC, which is less physiological and possibly affects interaction of virus-antibody-target cell.

The PBMC-based assay is regarded as a gold standard assay as the target cell is a natural target cell of HIV [274]. The assay accesses not only the viral attachment and entry but also cell-to-cell transmission [401]. Moreover, the outcomes from the PBMC assay may be more predictive of *in vivo* results [274]. We have noticed that the assay displayed higher sensitivity than the TZM-bl assay for detection of neutralization activity to block cell-to-cell transmission. Despite of the advantages, the assay is time consuming and less consistent compared to the TZM-bl assay.

Considering the pros and cons, we have decided to use the PBMC assay as a standard neutralization assay for bNAbs evaluation of inhibition of cell-to-cell transmission. As we will further evaluate the bNAbs efficacy *in vivo* in macaques, the use of macaque PBMC rather than human PBMC would have, however, represented a more physiologically relevant system.

5.3 Implication of progesterone treatment of cynomolgus macaques to facilitate cell-to-cell transmission

In the study performed to evaluate *in vivo* cell-mediated transmission (see **chapter 4.3**), the inoculated female macaques have been treated with Medroxyprogesterone acetate (DPMA, Depo-Provera®) 1 month before the challenge. It has been reported that DPMA treatment in women and female

macaques induced a thinning of the vaginal epithelium, imitating the luteal phase of the menstrual cycle [402,403]. Certainly, this does not represent the general situation of individuals exposed to HIV-1. Moreover, progesterone implants not only increased the susceptibility to SIV infection in macaques following vaginal challenge [404], but also induces changes in mucosal immune system, particularly on NK and T cells activation [405]. The genetic diversity of the transmitter/founder virus population was higher in treated female macaques, suggesting an increase of the number of variants that crossed the epithelial barrier. Although, progesterone treatment possibly introduced a potential bias, we considered that it was not a major concern for our study. However, future work will be needed to address the influence of the menstrual cycle in transmission of infection after exposure of the cervix or vagina to cell-associated SIV.

Chapter 6 Discussion and perspectives

6.1 General discussion

6.1.1 Determination of the immunological response in semen during SIV/HIV-1 infection

a) Seminal cytokine/chemokine modulation during infection

Many studies have reported that HIV-1 infection induced a dysregulation of cytokine profiles in the periphery and in the semen [178,406–408]. To understand what the impact of SIV infection on the seminal cytokine network is, we determined the level of 29 cytokines, chemokines and growth factors in semen.

We identified 7 molecules (IL-8, IL-13, IL-15, IL-18, IFN- γ , RANTES and IP-10) that were affected by SIV infection: significantly increased in SIV⁺ animals compared to SIV⁻ animals. Among them, IL-8, IL-13, IL-15, IL-18 and RANTES positively associated with blood viral load (BVL) and seminal viral load (SVL). Additionally, IL-1 β , IL-4 and TGF β 1-3 also correlated to BVL and SVL. The correlation of SVL/BVL and seminal cytokines/chemokines is depicted in **Table 11**.

Table 11: Correlation between viral loads and seminal inflammatory molecules.

Colors indicate classification: **pro-inflammatory**, **chemokines**, Th-1 cytokines, **Th-2 cytokines** and **immunomodulators**.

	Correlated to BVL and/or SVL	No correlation to VL
Upregulated during acute infection	IL-8, IL-15, IL-18, RANTES, IL-13,	IFN- γ
Unchanged during acute infection	IL-1 β , IL-4, TGF β 1-3	TNF- α , IL-2, IL-5

This inflammatory status induced by SIV infection may favor mucosal dissemination, especially through cell-mediated transmission. Pro-inflammatory cytokines, like IL-8, IL-15 and IL-18, trigger infected cells activation. Consequently, the increase production of them can boost viral replication [409]. Due to the fact that

TGF- β displays strong immunosuppressive activity, the increased levels of TGF- β may modulate the innate immune response to the pathogen [110,111,129,410], which affects the host immune response. Also high levels of chemokines, like RANTES, may induce a migration of mucosal resident virus target cells towards the epithelial surface along a chemokine gradient [411]. Although, RANTES has potent anti-viral properties, notably by inhibiting by competition the fixation by viral particles to their co-receptor CCR5 [412–414], this activity may not affect cell-to-cell transmission. While IFN- γ displays inhibitory effect on HIV-1, including antiviral, anti-proliferation, immunomodulation, Th2 cytokines (IL-4, IL-5 and IL-13) exhibited both stimulatory and inhibitory effect on HIV-1 replication [409]. As many upregulated molecules have a stimulatory effect on HIV-1 replication, the inhibitory activity of IFN- γ and Th2 cytokines may be inefficient.

Many studies have used different experimental approaches to investigate cytokine productions by cells from HIV⁺ subjects or from *in vitro* HIV-1 culture [409]. According to the experimental design used, the observed cytokine effects *in vitro* may be unable to predict the effect mediated by cytokines/chemokines presence in seminal plasma *in vivo*. The modulation of cytokines levels in semen that we observed could possibly influence the recruitment and activation of viral target cells and generates an environment that may facilitate HIV-1/SIV sexual transmission. The answer of this open question remains to be explored.

b) Cellular response in semen

i) Phenotype of seminal CD8⁺ T cells

Systemic CD8⁺ T cell responses are involved in host HIV-1 immune control by controlling viral replication and delaying disease onset [415–418]. Because of the plausible capacity of CD8⁺ T cells to modulate HIV-1 infectivity, we provide here an extensive characterization of seminal CD8⁺ T cells. We studied their expression of differentiation, activation, migration and adhesion markers.

Based on our analysis, seminal CD8⁺ T cells exhibited a phenotype that is typically found in mucosal resident cells. They displayed a memory phenotype

(CD95⁺) with large proportion of cells expressing activation markers (CD69 and HLA-DR), the migration marker CXCR3 and the integrin LFA-1. Of note, the differentiation profile and expression of HLA-DR and LFA-1 was not influenced by SIV infection. On the contrary, the proportion of CD69⁺, CCR5⁺ and CXCR3⁺ cells were elevated in SIV⁺ animals. Following the infection, we observed a significantly durable increase of CD69⁺ cells, which could be a marker of the generalized immune activation induced by HIV-1/SIV infection. In HIV⁺ men, the activation of seminal T cells and a correlation between the proportion of CD69⁺ T cells and SVL has been described [419]. Although, we did not find a correlation because of the modest number of studied macaques, we observed a correlation between the percentage of CD69⁺ CD8⁺ T cells in blood and in seminal plasma. Similar observations have been done in human but regarding the proportion of CD45RA⁻CD38⁺CD8⁺ T cells [420]. Additionally, the percentage of CCR5⁺CXCR3⁺ cells significantly increased in infected macaques. These cells tend to migrate towards tissues in which inflammation occurs [421].

Our result suggests that the activation of seminal CD8⁺ T cells occurs in the male genital tract, implying T cell compartmentalization between blood and semen.

ii) SIV specific CD8⁺ T cell response

It has been reported that elite controllers and long-term non-progressor HIV-1⁺ patients displayed highly polyfunctional T cells [422,423]. Moreover, SIV infected rhesus macaques that were depleted of CD8⁺ T cells exhibited the rising of plasma viral load, indicating that CD8⁺ T cells may function in controlling viral replication [415]. According to the prospective role of CD8⁺ T cells, we evaluated SIV specific CD8⁺ T cells response in macaques during SIV infection.

We showed that 3 out of 7 macaques showed a detectable systemic and semen SIV-specific T cell response. They had a high proportion of IFN- γ /MIP-1 β /TNF responses, which were about 10-fold higher in semen than in blood. Despite the detection of SIV-specific response in semen, this activity was not correlated with control of viral shedding, either in blood or in semen, which is similar to what has been reported in chronic HIV⁺ patients [184]. The profile of HIV specific CD8⁺ T cell

response in blood of progressor is less functional compared to non-progressor [423]. It would be interesting to elucidate the profile of semen SIV-specific T cell responses in progressor and elite controller macaques. Although our results suggest that the SIV-specific T cell response did not affect viral replication, the immune activation mediated by CD8⁺ T cells probably has an influence on the mucosa of semen receiver.

Due to the limited number of studied macaques, we were unable to observe a significant correlation between specific T cell responses and local viral shedding. It would be interesting to increase the number of animals in future studies. Moreover, it remains to assess whether semen SIV-specific T cells are able to kill semen infected cells, which possibly influences semen infectivity.

c) Humoral response in semen

HIV-1 specific antibodies could prevent or partially control mucosal HIV-1/SIV entry [213,224]. Using western blots, HIV-specific antibodies were detected in semen [424]. The Ab may form an immune complex with HIV cell free virus, which was possibly an explanation of the low sexual transmission rate and semen infectivity *in vitro* [164,173]. As seminal HIV-1 specific antibody activity remains poorly described, our study explored SIV-specific Abs in terms of quantity and function.

We measured the level of SIV-specific Ab titers in blood and semen. The level of total Abs (IgG, IgM and IgA) at day 51 p.i. was lower than at chronic stage (day 148 p.i.). Moreover, Ab level at chronic phase was significant higher in blood than semen. A positive correlation between semen SIV-specific Ab titers and semen viral RNA load was observed, suggesting that the local humoral response fail to control viral shedding. We further investigated the neutralizing activity of seminal SIV-specific Abs. We observed a weak neutralizing activity in blood and semen at chronic stage of infection.

As reported by others [425], we observed a toxic effect at low seminal plasma dilution. We were unable however to purify IgGs from semen samples because of the low amount available. For the same reason, we did not analyze the presence of IgA in seminal plasma in our study. We cannot exclude the presence of anti-SIV-IgA that

mediates a protective effect (through inhibition of viral transcytosis or a neutralizing effect). However, semen has been repeatedly reported to contain mostly IgG [213–215]. Nevertheless, these aspects warrant further study.

In the RV144 vaccine trial, the presence of Abs that mediate ADCC was reported to correlate with protection [426]. Moreover, ADCC activity has been demonstrated in cervico-vaginal fluids (CVF) from HIV-1 infected women and shown to be associated with lower genital viral load [248,427]. ADCC mediated by IgA in CVF from exposed-noninfected (ESN) women and breastmilk is associated with a lower risk of sexual and mother-to-child transmission, respectively [428,429]. Ruiz *et al* [430] recently reported that the magnitude of ADCC responses was elevated within chronically HIV-1-infected individuals who did not transmit infection to their heterosexual ESN partners. Whether these responses were also elevated at the most relevant sites of heterosexual transmission, such as genitorectal mucosal sites, warrants further investigation. Additionally, studies on HIV controllers have reported a higher level of Abs with ADCC activity than in progressor patients [431]. However, little is known about ADCC activity in the MGT and Fc-mediated antibody functions have been mostly overlooked in semen. Only one study reported the presence of ADCC Abs in the semen of HIV-1-infected individuals [223]. The presence of ADCC-mediating Abs has not been previously evaluated in the semen of macaques infected with SIV.

Thus, we evaluated ADCC function of semen SIV-specific IgG by ELISA based FcγRIIIa binding assay. In semen at 51 dpi, half of macaques had Ab that were able to engage the FcγRIIIa. Their activity increased and was detected in 4 out of 6 macaques in the samples at chronic stage. We also observed a positive correlation between FcγRIIIa binding activity and the level of Ab titers in both blood and semen.

d) Potential of seminal HIV-1/SIV infected leukocytes to transmit infection

We demonstrated that seminal leukocytes collected from SHIV⁺ macaques were able to transfer infection *in vitro* to susceptible target cells (PBMC and TZM-bl). Although the purification method used for semen leukocyte sorting may not get rid of 100% spermatozoa, we consider that it may not be a major concern as

spermatozoa are not widely supported as a source of virus [11,122,149]. Our results supported a prospective role of seminal leukocytes on HIV-1/SIV CAV transmission.

As we used the totality of cells to perform the *in vitro* neutralization assay, we could not systematically determine either proviral DNA or the phenotype of seminal leukocytes. Seminal leukocytes carry HIV-1/SIV proviral DNA and have the potential to transmit infection. The prevalence of proviral DNA ranged from 21% to 65% in semen of HIV⁺ subjects [72]. Although the levels of HIV RNA and DNA were decreased following ART treatment, proviral DNA as CAV was present in semen for several months [432,433] and exhibited *in vitro* infectivity [434]. Moreover, our group reported the presence of infected lymphocytes and macrophages in the semen of SIVmac215-infected macaques during progressive infection [73]. According to these results, we could imply that the transfer of infection in our *in vitro* assay was mediated by infected seminal leukocytes. Moreover, we observed that seminal viral load possibly predicted the capability of seminal leukocytes to transmit infection *in vitro* (**Table 12**).

Table 12: Seminal plasma viral load (SVL), blood viral load (BVL) and capability of CD4⁺ semen cells from SHIV_{162P3} infected cynomolgus macaques to transfer infection *in vitro*

Macaque	Days post infection	SIV RNA copies/mL		Efficiency to transmit infection	
		SVL	BVL	TZM-bl	PBMC
BA865F	0	0	1	ND	
	11	1.34E+06	1.23E+06	Yes	ND
	13	4.14E+04	4.93E+05	No	
CI901	0	0	0	ND	
	11	1.16E+05	1.65E+05	No	ND
	13	1.08E+05	3.08E+04	No	
CB804D	0	0	1		ND
	14	2.23E+03	2.84E+06	ND	No
	28	4.15	1.22E+04		ND
CA709F	0	0	0		ND
	14	1.26E+02	3.33E+05	ND	No
MF1414	0	0	0		ND
	14	2.9E+05	1.22E+06	ND	Yes
	28	2.31E+02	7.47E+03		ND

ND means not done.

Although it remains to investigate which cell type is the main source of HIV-1/SIV CAV in semen, some laboratories started to elucidate the infectivity of semen cells. Our group demonstrated that CD4⁺ T cells and macrophages sorted from semen of cynomolgus macaques at acute and chronic phase of SIVmac251 infection could transfer infection to CEMx174 cells [73]. Macrophages and T cells purified from HIV⁺ semen could transfer infection to PBMC *in vitro* [122]. We were unable to define the population of seminal leukocytes used in *in vitro* assay; however, we may assume that both CD4⁺ T lymphocytes and/or macrophages contributed to CAV transmission

to TZM-bl and PBMC. On the base of the phenotype characterization and seminal leukocyte infectivity *in vitro*, we hypothesised that T lymphocytes and macrophages may play a role in CAV transmission during sexual intercourse as both cells endowed with adhesion and migration markers [73]. In spite of the fact that there is no report of the infectivity of human seminal DCs, our laboratory reported that DCs were also present in the semen of SIV⁺ macaques at all stage of infection [73]. According to this observation, we should not exclude a prospective role of DCs in CAV transmission.

Seminal cells derived from HIV⁺ subjects demonstrated in *ex vivo* culture that they were able to penetrate ectocervical epithelium but remained trapped in cervical mucus at endocervical surface [435]. It remains to be investigated whether similar phenomenon of trapped seminal cells could be displayed *in vivo*. As there is no empirical evidence that seminal leukocyte can transmit HIV-1 CAV across mucosal barrier *in vivo* up to date, it would be our main interest to provide evidences of semen cells crossing the mucosal barrier on the FRT or of the rectum in macaque models.

e) Plausible impact of HIV-1/SIV⁺ semen exposure on female reproductive tract

Our observations together with human studies performed by others shown that during HIV-1/SIV infection the seminal compartment is highly affected, with modifications in terms of cytokine/chemokine, antibodies and leukocytes phenotype and function. None of the immune responses we analyzed could control viral shedding. In the presence of seminal fluid, the innate antiviral activity of cervico-vaginal lavage fluids declined, possibly indicating that factors in the seminal fluid affected the FRT immune response [436]. All these observations suggest that immunological modifications of semen during SIV infection might influence the efficiency of transmission across the FRT during sexual intercourse. It remains to be deciphered whether and/or how HIV-1 transmission within the FRT could be affected by changes in the content of semen immunomodulatory factors.

Seminal plasma contained many immunological factors that could favor or inhibit HIV-1 transmission [105,437,438]. In the context of cell free virus transmission, many studies reported that factors present in the seminal plasma could

inactivate HIV-1, including anti-HIV antibodies [439,440], X4/R5 chemokines [129], SLPI, lactoferrin, and defensins [441]. Despite of these studies implying a viral inhibitory effect of seminal plasma, controversial results have been reported about the impact of seminal plasma on SIV infection. Using NHP models, some studies have shown that in low dose SIV cell-free inoculations, the presence of seminal plasma could favor mucosal transmission [187,391]. In contrast, other NHP studies reported that the presence of semen during SIV vaginal inoculation had no effect on the infection potency [442,443]. Additionally, a recent study has shown that 20 weeks repeated semen exposure of rhesus macaques determined a reduction of the transmission rate of SIVmac251 cell free infection [444]. In spite of those studies focusing on cell-free virus, the influence of seminal plasma on the infectivity of semen leukocytes should also be taken into consideration. More works need to be done to clarify the effect of seminal plasma not only on HIV-1/SIV cell free but also on HIV-1/SIV CAV transmission.

In addition, leukocytes may be affected by the presence of semen. The slightly basic pH of semen in humans and macaques should not impact the vitality of leukocytes [108] but other factors in semen possibly have a cytotoxic effect on the cells [105]. Moreover, it has been reported that seminal plasma of HIV negative donors could suppress CD4 expression by blood T cells, whereas blood T cells exposed to HIV seminal plasma increased CCR5 expression, which could favor the transmission of R5-tropic strains [209]. Furthermore, seminal leukocytes infectivity is probably raised by the strong seminal inflammation occurring during the acute phase of HIV-1/SIV infection. As we still do not have a conclusive information of the impact of seminal plasma on seminal leukocytes, more work needs to be done to investigate the activity of seminal plasma on virus-infected cells in semen and on CAV HIV-1/ SIV infectivity *in vivo*.

In vitro and *in vivo* studies demonstrated that semen was able to shape the composition of immune cell populations within the FRT [445]. Whether similar effects could be observed in HIV-1/SIV sexual transmission need to be further evaluated. If seminal plasma affects semen infectivity, this could be an issue to

consider when adding seminal plasma to infected cells in *in vivo* models of male-to-female transmission.

6.1.2 Potential of bNAbs to prevent cell-to-cell heterosexual transmission

bNAbs gained interest as a promising tool for HIV-1 treatment as being able to potentially prevent cell-free infection in *in vivo* models [314–319]. In spite of the achievement to control cell-free infection, bNAbs in the context of CAV transmission were poorly investigated.

Our *in vitro* results shown that a subset of bNAbs could efficiently prevent cell-to-cell transmission of SHIV_{162P3}, which is a “neutralization-resistant”, tier 2 virus. Similarly to what has been reported by others, we observed that bNAbs could inhibit cell-free infection easier than cell-associated transmission [337–339,344]. While the improvement of protection against cell-to-cell transmission by 1st generation bNAbs (2F5, 4E10 and 2G12) was clear when the bNAbs were used in combination, this phenomenon was not so clearly observed when we used 2nd generation bNAbs. This additive potency may be explained by the fact that combination of bNAbs targeting different epitopes of the virus could possibly inhibit the contact between the infected cells and susceptible cells better than the individual antibody did. Indeed, it was described that stabilization of the blocking by Abs needed many molecules of Abs to prevent a contact between the virus and the target cells [446]. Moreover, bNAbs targeting distinct epitopes displayed an independent binding [304,447], implying that possibly no cross-competition between those bNAbs occurred [448]. This may explain why we did not observe an antagonized effect when combining 2nd generation bNAbs.

Despite of the non-additive effect observed when combination of 2nd generation bNAbs were used (10-1074, PGT128, PGT151 and N6), the combination of bNAbs may give more protection coverage and potency against diversity of HIV-1 strains. To prove this hypothesis, we would need to prepare stocks of splenocytes from macaques infected with different SHIVs and use them as CAV source in *in vitro* and *in vivo* assays.

The *in vitro* experiments we performed allowed us to identify 10-1074 as the most potent bNAbs inhibiting cell-to-cell transmission of SHIV_{162P3}. Thus, this Ab has been selected to further evaluate its efficacy to block cell-to-cell transmission *in vivo* using SHIV_{162P3}-infected splenocytes as a viral challenge stock. 10-1074 displayed high breadth and potency against various viral strains in cell-free assays. The Ab was able to neutralize 77.7% of primary HIV-1 isolates from 179 HIV-1⁺ individuals, with a mean IC80 of 0.67 µg/mL [328]. In TZM-bl neutralization assay, the Ab could neutralize 60.5 % of 306 tested HIV-1 pseudoviruses, composing of 13 subtypes, at an average IC80 of 0.18 µg/mL and neutralize up to 88.5% of 26 clade B strains with a mean IC80 of 0.13 µg/mL. As the SHIV_{162P3} virus that we used is also a clade B strain, it is not surprising that this Ab was able to inhibit SHIV_{162P3} CAV transmission. In addition to its efficacy *in vitro*, the potency of 10-1074 against cell free viral challenge has proven in NHP studies. The Ab could protect rhesus macaques from SHIV_{AD8EO} rectal challenge [316]. When used in combination with 3BNC117 and PGT121, the Ab cocktail was able to suppress viremia to undetectable level in SHIV_{162P3} or SHIV_{AD8} chronically infected rhesus macaques [320,321]. In human clinical trials of 10-1074, the Ab displayed a favorable potency by inducing a rapid decrease of viremia in 10-1074-sensitive patients, however, the emergence of escaped virus occurred after the first weeks of infusion [328].

Interestingly, a combination of 1st generation bNAbs (2F5+2G12+4E10) and the individual Ab 10-1074 were capable to prevent cell-to-cell transmission mediated by seminal leukocytes in TZM-bl and PBMC assay respectively. Although our results need to be confirmed in a larger cohort, they proof the potential of bNAb to prevention HIV-1 sexual transmission.

6.1.3 *In vivo* inoculation of splenocytes collected from SHIV_{162P3} infected macaques

In the last part of this work, we assessed the capacity of splenocytes to transmit infection *in vivo*. The previous work of the laboratory proved CAV mucosal transmission using SIVmac251 infected splenocytes as a viral challenge stock. The numbers of DNA copies to successfully transmit infection by vaginal exposure was

estimated at $6.69 \times 10^5 \pm 2.08$ copies of SIV DNA [71]. The inoculum used in the case of SHIV-infection contained $4.73E+05$ or $6.15E+05$ copies of SIV DNA. At each experiment, 10^7 lived SHIV_{162P3}-infected splenocytes obtained from 2 different stocks were inoculated. We attained establishment of systemic infection following intravaginal inoculations in two female cynomolgus macaques, treated with progesterone 30 days before challenge. The inoculated macaques became infected 1 and 2 weeks after the first splenocytes challenge.

For the challenge stocks, we pooled the cells from two out of three different splenocyte stocks in order to assure the completion of the future challenge study using the same splenocyte stocks. Putting together the immune cells of different animals may represent a hindrance of our experiments. It may induce cellular stress, aggregation and mortality, thereby limiting the infectivity of the inoculated cells. However, when used *in vitro* the pooled splenocytes transmitted infection with an efficiency compared to splenocytes from animal AX450, possibly implying that mixing different splenocytes had no impact on their infectivity *in vivo*.

Indeed, HIV-1 mucosal transmission is a rare event [85], and when it happens, the transmitter founder virus population is very small [14,449,450]. According to this fact, the high dose inoculum used in cell-free challenge is generally high to ensure successful establishment of viral infection upon the viral challenge. In contrast, our model of CAV mucosal transmission highlighted that AID50 dose used to infect animal models by mucosal exposure do not need to be high. Our results are concordant with the fact that HIV-1 CAV transmission is more potent compared to cell-free virus transmission [5,85,86], implying that possibly lower inoculation doses could establish systemic infection. To confirm that cell free virus is less potent in transmitting infection than CAV transmission via mucosal route, it would be interesting to intravaginally inoculate female macaques with cell-free SHIV_{162P3} using similar AID50 dose as CAV challenge.

Another interesting evolution of the project might be to inoculate splenocytes in the presence of seminal plasma in order to better mimic what happen during natural transmission of the virus. Moreover, challenging the macaques with

stocks of splenocytes expressing other strains of SHIV, such as SHIV 325C [451], SHIV-327C [452] and/or SHIV encoding transmitted/founder HIV-1 envelope such as SHIV-AE6 [453], SHIV 375 mutation [454], will make our model more relevant as it would reflect the variety of HIV-1 strains found in natural infection. Finally, as HIV-1 infection via the rectal route represented the highest estimated risk of sexual transmission [7], it will be interesting to further develop models of rectal challenge using splenocytes as a CAV challenge stock and test the inhibitory efficacy of bNAbs.

6.2 Perspectives

In this study we have characterized the anti-viral immune response that take place against SIV infection in the semen compartment and provided evidences of factors that can potentially modulate semen infectivity. Moreover, we have demonstrated that infected semen cells can transmit infection *in vitro* and that *in vivo* transmission can be initiated by donor cells (splenocytes) in the context of SHIV_{162P3} transmission. Together with previous results obtained by our group [71,73], we provided strong evidences that semen leukocytes may play a role in HIV-1 sexual transmission, that seminal plasma and the inflammatory environment of the MGT during infection have an impact on transmission, and finally that bNAbs represent a valuable tool to prevent CAV transmission.

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

6.2.1 Short-term perspectives

a) 10-1074 bNAb efficacy trial against SHIV_{162P3} cell-to-cell transmission (intravaginal challenge) in cynomolgus macaques

The success of the pilot study we conducted to induce systemic infection of macaques intravaginally inoculated with SHIV_{162P3} infected splenocytes (see **chapter 4.3**) and the *in vitro* results of bNAbs-mediated protection against cell-to-cell SHIV transmission, prompt us to evaluate the ability of 10-1074 bNAb to inhibit cell-to-cell transmission *in vivo*.

Female cynomolgus macaque will be treated with progesterone at least 30 days before the challenge. The animals will be divided into 2 groups (treatment and control group), including 6 macaques per group. 10-1074 bNAb will be prepared as an HEC gel formulation to be vaginally applied at a concentration of 5 mg/g of gel. The gel will be applied one hour before challenge. We will use the same challenge strategy used in the pilot study, i.e. mixing splenocytes from macaques CDJ067, CEB071 and CB790B. During the *in vivo* study, blood and vaginal fluid will be collected from the animals during the follow up to monitor viral loads and define their immune response and neutralization activity. The pharmacokinetic study to evaluate the stability of the 10-1074-gel is currently ongoing.

b) Development of the in vivo model of rectal CAV transmission

Due to the fact that HIV-1 infection via rectal route represent the highest estimated risk of sexual transmission [7], our CAV model using splenocytes could be further implemented to study rectal transmission.

As SHIV_{162P3} infected splenocytes could transmit infection following intravaginal challenge, the same approach could be used for rectal inoculation. If we achieve to set up the CAV rectal transmission model using infected splenocytes, it will be interesting to test the efficacy of the bNAbs panel in this model.

c) Development of ex-vivo model for CAV transmission

We demonstrated here that HIV-1/SIV infection *in vitro* could be mediated by a source of CAV like splenocytes and semen leukocytes. It would be interesting to investigate the mechanisms underlying CAV transmission using *ex vivo* explant models. Those kind of models would for instance allow to investigate if the 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), or transmigration through the mucosa, could initiate and establish cell-to-cell transmission.

To achieve this purpose, we might develop an *ex vivo* model using tissue explants (from macaque colo-rectal or cervico-vaginal tissues). The source of cell-

associated virus will be SHIV_{162P3} infected cells, either splenocytes or semen leukocytes collected from cynomolgus macaque. This kind of experimental model allow also to study the interaction between bNAbs and infected cells and to determine the ability of bNAbs to prevent cell-to-cell transmission. Moreover, it allows to consider the buffering effect of seminal plasma.

d) Improvement of CAV transmission model by preparing infected splenocytes from various SHIV strains

In this study we have used SHIV_{162P3} infected splenocyte stocks. SHIV_{162P3} is a clade B strain which is an appropriate strain, as HIV-1 clade B is the major circulating strain in North America and Western Europe [274]. However, using individual viral strain did not represent the diversity of HIV-1 infection. In HIV pandemic, not only clade B virus but also clade A, C, D, CRF01_AE and CRF01_AE are responsible for the epidemic [274]. Moreover, a recent study demonstrated that single bNAbs decreased their protection activity when double strains of SHIV_{162P3} and SHIV-325c virus were present in the viral inoculum in rhesus macaques [455]. According to this, we need to improve the infected cell stocks to better represent the diversity of natural infection.

To reach this goal, we need to produce stock of splenocytes collected from cynomolgus macaques infected with other strains of SHIV, such as SHIV-325C [451], SHIV-327C [452] and/or SHIV encoding transmitted/founder HIV-1 envelope such as SHIV-AE6 [453], SHIV 375 mutation [454]. This will allow us to assess the coverage potency of the bNAb panel. Furthermore, this type of CAV transmission model will closely mimic the diversity of cell-to-cell HIV transmission and allow us to further investigate the underlying mechanisms.

6.2.2 Long-term perspectives

a) Deciphering mucosal CAV transmission mechanisms: in vivo inoculation of sorted semen leukocytes

We have evidences that SHIV infected splenocytes were able to initiate infection in macaques after vaginal exposure. However, semen cell composition is

different compared to the inoculum of splenocytes we used, among which a majority of lymphocytes and very few macrophages are found. A more relevant model would assess the capacity of semen leukocytes to transmit infection *in vivo*. Moreover, if we could confirm that semen leukocytes are able to transmit infection *in vivo* after mucosal exposure, it would be interesting to assess the migratory and dissemination properties of the cells and to determine which type of cells are able to transmit infection: CD4⁺ T cells and/or macrophages. To investigate the migration and dissemination of seminal leukocytes, we could label the cells with radioactive elements (¹⁸F or ¹¹¹In for example) and track them *in vivo* by imaging using Positron Emission Tomography – PET, that has been established in our laboratory. This technique will allow us to investigate the dynamics of the potential migration of the cells after the challenge. Similar experiments might be performed in presence of NAb.

However, the major constraints will be the number of seminal leukocytes collected and the variable semen infectivity state, which would greatly affect viral replication and transmission. Indeed, previous attempts performed in the laboratory up to now using both the intra-rectal and intra—vaginal transmission route were not successful.

b) Impact of the vaginal dysbiosis on the protective efficacy of a bNAb-containing gel

PrEP is currently considered as a promising preventive strategy against HIV-1 [456]. ARTs including tenofovir and dapivirine as topical PrEP could prevent HIV-1 infection in clinical trials [457–459]. In our future study, the 10-1074 antibody we'll be used as a gel to prevent vaginal mucosal CAV transmission by SHIV_{162P3} infected splenocytes. It has been reported that vaginal dysbiosis could affect the efficacy of topical tenofovir [460,461]. Whether this modification could also affect the protective effect of a topical 10-1074 gel, needs to be investigated.

Although the cause of genital inflammation remains unknown, genital inflammation is associated with vaginal microbiome modification and/or bacterial vaginosis (BV) [462,463]. BV affected the health of the FRT and increased the risk of

HIV-1 infection in women [464]. In our unit the composition of the cynomolgus macaques vaginal microbiota has been characterized [465]. Compared to human, the vaginal microbiome in cynomolgus macaques is similar to the community state type IV-A that usually is associated with dysbiosis [465]. In women, the most common bacteria genus is *Lactobacillus* spp. [466–469], which plays a role in maintaining the protective environment of the FRT against invading pathogens by producing lactic acid and bacteriocins [470,471]. Due to the discrepant composition between the vaginal microbiota in human and macaques, our laboratory is currently trying to induce a healthy vaginal flora in macaques by vaginal inoculation with lactobacillus. If we'll be successfully established this model, it will represent a tool to investigate the effect of a healthy and dysbiosis microbiota on the efficacy of the bNAb-containing gel.

Synthèse de thèse

Avec le nombre croissant de preuves empiriques et d'intérêt sur la transmission du virus intercellulaire (TVI) médiée par le sperme chez des sujets séropositifs pour le VIH, il est important de mieux comprendre la modulation dynamique du sperme pendant l'infection par le VIH-1 et de trouver les stratégies optimales pour empêcher la dissémination virale. Dans ce projet de thèse, nous avons utilisé des tests *in vitro* et le modèle de primates non humains d'infection de cynomolgus macaques pour répondre à des questions spécifiques. Plus précisément, les objectifs du projet sont d'étudier les réponses immunitaires dans le sperme pendant l'infection par le virus de l'immunodéficience simienne (VIS) et d'évaluer la capacité des anticorps neutralisants à large spectre (bNAbs) pour bloquer la TVI. Les deux objectifs sont divisés en 2 études.

Dans la première étude, nous avons étudié la réponse immunitaire innée et adaptative anti-VIS dans le sperme de cynomolgus macaque pendant l'infection par VISmac251. Nous avons mené une étude approfondie sur un total de 53 animaux. Nous avons analysé l'excrétion virale et les profils cytokiniques et chimiokines du liquide séminal. De plus, nous avons caractérisé le phénotype des lymphocytes T CD8⁺ du sperme et la réponse des cellules CD8⁺ T spécifiques au VIS en utilisant la stimulation *ex vivo* des cellules du sperme avec des peptides gag du VIS et le marquage intracellulaire avec des anticorps ciblant CD45RA, IL-2, IFN- γ , TNF et MIP-1 β . Pour déterminer la réponse en anticorps, nous avons quantifié les titres d'anticorps spécifiques au VIS dans le sperme de macaques à différents stades de l'infection. Nous avons également effectué un test de neutralisation *in vitro* et un test de liaison au Fc γ RIIIa, indicateur de la fonction ADCC de l'anticorps afin d'évaluer la fonction potentielle de l'anticorps spécifique du VIS.

Dans le sperme de macaque, nous avons observé que le niveau de nombreuses cytokines étaient affectées par une infection par le VIS. Plusieurs cytokines pro-inflammatoires (IL-8, IL-15, IL-18, IFN- γ , IP-10 et RANTES) et cell immunomodulatrice IL-13 se sont révélés significativement augmentés pendant l'infection primaire par rapport aux macaques non infectés et plusieurs d'entre eux

corrèlent avec la charge virale séminale et/ou sanguine. Cet environnement inflammatoire pourrait recruter des cellules cibles et moduler les cellules immunitaires. En effet, nous avons montré un niveau élevé de lymphocytes T CD8⁺ CD69⁺ activées préexistantes dans le tractus génital masculin, et qui est encore plus élevé lors d'une infection par le VIS. En parallèle, nous avons observé une régulation positive des lymphocytes T CD8⁺ CCR5⁺ CXCR3⁺, typique d'un phénotype Tc1, ce qui est en adéquation avec la capacité de ces cellules à migrer vers les tissus inflammatoires. Les réponses des lymphocytes T CD8⁺ spécifiques au VIS ou les réponses humorales ne parviennent pas à contrôler l'excrétion virale séminale. L'activité de neutralisation est généralement faible mais spécifique, comme évalué en utilisant un virus pseudotypé VSV comme contrôle. Dans 67% des macaques infectés, nous avons pu détecter des anticorps se liant au FcγRIIIa dans le sperme. Fait intéressant, l'activité de liaison au FcγRIIIa dans le sperme est positivement corrélée avec les titres anticorps spécifiques au VIS et la charge virale séminale.

Ainsi, l'incapacité à contrôler la réplication virale dans le sperme infecté par le VIS est associée à une inflammation générale et une activation immunitaire, qui pourrait refléter ce qui se passe dans le tractus génital masculin et qui conduirait à une transmission accrue du VIH-1/VIS.

Dans l'ensemble, notre étude met en évidence l'interaction complexe entre l'activation immunitaire et l'inflammation dans la modulation de l'infectiosité du sperme et du risque de transmission du VIH-1/ VIS. Ces observations sont pertinentes pour une meilleure compréhension de l'immunité naturelle au VIH-1 et des mécanismes qui régulent l'immunité dans le tractus génital masculin. Ces résultats peuvent contribuer à améliorer l'efficacité des mesures de prévention actuellement disponibles et conduire à l'identification de nouveaux déterminants de la transmission du VIH-1.

Dans la deuxième étude, nous avons évalué l'efficacité des bNAbs contre le virus de l'immunodéficience simienne humaine (VISH)_{162P3} TVI par rapport à une infection sans cellules. Nous avons développé des tests TVI *in vitro* en utilisant soit TZM-bl ou PBMC humain comme cellules cibles. Les cellules donneuses utilisées dans

le test étaient des leucocytes de sperme ou des splénocytes prélevés sur un cynomolgus macaque infecté par VISH_{162P3} à la phase aiguë de l'infection. Le panel d'bNAbs, y compris les anticorps de 1^{re} génération et de 2^e génération, a été sélectionné pour cibler différents épitopes de la boucle enveloppe VIH: V1/V2, boucle V3, site de liaison CD4, MPER de gp41 et région de pontage de gp120-gp41. La capacité des bNAbs à protéger du VISH_{162P3} TVI par les splénocytes a été testée *in vitro* dans un essai TZM-bl et un essai PBMC. Pour confirmer les données, l'activité neutralisante a été évaluée en utilisant des leucocytes de sperme CD45⁺.

L'inhibition de l'infection acellulaire a été obtenue avec 1 log de concentration inhibitrice (CI) 50 inférieure à celle du TVI, quel que soit le bNAbs utilisé. Les deux types de cellules donneuses du test ont montré leur infectiosité qui pourrait être inhibée par les candidats bNAbs. Une combinaison d'bNAbs de 1^{ère} génération 2F5+2G12+4E10 a inhibé la TVI avec une efficacité égale lorsque des leucocytes de sperme et des splénocytes ont été utilisés comme cellules donneuses dans un essai TZM-bl. La protection était fortement réduite lorsque les mêmes bNAbs étaient utilisés en double combinaison ou individuellement. Fait intéressant, les bNAbs de 2^e génération (10-1074, PGT128, PGT151 et N6), même lorsqu'ils sont utilisés individuellement, se sont révélés aussi efficaces contre la TVI que la triple combinaison des bNAbs de 1^{re} génération. De plus, le bNAbs anti-V3 10-1074 étaient capables d'inhiber la TVI médiée par les leucocytes de sperme et les splénocytes avec la plus grande efficacité.

En conclusion, nous avons démontré qu'un sous-ensemble de bNAbs pourrait prévenir efficacement VISH_{162P3} TVI *in vitro*. Sur la base de ces résultats, 10-1074 a été sélectionné pour évaluer son potentiel d'inhiber *in vivo* VISH_{162P3} TVI après provocation intravaginale de cynomolgus macaques.

Pour atteindre cet objectif, la première étape à accomplir consiste à valider l'infectiosité des splénocytes infectés par VISH_{162P3} après l'inoculation vaginale des animaux. Afin de constituer un stock de cellules spléniques infectées, sept mâles cynomolgus macaques ont été infectés par le virus VISH_{162P3} à 5000 DIA50 (Dose Infectieuse Animal à 50%) par voie intraveineuse. Les animaux ont été euthanasiés

au pic de la virémie sanguine. Parmi ces animaux, l'ADN viral le plus élevé a été trouvé chez trois macaques (CB790B, CDJ067 et CEB071) avec des valeurs de 8.30×10^4 , 4.74×10^4 et 1.15×10^4 copies/million de cellules. De plus, les splénocytes de ces macaques ont été les plus efficaces pour la transmission de l'infection *in vitro* aux cellules cibles TZM-bl et PBMC. Ainsi, ces splénocytes ont été sélectionnés pour la suite de nos études.

Nous avons réalisé une étude pilote pour confirmer *in vivo* l'infectiosité de ces splénocytes de macaques CB790B, CDJ067 et CEB071. Deux jeune femelles macaques cynomolgus (CFF007 et CFC037) ont été incluse dans cette étude. Nous avons prévu d'effectuer le challenge des animaux par voie intravaginale chaque semaine pendant 7 semaines maximum (8 infections maximum). Les stimulations cesseront lorsque l'animal montrera une virémie mesurable sur deux mesures consécutives. Les femelles ont été stimulés avec 1×10^7 de splénocytes décongelés 1 heure avant l'inoculation.

Les résultats de cette étude ont montré que CFC037 et CFF007 présentaient respectivement une virémie détectable lors de la semaine 1 et 2 après la première inoculation. Le macaque CFC037 a démontré une infection rapide avec une charge d'ARN viral sanguin détectable (200 copies/ml) une semaine après la 1ère inoculation, suivie d'un pic d'infection au 14e jour puis d'une diminution et d'une stabilisation jusqu'à la fin de l'étude (84 jours après la première inoculation). Une cinétique similaire a également été observée pour le macaque CFF007, avec une virémie détectable de 10 copies/ml après la deuxième inoculation et un pic d'infection atteint après trois stimulations. Fait intéressant, le pic de virémie était comparable chez les deux macaques, avec des valeurs de 2.80×10^6 et 2.82×10^6 copies d'ARN/ml pour CFC037 et CFF007, respectivement. Sur la base de ces résultats, nous avons mis au point un modèle *in vivo* de VISH TVI qui sera utilisé pour évaluer la protection conférée par un gel contenant un anticorps neutralisant anti-V3 10-1074.

Dans ce projet de thèse, nous avons caractérisé la réponse immunitaire antivirale qui a lieu contre l'infection VIS dans le sperme et identifiés des facteurs qui

peuvent potentiellement moduler l'infectiosité du sperme. De plus, nous avons démontré que les cellules de sperme infectées peuvent transmettre une infection *in vitro* et que la transmission *in vivo* peut être initiée par des cellules donneuses (splénocytes) dans le contexte de la transmission VISH_{162P3}. Avec les résultats précédents obtenus par notre groupe, nous avons fourni des preuves solides que les leucocytes du sperme peuvent jouer un rôle dans la transmission sexuelle du VIH-1, que le plasma séminal et l'environnement inflammatoire du tractus génital masculin pendant l'infection ont un impact sur la transmission, et enfin que les bNAbs représentent un outil précieux pour prévenir la TVI.

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Titre: Réponse immunitaire antivirale du sperme de macaque cynomolgus et inhibition de la transmission intercellulaire par des anticorps neutralisant à large spectre dans un modèle d'infection SIV/SHIV

Mots clés: VIH-1, sperme, macaque, transmission intercellulaire, bNAbs

Résumé: La transmission sexuelle du VIH-1 se fait principalement via du sperme infecté contaminé, contenant à la fois des virions libres et des leucocytes infectés. Les facteurs présents dans le liquide séminal peuvent aussi moduler l'infektivité du sperme et la réponse immunitaire de l'hôte. Ainsi, le macaque infecté par le virus de l'immunodéficience simienne (VIS) sera utilisé comme modèle expérimental afin d'étudier l'infektivité des cellules séminales, les réponses immunitaires anti-virales et évaluer l'effet inhibiteur d'anticorps neutralisant à large spectre (bNAbs) sur la transmission du virus intercellulaire (TVI). Chez les macaques infectés avec le SIVmac251, les réponses immunitaires innées et adaptatives spécifiques du VIS ont été étudiées dans le sperme, en se focalisant sur les réponses T CD8, humorale et sur l'expression des cytokines, chimiokines et facteurs de croissance. L'infection VIS induit l'expression de cytokines pro-inflammatoires et immuno-régulatrices dans le sperme concordant avec une augmentation des cellules activées T CD8⁺ CD69⁺ et T CD8⁺ CXCR3⁺ CCR5⁺. Ni les cellules T CD8⁺ spécifiques du VIS, ni la réponse humorale ne permettent le contrôle de la dissémination du virus dans le

sperme. L'absence de contrôle de la réplication virale dans le sperme infecté VIS est associée à une inflammation générale et à une activation immunitaire, pouvant refléter ce qui se produit dans le tractus reproducteur masculin, et qui pourrait mener à l'augmentation de la transmission VIH-1/VIS.

De plus, nous avons développé un modèle d'étude *in vitro* de TVI en utilisant soit la lignée cellulaire TZM-bl, soit des PBMC humains comme cellules cibles, et des splénocytes ou des leucocytes CD45⁺ du spermes infectés avec le SHIV_{163P3} comme cellules infectieuses. Ce modèle nous a permis d'évaluer l'inhibition de TVI par des bNAbs. Nous avons testé 4 bNAbs de 1^{ère} génération et 8 bNAbs de 2^{nde} génération. La combinaison de bNAbs de 1^{ère} génération (2F5+2G12+4E10) permet d'inhiber TVI, alors qu'en combinaison double ou seule, aucune inhibition de la transmission n'est observée. Cependant, les bNAbs de 2^{nde} génération peuvent, seules, induire une inhibition de TVI aussi efficacement qu'en combinaison. Ainsi, un bNAbs de 2^{nde} génération anti-V3 a été sélectionné afin de tester son effet inhibiteur de TVI dans un modèle *in vivo*.

Title: Anti-viral immune response in the semen of cynomolgus macaques and inhibition of cell to cell transmission by broadly neutralizing antibodies in an SIV/SHIV model of infection

Keywords: HIV, semen, macaque, cell-to-cell transmission, bNAbs

Abstract: HIV-1 sexual transmission occurs mostly through contaminated semen, which contains both free virions and infected leukocytes. Moreover, factors in seminal plasma (SP) can influence both semen infectivity and host's response. Therefore, we used the experimental model of Simian Immunodeficiency Virus (SIV) infection of macaques, to investigate semen cells infectivity and the antiviral immune responses and to evaluate the potency of broadly neutralizing antibodies (bNAbs) to block cell-to-cell virus transmission.

In SIVmac251 infected cynomolgus macaques, we investigated SIV-specific innate and adaptive responses in semen, including CD8⁺ T cell response, humoral response and levels of cytokines, chemokines and growth factors. SIV infection induced pro-inflammatory and immunoregulatory cytokines in semen and a concomitant upregulation of activated CD69⁺ CD8⁺ T cells and CCR5⁺ CXCR3⁺ CD8⁺ T cells. Neither SIV-specific CD8⁺ T-cell responses nor humoral responses controlled seminal viral

shedding. Failure to control viral replication in SIV-infected semen is related to a general inflammation and immune activation, which possibly mirrors what happen in the male genital tract and which could lead to enhanced HIV/SIV transmission.

Moreover, we developed cell-to-cell transmission assays, using either TZM-bl or human PBMC as target cells and SHIV_{163P3}-infected splenocytes and CD45⁺ semen leukocytes as donor cells, and evaluated bNAbs-mediated inhibition. The bNAbs panel included four 1st generation bNAbs and eight 2nd generation bNAbs. A combination of 1st generation bNAbs (2F5+2G12+4E10) was able to efficiently inhibit CAV transmission, while double combination or single bNAbs showed reduced potency. Of note, individual 2nd generation bNAbs inhibited transmission as efficiently as bNAbs combinations. An anti-V3 bNAbs has been selected to evaluate its potential to block cell-to-cell transmission *in vivo*.

