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HIV TARGET CELLS AND INNATE IMMUNE FACTORS IN THE HUMAN FEMALE GENITAL TRACT

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To my family

ABSTRACT

World-wide, human immunodeficiency virus (HIV) transmission most often takes place in the genital tract during heterosexual intercourse, but the site where HIV most commonly establishes primary infection in the female genital tract remains unknown. Several factors including viral load in the HIV-infected individual, presence of genital infections, genotype and possibly sex hormone levels influence the risk of HIV acquisition. In addition, cationic polypeptides with anti-HIV activity *in vitro* including secretory leukocyte protease inhibitor (SLPI), Elafin/Trappin-2, human neutrophil peptide (HNP) 1-3 and LL-37 may play dual roles *in vivo* with both an antiviral effect and target cell-recruiting properties.

For this thesis, we investigated the distribution of potential HIV target cells in three anatomical regions (ectocervix, endocervix and endometrium) of the female genital tract by immunostaining, computerized image analysis and confocal microscopy. Furthermore, innate immune peptides were quantified in genital secretions using ELISA and in ectocervical tissue using real-time RT-PCR and immunohistochemistry. To characterize antigen presenting cell subsets and their expression of HIV-binding C-type lectin receptors (CLRs) ectocervical tissue biopsies were collected from HIV-negative women at low-risk of HIV infection and HIV-negative female sex workers considered to be at high-risk of acquiring such infection. Distinct cell populations were identified including CD1a⁺ Langerin⁺ Langerhans cells (LCs) in the epithelium and CD11c⁺ DC-SIGN⁺ myeloid dendritic cells (mDCs), CD68⁺ DC-SIGN⁺ Mannose receptor⁺ (MR⁺) mDCs and/or macrophages as well as CD123⁺ plasmacytoid dendritic cells (pDCs) in the submucosa of the ectocervix. The high-risk women had significantly higher expression of CLRs than the low-risk subjects. To map the distribution of potential cellular targets and receptors binding to HIV in the endocervix and endometrium, tissue biopsies were collected from HIV-uninfected low-risk women undergoing hysterectomy. LCs were localized mainly in the columnar epithelium, whereas CD4⁺ CCR5⁺ T cells were present both within and adjacent to the endometrial and endocervical epithelium. CD11c⁺ DC-SIGN⁺ MR⁺ and CD68⁺ DC-SIGN⁺ MR⁺ mDCs and macrophages were confined to the submucosa of both tissue types but were localized in close proximity to the epithelial surface. To assess the expression of SLPI, Trappin-2, HNP 1-3 and LL-37, genital fluid samples and ectocervical biopsies were collected from HIV-uninfected low-risk woman and HIV-uninfected sex workers (HIV high-risk). LL-37 and Trappin-2 levels were significantly lower among low-risk women currently using combined oral contraception. Compartmentalization of the investigated factors classed HNP 1-3 as the most abundant factor in genital fluids of low-risk woman, whereas SLPI had the highest expression in ectocervical tissue. No association between tissue expression and soluble levels of the investigated factors was seen at the individual level in either HIV low-risk or high-risk women.

In summary, the spatial distribution of potential HIV target cells and innate immune factors may play an important role in HIV transmission events. Thus, a better understanding of this environment may contribute to designing HIV-inhibiting compounds.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. Taha Hirbod, TOVE KALDENSJÖ, Lucia Lopalco, Elin Klareskog, Sonia Andersson, Caterina Uberti-Foppa, Davide Ferrari, Mara Manghi, Jan Andersson, Karin Loré and Kristina Broliden.
Abundant and superficial expression of C-type lectin receptors in extocervix of women at risk of HIV infection
J Acquir Immune Defic Syndr. 2009;51:239-247
- II. TOVE KALDENSJÖ, Pernilla Petersson, Anna Tolf, Gareth Morgan, Kristina Broliden and Taha Hirbod.
Detection of intraepithelial and stromal Langerin and CCR5 positive cells in the human endometrium: potential targets for HIV infection
PLoS ONE. 2011 6(6): e21344. Epub 2011 Jun 29.
- III. Taha Hirbod, TOVE KALDENSJÖ and Kristina Broliden.
In situ distribution of HIV-binding CCR5 and C-type lectin receptors in the human endocervical mucosa
PLoS ONE. 2011 6(9): e25551. Epub 2011 Sep 30.
- IV. TOVE KALDENSJÖ, Taha Hirbod, Walter Jaoko, Joshua Kimani, Terry B Ball and Kristina Broliden.
Oral contraceptive use down-regulates LL-37 and Trappin-2 in the cervical region of the human female genital tract
In manuscript

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ART	Anti-Retroviral treatment
CCR5	Cysteine-cysteine chemokine receptor 5
CLR	C-type lectin receptor
CR	Complement receptor
CTLs	Cytotoxic T Lymphocytes
CVS	Cervicovaginal secretions
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
Env	Envelope
ELISA	Enzyme-linked immunosorbent assay
FGT	Female genital tract
HIV	Human immunodeficiency virus
HESN	HIV exposed seronegative
HNP1-3	Human neutrophil peptide 1-3
HPV	Human Papilloma virus
HR-HPV	High-risk (oncogenic) HPV
HSV-2	Herpes simplex virus type 2
ICAM-3	Intercellular adhesion molecule-3
IFN- α	Interferon-alpha
LC	Langerhans cell
LR-HPV	Low-risk HPV
mRNA	Messenger RNA
mDC	Myeloid DC
MR	Mannose receptor
NF- κ B	Nuclear factor κ B
NK cells	Natural killer cells
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RT	Reverse transcriptase
SLPI	Secretory leukocyte protease inhibitor
STI	Sexually transmitted infection
TLR	Toll-like receptor
UNAIDS	Joint United Nations programme on HIV/AIDS
WHO	World Health Organization

1 INTRODUCTION

1.1 THE HIV/AIDS EPIDEMIC

A report published in June 1981 described five previously healthy gay men that had developed *Pneumocystis pneumonia*, a disease normally associated with severe immunosuppression (MS Gottlieb, 1981). The patients also displayed other opportunistic infections and symptoms including fever and weight loss. This and other medical reports led to the definition of acquired immune deficiency syndrome (AIDS) (Gottlieb et al., 1981, Masur et al., 1981). In 1983 a virus was identified as the causative agent of AIDS and was later named human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983). Since the start of the global HIV epidemic in the 1980's, 25 million individuals have died due to the infection and it is estimated that more than 33 million people are living with HIV today (UNAIDS.org, 2010). The epidemic is thought to have peaked in 1999, but still 2.6 million individuals were infected during 2009 (UNAIDS.org, 2010). Since the introduction of effective antiretroviral treatment (ART) against HIV during 1995 and 1996 (Richman, 2001), the development of AIDS can be thwarted, but there exist no definitive cure or vaccine against HIV infection. Furthermore, ART only reaches approximately one third of all people in need of it (UNAIDS.org, 2010).

HIV can be spread by sexual contact, as blood-borne infection (contaminated blood product or shared needles) or from an infected mother to her child (during pregnancy or breastfeeding), but the major transmission route varies between different regions in the world. In Sub-Saharan Africa, the epicenter of the HIV epidemic (Haase, 2010), HIV infection is primarily transmitted via unprotected sexual intercourse and mother to child transmission (UNAIDS.org, 2010). In this region, 68% (22.5 million) of all HIV infected individuals live and the proportion of infected women is higher. Among young people aged 15-24 years women are as much as eight times more likely than men to be HIV-positive. In contrast, intravenous drug users (IVDU) and sex workers are the main affected in the epidemics of Eastern Europe and Central Asia (UNAIDS.org, 2010).

In Sweden, the incidence of new HIV infections has increased slowly over the last ten years and today 5300 individuals are living with HIV (www.smittskyddsinstitutet.se, 2011). The major group of newly infected represents individuals infected heterosexually before arrival to Sweden followed by men who have sex with men (MSM) infected within Sweden.

1.2 THE HUMAN IMMUNODEFICIENCY VIRUS

1.2.1 Origin of HIV

The most common explanation for the origin of HIV is the transfer of Simian Immunodeficiency virus (SIV) from African monkeys to humans as early as 100 years ago (Levy, 2007). There are two separate types of HIV; HIV-1 and HIV-2, which differs in their phylogenetic (evolutionary) relationship with other primate lentiviruses. HIV-1 is believed to stem from SIV from chimpanzees (SIVcpz) (Hahn et al., 2000) and HIV-2 to originate from another primate species in Africa, the sooty mangabeys (SIVsmm) (Silvestri et al., 2007). Despite the name of the virus, SIV infection in their natural hosts (i.e. chimpanzees and sooty mangabeys) does not cause clinical disease. In contrast, SIV infection in a non-natural host, for example in the rhesus macaque monkeys of Asian origin, causes an illness similar to AIDS (simian AIDS). This animal model of AIDS is now widely used for HIV research purposes for the study of various aspects of the infection. The different disease outcome in natural and non-natural SIV host may partly be explained by the lower levels of immune activation seen in the natural hosts (Silvestri et al., 2007). The earliest documented evidence of HIV-1 infection in humans came from an African seropositive sample collected in 1959 in Congo (Zhu et al., 1998), but the epidemic started spreading over the world during the 1980ies. Several factors have been proposed for this delay, including urbanization, deforestation, increased international travelling, prostitution and most likely the use of needle injecting devices (Levy, 2007).

Both HIV-1 and HIV-2 are subclassified into groups, based on phylogenetic criteria. HIV-1, the virus responsible for the global HIV epidemic, consists of group M (main), O (outlier) and N (non M, non O) (Levy, 2007). The predominant M group is further divided into 11 subtypes (or clades) that are differently distributed geographically. Furthermore, recombinant viruses often emerge in populations where multiple clades cocirculate and these so called circulating recombinant forms (CRF) represent over 20% of all new infections (Hemelaar et al., 2011). HIV-2 infection is most prevalent in West African nations, but is also reported in countries with historical and socioeconomic ties with this region. Compared with HIV-1, HIV-2 is considered to be less infectious and the progression to AIDS is slower in HIV-2 infected individuals (Campbell-Yesufu and Gandhi, 2011).

1.2.2 Viral structure

HIV is a member of the genus Lentivirus in the Retrovirus family. The HIV (onwards HIV-1 is referred to as HIV) particle is 100 nm in diameter, which is around 60 times smaller than a red blood cell. The viral genome contains 9 genes (encoding 15 proteins): the structural Env, Gag and Pol, the regulatory Rev and Tat and the accessory Vif, Vpr, Nef and Vpu. The viral envelope is of host origin, but in addition to host

cellular protein also the virally derived proteins (envelope glycoproteins) gp41 (transmembrane protein) and gp120 (outer surface protein) are embedded in the lipid bilayer (Sierra et al., 2005). The coneshaped core is composed of the gag p24 capsid protein. Inside the capsid are two identical copies of single stranded RNA molecules, the enzymes reverse transcriptase (RT), viral protease, integrase and Vif, Vpr, Vif, Vpr, Nef and Vpr. By RT the viral RNA is reverse transcribed into DNA, which subsequently is incorporated into the host cell DNA by the viral integrase. The RT enzyme is very error prone and it has been estimated that up to 10 base changes in the HIV genome can occur per replicative cycle (Levy, 2007). This in combination with high rates of viral replication is basis for the vast genetic variability seen for HIV which in turn enables the virus to evade the immune system and develop resistance to pharmacological treatments.

HIV infection of target cells requires fusion of the viral membrane and cell membrane by sequential interaction of the viral gp120 firstly with cell surface CD4 and secondly with one of two co-receptors, chemokine (CC) receptor molecules CXCR4 or CCR5. Based on co-receptor usage the HIV variants are divided into R5 (using CCR5), X4 (using CXCR4) or R5X4 (can use both) strains (Berger et al., 1998). Both R5 and X4 HIV are present in body fluids including semen, blood, cervicovaginal and rectal secretions, however R5 HIV is responsible for primary transmission events and dominate the early stages of HIV disease (Grivel et al., 2011). The mechanisms behind this selection of R5 HIV in initial transmission and infection are not entirely understood but may be partly explained by reduced glycosylation of HIV envelope protein (Derdeyn et al., 2004, Liu et al., 2008). This will be discussed more in the CLR section of this thesis. X4 viruses may evolve at later stages of the HIV infection and this is associated with a more rapid loss of CD4⁺T cells and accelerated progression to AIDS (Connor et al., 1997). Of note, also other receptors and molecules including, complement receptors (CRs) (Doepper et al., 2002), Fc receptors (FcRs)(Willey and Aasa-Chapman, 2008), Syndecan-3 (de Witte et al., 2007a) and C-type lectin receptors (CLRs) (Turville et al., 2002) have the capacity of HIV binding, as will be described later.

1.2.3 Natural course of HIV infection

HIV is primarily a mucosal infection and transmission most often takes place in the genital tract during heterosexual intercourse. Based on non-human primate studies of mucosal SIV-infection, it is believed that the virus initially establish a small founder population of infected cells in the submucosa which undergoes local expansion during the first week of infection. This expansion generates virus and infected cells which subsequently disseminate and establish infection in secondary lymphoid organs and then spreads through the blood stream. During the second week of infection a systemic infection is established with the virus replicating rapidly in the lymphoid organs (Haase, 2010). This is the basis behind the suggestion for prevention strategies during the first week of infection, which could target either the small founder population or the local expansion, before systemic infection is established.

Figure 1 illustrates the three phases of HIV infection; the acute or primary phase, the chronic phase and AIDS. Summarized, viral RNA can be detected around a week after infection and usually peak near the end of the second week. Primary infection with HIV (PHI) can be associated with clinical symptoms which are usually seen within 2-4 weeks following infection (Lindback et al., 2000). Usually over 50% of infected individuals exhibit clinical symptoms including malaise, swollen lymph glands, sore throat and fever (Tindall et al., 1988, Cooper et al., 1985). During the acute phase of HIV infection the number of CD4⁺ T cells declines throughout the body. In the gastrointestinal tract, which harbors the majority of total body T lymphocytes, a marked depletion of memory CD4⁺ T cells is seen during acute infection (Brenchley et al., 2004, Mattapallil et al., 2005). Moreover, the CD4⁺ T cells in the gastrointestinal tract may not be restored following the initiation of antiretroviral treatment (Guadalupe et al., 2006). This loss of CD4⁺ T cells, the main target cell for the virus, in combination with the appearance of HIV-specific CD8⁺ cytotoxic T lymphocytes (CTLs) and possibly HIV-specific CD4⁺ T cells and HIV neutralizing antibodies, are thought to be responsible for the decline in viral load to a viral set point (McMichael and Rowland-Jones, 2001, Mascola, 2003). During the chronic stage a steady state of viral production is present and a gradual loss of CD4⁺ T cells take place. Without antiretroviral treatment, the time from infection to AIDS development is approximately 8 years.

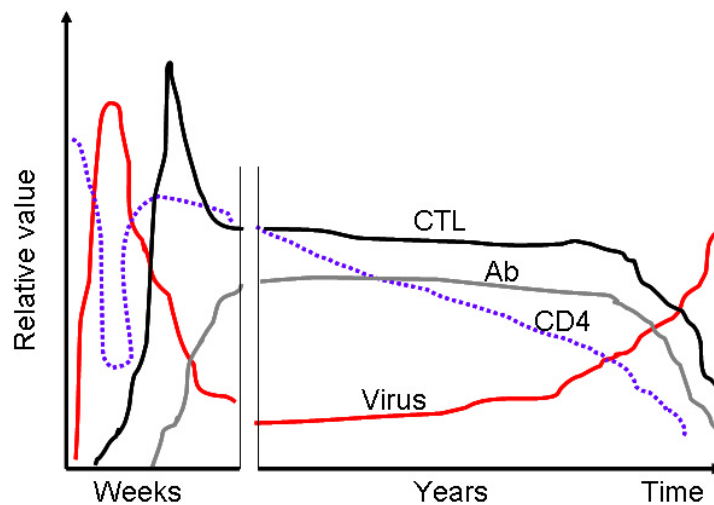


Figure 1.

Schematic illustration of the natural course of a typical HIV-1 infection showing viral load (red line), CD4⁺ T cell counts (purple dotted line), CTL response (black line) and Ab response (grey line).

1.3 THE FEMALE GENITAL TRACT

The female genital tract (FGT) consists of the ovaries, the fallopian tubes, the uterus, the cervix uteri and the vagina (Figure 2). The vagina and ectocervix are constantly exposed to the external environment and are rich in resident microbial flora, which in a healthy vaginal mucosa is dominated by lactobacilli strains. The flora contributes in lowering the vaginal pH which in turn protects against foreign pathogens (Kaushic, 2011). In comparison, the upper parts of the FGT (i.e. ovaries, fallopian tubes, uterus and endocervix) are less exposed to the external environment (Wira and Fahey, 2008).

The mucosa of the uterus is called the endometrium and it is covered with a single layer columnar epithelium (a, figure 2). The endometrium is divided into the functional (superficial) layer and the basal layer. The functional layer varies with the changes in sex hormones during the menstrual cycle and is shed during the menstrual bleeding. Below the endometrium is a layer of smooth muscle called the myometrium (Kurman, 2002). The cervix uteri is the part of the uterus that protrudes into the vagina. It is further divided into the outside portion called the ectocervix and the inside portion called the endocervix. The ectocervix (and the vagina) are lined with a non-keratinized multi-layered squamous epithelium (c, figure 2), whereas the endocervix is covered with a single layer mucin-secreting columnar epithelium (a, figure 2). The epithelium of the endocervix is richly folded to increase the surface area and these cleft-like infoldings give an impression of glands. Moreover, the columnar epithelium may protrude outside the cervical canal (onto the ectocervix), so called cervical ectopy (Jacobson et al., 2000). This condition is a result of hormonal influence and is mostly seen in teens, during pregnancy and in women using hormonal contraception. The columnar epithelium in the ectopy will gradually be replaced by squamous epithelium through a process known as metaplasia, which will take place in the transformation zone (TZ) (b, figure 2) (Jacobson et al., 2000).

1.3.1 Cervicovaginal fluid

The epithelial cells of the FGT produce a hydrophilic surface layer called the glycocalyx and thick hydrophobic mucus layer (Kaushic et al., 2010). Together with secretions from the cervical vestibular glands, plasma transudate and endometrial and oviductal fluids (Elstein, 1978, Huggins and Preti, 1981) they compose the cervicovaginal genital fluids. The character of the mucus from the cervical vestibular glands varies during the menstrual cycle between a transport medium for spermatozoa at time of ovulation to a barrier for fertilization at other times (Khalidoun Sharif, 2006). Although more penetrable at time of ovulation, the cervical mucus is never totally impenetrable. This has been shown experimentally by using radiolabeled microspheres which entered the uterus within minutes following intravaginal application both during the proliferative and secretory menstrual cycle phase. However, entry to the fallopian tubes was only observed during the proliferative phase (Zervomanolakis et al., 2007, Kunz et al., 1996). The mucus itself physically protect against invading pathogens

including HIV (Kaushic et al., 2010, Maher et al., 2005). In addition, the genital secretions contain cationic polypeptides with antimicrobial activity. These polypeptides are produced by epithelial cells and various immune cells throughout the genital tract and will be discussed more thoroughly later in this thesis.

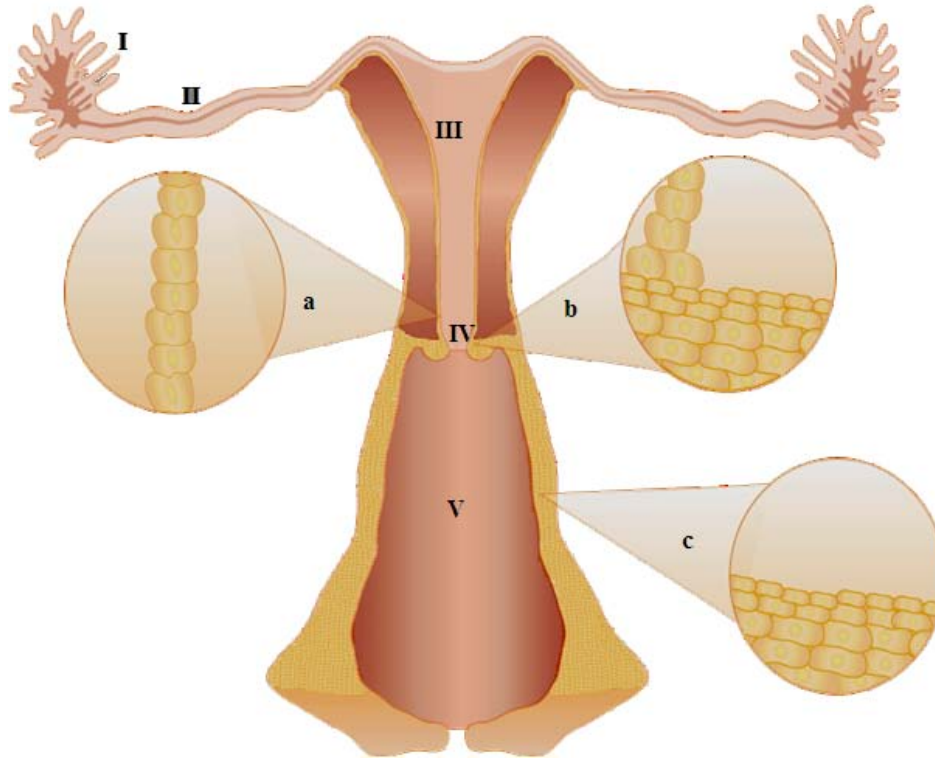


Figure 2

The female genital tract. (I) Ovaries, (II) the fallopian tubes, (III) the uterus, (IV) the cervix uteri and (V) the vagina. (a)The uterus and endocervix are lined with a single layer columnar epithelium, whereas (c) the ectocervix and vagina are covered with a multilayered squamous epithelium. (b)Transformation zone. Copyright of Sam Hirbod, illustrator.

1.4 THE MUCOSAL IMMUNE SYSTEM

The mucosal immune system in the female genital tract is uniquely adapted to the highly specialized functions of reproduction, whilst concurrently protecting against foreign pathogens. The human immune system consists of immune cells and soluble molecules such as cytokines, chemokines, proteins of the complement system, cationic polypeptides and antibodies. The immune system can be divided into the innate and adaptive arms of which the innate is believed to have developed prior to the adaptive (Janeway, 2005). The innate immune system represents the first line of defense against pathogens and provides the necessary alert signal for the adaptive immune system to the fact that an infectious pathogen has attacked the body. The innate immune system recognize non-self pathogens by their pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Geijtenbeek and Gringhuis, 2009). The innate response is rapid, whereas the adaptive response takes longer time to develop (Janeway, 2005). This is because adaptive immunity generates and clonally expands cells with specificity of foreign epitopes. Some of these cells are long-lived and provide immunological memory. Considering all components and the complexity of the immune system, all aspects will not be discussed here. Rather, the aspects relevant to this thesis will be introduced.

1.4.1 Dendritic cells

DCs consist of a heterogeneous cell population found in blood, lymphoid tissue and various tissues including the FGT mucosa. DCs can be of both myeloid (myeloid DCs, mDCs) and lymphoid lineage (plasmacytoid DCs, pDCs) (Donaghy et al., 2006, Robinson et al., 1999). Of the two, mDCs are more frequent than pDCs (Larsson, 2005). Mucosal mDCs express CD11c, an α integrin subunit which together with a β integrin subunit (CD18) form the complement receptor 4 (Bilsland et al., 1994). The mDCs are further subdivided into Langerhans cells (LCs), generally found within the epithelium, and interstitial mDCs localized in the submucosa. The pDCs are found in blood and lymphoid organs (Siegal et al., 1999, Grouard et al., 1997) as well as in other tissues (Donaghy et al., 2009, Jahnsen et al., 2000, Wollenberg et al., 2002) including the FGT mucosa (Papers I-III). The pDCs express CD123 (interleukin-3 receptor) and the C-type lectin BDCA-2 (Dzionek et al., 2001).

Three stages of maturation exist for DCs; precursors, immature and mature, and their localization and function in the body are dependent on the state of maturation (Turville et al., 2003). Immature DCs are distributed in the tissue where they act as sentinels and survey the surroundings for evidence of pathogens. Immature DCs are experts on antigen uptake and express an array of receptors on their surface for this purpose, including different C-type lectin receptors (CLRs), CRs and FcRs. The DCs are capable of antigen uptake either via receptor mediated endocytosis or through the receptor-independent pathway of macropinocytosis (Janeway, 2005). During endocytosis, the

DC engulfs the pathogen, whereas whole droplets of fluid are captured during micropinocytosis. DCs mature in response to both endogenous and exogenous stimuli and migrate to lymphoid organs (Donaghy et al., 2006). Mature DCs are highly efficient at antigen presentation and activation of T cells. During the maturation process the phenotype of the DC changes and molecules important for antigen presentation such as MHC class II, co-stimulatory and adhesion molecules are up-regulated, whereas others, including CLR and CCR5, are down-regulated.

The pDCs are functionally distinct from mDCs. The pDCs are the professional type 1 interferon producing cells but are also capable of antigen presentation (Siegal et al., 1999). The pDCs produce IFN- α in response to viruses, including HIV (Fonteneau et al., 2004).

1.4.2 The role of DCs in HIV infection

DCs have been proposed to play a number of roles in HIV pathogenesis, including infection in the genital mucosa, transport of virus to lymphoid tissue and transmission of virus to T lymphocytes (Donaghy et al., 2006). All DC subtypes express CD4 and the co-receptors CCR5 and CXCR4 to varying degrees (Larsson, 2005), in addition to other receptors capable of interaction with HIV.

1.4.2.1 HIV infection of mucosal DCs

Both intraepithelial LCs and submucosal mDCs have been proposed as targets for HIV. In the rhesus macaque animal model, SIV⁺ LCs were detected after vaginal exposure of SIV (Hu et al., 2000). In humans, vaginal LCs internalized HIV into cytoplasmic organelles but were not productively infected (Hladik et al., 2007), whereas infection of skin LCs has been observed (Kawamura et al., 2000). Mucosal LCs may play a protective role against sexual HIV acquisition as suggested by a study by de Witte et al, where it was shown that HIV uptake via the CLR Langerin in immature skin LCs directed the virus to the organelle Birbeck granules, where the virus was degraded (de Witte et al., 2007b). However, under inflammatory conditions (de Jong et al., 2008), at high viral concentrations, or in the presence of anti-Langerin antibodies the LCs could be infected. This suggests that certain conditions may be required for the protective function of LCs. Interstitial DCs in the submucosa express other CLR capable of HIV binding, which will be discussed later in this thesis. Submucosal DCs mediated viral transmission to T cells in human skin explants (Reece et al., 1998) and infected DCs were observed following vaginal exposure of SIV in rhesus macaques (Spira et al., 1996).

1.4.2.2 Trans and cis transmission to T cells

CLR mediated binding of HIV to DCs may result in either trans or cis transmission to T cells. The CLRs for which this has been described are DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2000, Burleigh et al., 2006) and DC-immunoreceptor (DCIR) (Lambert et al., 2008). The DC is not infected by HIV during trans transmission, but captures and carries the virus with a CLR and mediates local transfer of virus to T lymphocytes (Turville et al., 2003). In contrast, the DC is productively infected during cis transmission and newly synthesized virions are subsequently transferred to T cells. In cis transmission, the initial capture of HIV via a CLR facilitates entry of HIV into the cytoplasm by the conventional CD4/CCR5 fusion pathway (Cunningham et al., 2008). Of the two, trans transmission is believed to be the first to occur, since the *novo* production of virus is seen approximately 24 h after HIV binding.

1.4.2.3 Other aspects of HIV and DC interaction

Several DC subsets can be HIV infected *in vitro*, but the level of productive infection in DCs is much lower as compared to T lymphocytes (Larsson, 2005). Immature DCs are more susceptible to productive HIV infection as compared to mature DCs, which may be due to the expression level of CD4 and the co-receptors, intrinsic antiviral properties of the DC as well as metabolic activity. HIV infection and replication is enhanced when DCs are clustered with CD4⁺ T cells (Pope et al., 1994) by the formation of an infectious synapse between the cells which results in very efficient viral spread (McDonald et al., 2003). In the infectious synapse, mature DCs are more efficient of viral transfer to T cells than immature DCs. Furthermore, immature and mature DCs differ with regard to viral storage. Immature DCs store HIV in small vesicles containing few virions, whereas mature DCs have a single or a few large vesicles with high quantity of virions (Frank et al., 2002). HIV infection induces alterations in DC function. During acute HIV infection the number of DCs in lymphoid tissue increases, but their expression of the co-stimulatory molecules CD80 and CD86 are reduced (Lore et al., 2002). DC maturation is impaired and HIV-infected DCs express less MHC I and CD4 and secrete the immunosuppressive cytokine IL-10 (Larsson, 2005). In combination, these alterations may impair the DC ability to activate T cells.

1.4.3 Macrophages

Macrophages are widely distributed in the tissues of the body, where their main function is phagocytosis of pathogens and subsequent destruction in intracellular vesicles. Macrophages also have the capacity of antigen presentation to T cells. Macrophages express the conventional receptors for HIV binding and fusion (CD4, CCR5 and CXCR4) as well as CLRs, CRs and FcRs. For example, Mannose Receptor (MR), a CLR expressed on both macrophages and DCs, has been found to bind and mediate transfer of HIV to T lymphocytes (Nguyen and Hildreth, 2003). However, MR interaction with HIV did not lead to a productive infection of the macrophage; rather

the viral uptake was by phagocytosis (Trujillo et al., 2007). It is suggested that this noninfectious route of entry may contribute to CTL activation.

1.4.4 C-type lectin receptors (CLRs)

The CLRs are predominantly expressed by antigen presenting cells (APCs), such as macrophages and DCs, and recognize carbohydrates in a calcium-dependent manner via their carbohydrate recognition domain (CRD). The transmembrane CLRs are separated into type I and II; type I have several CRDs, whereas type II have a single CRD (Gijzen et al., 2006). In addition to mediating endocytic activity after recognizing glycosylated ligands on pathogens, the CLRs mediate cell-cell adhesion and clearance of apoptotic cells when binding to endogenous carbohydrates (Gijzen et al., 2006). HIV cloaks its envelope protein in N-linked glycans to reduce antibody recognition (Doores et al., 2010) and all the CLRs with HIV binding capacity bind to these high mannose structures on gp120. Four CLRs have been reported to bind HIV: MR (Larkin et al., 1989, Turville et al., 2001), DC-SIGN (Geijtenbeek et al., 2000), DCIR (Lambert et al., 2008) and Langerin (Turville et al., 2002). However, as described for Langerin and DC-SIGN, the consequences of HIV uptake via CLRs may differ between different CLRs. Furthermore, Langerin polymorphisms exist which result in different carbohydrate-binding capacities, whereas the DC-SIGN gene is highly conserved (Ward et al., 2006). The CLRs are expressed by different APC subtypes; Langerin is exclusively expressed by Langerhans cells (Valladeau et al., 2000), whereas DC-SIGN and MR are expressed by interstitial DCs and macrophages in the submucosa (de Witte et al., 2008, Geijtenbeek et al., 2000, Kamada et al., 2008). DCIR is expressed by APC, but its expression on mucosal DC subsets in vivo remains to be established (Lambert et al., 2008). The CLR BDCA-2 is expressed by pDCs, but has not been associated with binding affinity to HIV. BDCA-2 is involved in endocytic antigen uptake and subsequent presentation to T cells, but may also suppress induction of interferon α/β production (Dzionek et al., 2001).

As mentioned previously, the majority of HIV variants establishing primary infection are R5 strains, independently of the route of transmission. Furthermore, it has been observed that the transmitted R5 virus often has reduced gp120 glycosylation (Derdeyn et al., 2004, Liu et al., 2008). This would hypothetically make the virus less susceptible to recognition by CLRs, but more sensible to neutralizing antibodies. However, avoidance of DC stimulation through CLR binding may be more important to the establishment of primary infection than the capacity of avoiding neutralizing antibodies in a population never previously exposed to HIV. Another theory involves the alpha-4 beta-7 ($\alpha 4\beta 7$) gut homing integrin, which is expressed on highly susceptible CD4⁺ T cells in the mucosa (Cicala et al., 2009). It is suggested that the $\alpha 4\beta 7$ integrin binds more efficiently to viruses with reduced glycosylation (Grivel et al., 2011).

1.4.5 Toll-like receptors (TLRs)

TLRs were the first Pattern recognition receptors (PRRs) to be identified. A total of ten TLRs have been described in humans and each recognizes distinct PAMPs from viruses, bacteria, fungi and parasites. The TLRs are associated with intracellular vesicles or expressed on the cell surface of innate immune cells including DCs and macrophages (Kawai and Akira, 2011). Also FGT epithelial cells express TLRs (Kaushic et al., 2010). PAMP recognition by TLR induces the secretion of inflammatory cytokines, type I interferons, chemokines and antimicrobial peptides, often mediated by the activation of the transcription factor NF- κ B (Kawai and Akira, 2011). These responses in turn recruit neutrophils and activate macrophages. Moreover, TLR signaling leads to DC maturation with up-regulation of co-stimulatory molecules necessary for activation of the adaptive immune response (Janeway, 2005).

1.4.6 Cationic polypeptides with antimicrobial activity

Cationic polypeptides are present in genital secretions and in genital tissue, where they constitute part of the innate host defense of the mucosa. These peptides exist both in plants and animals, indicating both the importance and senescence of this part of the innate immunity. The complete repertoire of cationic polypeptides in FGT secretions is unknown, but by using a proteomic approach 20 different peptides were found in vaginal fluid (Venkataraman et al., 2005). The peptides differ with regard to cellular source and how they exert their antimicrobial activity, but also have common properties including amphipathicity (spatial separation of hydrophilic and hydrophobic residues) and cationicity (a net positive charge at physiological pH) (Cole and Cole, 2008), which are important to mediate peptide insertion into anionic microbial membranes and prevent membrane-membrane interactions.

Several of these peptides have anti-HIV activity in vitro, but their role in mucosal HIV susceptibility in vivo is more complex. Since they also exert chemotactic and immunomodulatory effects on immune cells, they may in fact increase HIV susceptibility (Borrow et al., 2010). Whether the pro-or anti-HIV activity predominates may be dependent on the cellular origin of the innate factors as well as their distribution in the tissue or in the genital secretions (Broliden, 2010). Furthermore, several factors influence the levels of the cationic polypeptides, including hormonal changes during the menstrual cycle (Wira et al., 2011), inflammatory conditions (Wiesenfeld et al., 2002, Valore et al., 2006) and genetic polymorphisms (Linzmeier and Ganz, 2005, Chowdhury et al., 2006).

1.4.6.1 Defensins

In mammals, defensins are classified into three subfamilies, the alpha-, beta- and theta-defensins (α -, β - and θ -defensins) (Cole, 2006). All classes of defensins exhibit antimicrobial activity against several bacteria, fungi and viruses including HIV in vitro.

However, the mature form of θ -defensins is not produced in humans, due to a premature termination codon which inhibits its complete translation.

The majority of the α -defensins, human neutrophil peptides (HNP) 1-3, is synthesized by neutrophil precursor cells in the bone marrow and stored in primary granules of neutrophils in their mature form. However, also other immune cells including NK cells and macrophages may produce α -defensins (Klotman and Chang, 2006). The level of HNP 1-3 expression is correlated to gene copy numbers, which can range from 5 to 14 copies per genome between individuals (Linzmeier and Ganz, 2005). HNP 1-3 may recruit potential HIV target cells since it has chemotactic effect on T cells, monocytes and DCs (Territo et al., 1989, Yang et al., 2000), but also display anti-HIV activity by a direct effect on the virion or by inhibiting HIV at the steps of nuclear import and transcription in the target cells (Chang et al., 2005). The complexity of the antiviral effects of cationic peptides in vivo is illustrated by a prospective study of HIV-exposed uninfected female sex workers (Levinson et al., 2009). Here, a significant association was found between HIV neutralizing capacity and elevated levels of HNP 1-3 and the cathelicidin LL-37 in genital secretions. However, genital infections (*Chlamydia Trachomatis* and *Neisseria Gonorrhoea*) as well as HIV acquisition also correlated significantly with higher levels of HNP 1-3 and LL-37. It may be speculated that the antiviral activity of HNP 1-3 and LL-37 was overwhelmed by their chemoattracting activities and/or excessive HIV exposure in this case.

1.4.6.2 Cathelicidins

LL-37 is the only member of cathelicidins in humans. It is formed after proteolytic cleavage of the precursor hCAP-18, and is found in specific granules of neutrophils and in epithelial cells at various mucosal sites including the female genital tract (Frohm Nilsson et al., 1999). In the FGT, the cleavage of hCAP-18 into ALL-38 (a peptide with similar anti-bacterial activity as LL-37) is mediated by the seminal protease gastricsin. This process is pH dependent, since gastricsin is functionally active in the low pH milieu of vaginal fluid but inactive at neutral or basic pH (Sorensen et al., 2003). LL-37 has a broad spectrum of antimicrobial activity including anti-HIV activity in vitro (Bergman et al., 2007). The HIV inhibitory activity is believed to be through an effect on the targets cells. Furthermore, LL-37 exerts chemotactic activity on T cells, monocytes and neutrophils (De et al., 2000, Agerberth et al., 2000) and induces the expression and extracellular release of HNP 1-3 from neutrophils (Zheng et al., 2007).

1.4.6.3 Secretory leukocyte protease inhibitor and Elafin

Other peptides with anti-HIV activity in vitro are the serine protease inhibitors; secretory leukocyte protease inhibitor (SLPI) and Elafin. They are members of the whey acidic protein family and share 40% homology. However, the gene encoding for Elafin is highly polymorphic, whereas the SLPI gene is stable (Chowdhury et al., 2006). SLPI and Elafin are produced by epithelial cells at various mucosal surfaces as well as by neutrophils and macrophages (Pfundt et al., 1996, King et al., 2003a). Elafin is the mature peptide formed after cleavage of the precursor Trappin-2. However, as no specific Elafin antibody exists, Trappin-2 and Elafin are often measured simultaneously

in laboratory settings. Furthermore, in many cases Trappin-2 displays similar capacity of antimicrobial activity and protection against proteolysis as Elafin (Moreau et al., 2008, Bourbonnais et al., 2000).

A major function of SLPI, Trappin-2 and Elafin is to protect against excessive proteolysis following neutrophil activation (Moreau et al., 2008). Moreover, SLPI, Elafin and Trappin-2 display anti-inflammatory activity for example via prevention of NF- κ B activation and suppression of LPS-induced production of cytokines (Moreau et al., 2008). In addition, they have broad spectrum anti-microbial activity and have been shown to inhibit HIV in vitro (Kazmi et al., 2006, McNeely et al., 1995, Ghosh et al., 2010). The anti-HIV activity of SLPI is mediated through binding of a co-factor (annexin II) important for stabilization of viral fusion (Ma et al., 2004), whereas Elafin is believed to interact directly with the virion (Ghosh et al., 2010). Furthermore, elevated levels of Elafin and Trappin-2, but not SLPI, have been associated with protection against mucosal HIV acquisition in an epidemiological study (Iqbal et al., 2009).

1.5 HIV TRANSMISSION FOLLOWING SEXUAL INTERCOURSE

World-wide, HIV transmission most often takes place in the genital tract during heterosexual intercourse, but HIV can also be transmitted via rectal and oral intercourse. Of the three, receptive anal intercourse carries the highest probability of infection (approximately 1:20-1:300) (Hladik and McElrath, 2008), which is attributed to the fragile single layer columnar epithelium and abundance of HIV target cells at this site. In comparison, the transmission probability for each sexual encounter following vaginal intercourse is estimated to 1:200-1:2000 (male-to female) and the lowest risk is attributed to the oral route (1:2500).

1.5.1 HIV infection in the female genital tract

Women appear to be more vulnerable to HIV infection following heterosexual intercourse, and this may be attributed to biological as well as social, economic and behavioral factors (Kaushic, 2009). Based on animal models (Kaizu et al., 2006, Salle et al., 2010), ex vivo human cervical explant studies (Gupta et al., 2002) and genetic sequencing (Zhu et al., 1996), it is proposed that both free virions and cell associated virus can establish mucosal HIV infection. Furthermore, sequence analysis of virus in acute infection show that in the vast majority of sexually acquired HIV infections in the FGT, the infection is initiated by a single transmitted founder virus (Keele and Derdeyn, 2009). However, in the case of genital inflammation, the number of virus that is transmitted may be increased (Haaland et al., 2009).

Where HIV most commonly establishes primary infection in the female genital tract remains unknown, but HIV can infect vaginal, ectocervical, endocervical and uterine mucosa (Hladik and Hope, 2009, Howell et al., 1997). The multilayered epithelium of the vagina and ectocervix offers, when intact, a better mechanical protection against invading pathogens compared with the single-layer epithelium of the endocervix and endometrium (Pope and Haase, 2003). However, the larger surface area of the vagina and ectocervix provide greater access for HIV entry. Genital transmission of HIV/SIV has been reported in a woman born without a uterus (Kell et al., 1992) and in monkeys after hysterectomy (Miller et al., 1992). The potential benefit of blocking HIV exposure to the cervix by the use of a diaphragm was investigated in a large randomized controlled prevention clinical trial in African women (Padian et al., 2007). No significant reduction in HIV acquisition was observed in women using a diaphragm compared with the control group; however the sexual partners of the women using a diaphragm reported lower condom use than those in the control group. In summary, these reports suggest the vaginal mucosa as the initial transmission site for HIV. However, in the rhesus macaque model, foci of SIV RNA⁺ cells were identified in the endocervix and transformation zone (Li et al., 2009).

Prior to the epithelium, the cervicovaginal secretions provide the first potential barrier for HIV. As mentioned before, the fluid contains antimicrobial cationic polypeptides

with anti-HIV activity. In addition, both virions and cell-bound HIV may be temporarily trapped by the cervical mucus (Maher et al., 2005). These two protective mechanisms may work synergistically, as the trapping may increase the possibility for soluble antiviral factors to attack the virus (Grivel et al., 2011).

If HIV reaches the epithelium, the virus may cross via several mechanisms. Epithelial damage, due to genital infections or after “normal” sexual intercourse (Norvell et al., 1984), is one possible entry mechanism. Furthermore, as described previously, the virus can be captured by intraepithelial LCs or the virus may penetrate the superficial layers of the epithelium and infect intraepithelial CD4⁺ T cells (Hladik et al., 2007). Moreover, virions may transcytose through single layer columnar epithelium or the basal layers of squamous epithelium (Hladik and Hope, 2009, Bomsel, 1997, Bobardt et al., 2007). HIV has been found to infect epithelial cells in vitro (Wu et al., 2003) and both endometrial and ectocervical epithelial cells may express CD4 and the chemokine-coreceptors (Yeaman et al., 2003, Yeaman et al., 2004). As mentioned previously, susceptible HIV target cells in the genital tract mucosa are CD4⁺ T cells, DCs including LCs, and macrophages (Spira et al., 1996, Hu et al., 2000, Hladik et al., 2007). Observations in human tissue explant models (Hladik et al., 2007, Saba et al., 2010) and in rhesus macaques (Haase, 2010) suggest that CD4⁺ T cells are the initial targets in HIV infection. Especially “resting” memory CD4⁺ T cells are the first cells to become infected, mainly due to the fact that they are more numerous than other potential target cells (Zhang et al., 1999, Zhang et al., 2004).

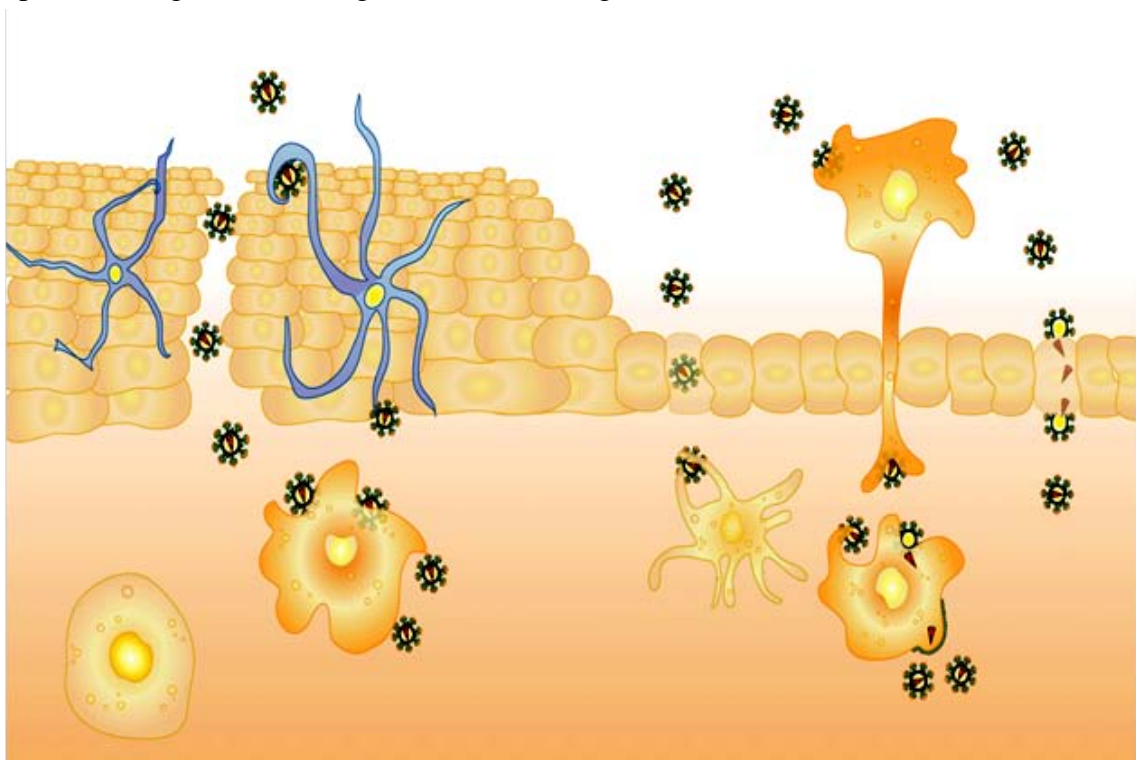


Figure 3

Illustration showing some of the suggested routes for HIV transmission across squamous and columnar epithelium; after epithelial damage, by capture by intraepithelial Langerhans cells, transcytosis of virions and direct infection of epithelial cells.

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1.5.2 Viral and host factors modulating HIV susceptibility

Several factors may influence the susceptibility of HIV infection in the FGT. A high viral load in the infected individual, which occurs during the early-stage of HIV infection, is associated with a higher risk of infection (Wawer et al., 2005). Sexually transmitted infections (STIs) in the non-infected subject increase the susceptibility, possibly by disrupting the epithelial barrier and/or by an increase in susceptible target cells (Laga et al., 1993, Plummer et al., 1991, Patterson et al., 1998). For example, following herpes simplex virus type 2 (HSV-2) infection, the number of potential HIV target cells was increased and persisted in the tissue despite of systemic antiviral treatment (Zhu et al., 2009). This may partly explain why HSV-2 treatment fails to reduce HIV acquisition. Moreover, concomitant HIV and STI infection increases viral load in genital secretions (Ghys et al., 1997, Cohen et al., 1997). In addition, factors in human semen may influence HIV susceptibility. A seminal peptide, named semen-derived enhancer of virus infection (SEVI), enhances HIV infection in vitro through formation of amyloid fibrils that capture and focus virus onto target cells (Munch et al., 2007). A genetic factor mediating decreased susceptibility against HIV is the mutation of the co-receptor CCR5 (CCR5 delta 32), which results in a non-functional receptor. Thus, a homozygous mutation results in a subsequent inability of R5 viruses to fuse with the target cell (Samson et al., 1996).

1.5.3 Sex hormone levels and HIV susceptibility

Several components of the mucosal immunity in the FGT vary with the changes in sex hormone levels during the menstrual cycle. Also exogenously applied sex hormones, such as the commonly used oral contraceptive pill, may influence the immune response. Wira et al have proposed a window of vulnerability for mucosal HIV acquisition 7-10 days following ovulation due to hormonal suppression on mucosal immunity (Wira and Fahey, 2008). Here, the menstrual cycle will be described briefly and some examples of hormonal regulation of the mucosal immunity will be discussed.

During the first half of the menstrual cycle (the proliferative or preovulatory phase) estradiol (estrogen) levels increases gradually, peak a few days before ovulation and thereafter transiently declines. Following ovulation, the other sex hormone, progesterone, increases together with estradiol for 7-10 days (the secretory or postovulatory phase) after which both decline to initiate menstruation. These changes create an optimal environment for fertilization, but also affect the immune system in the FGT. In this thesis, menstrual cycle stage based on self-reporting is described as preovulatory or postovulatory stage (Papers I and IV) whereas stage determined by endometrial dating is described as proliferative or secretory stage (Papers II and III).

At the level of the epithelium, progesterone implants are known to reduce vaginal epithelium thickness in rhesus macaques (Marx et al., 1996), but not in humans (Mauck et al., 1999). In women not using hormonal contraception, the number of vaginal epithelial cell layers was found to be the lowest in the secretory stage of the menstrual

cycle (Patton et al., 2000). Although statistically significant, the clinical significance of these changes was uncertain since they only consisted of one to two cell layers.

The number and functional capacity of potential HIV target cells may vary with the menstrual cycle. In the endometrium, lymphoid aggregates consisting of a core of B cells surrounded by CD8⁺ T cells and macrophages, increased in size over the menstrual cycle (Yeaman et al., 1997). Furthermore, the cytotoxic activity of the CD8⁺ T cells in the endometrium and fallopian tubes was found to be suppressed during the secretory phase (Wira and Fahey, 2008). However, in an extensive study characterizing T cell subsets, natural killer cells (NK cells), macrophages and DCs at several locations in the lower FGT, no effect of menstrual cycle stage on cellular abundance or localization was seen (Pudney et al., 2005). Several soluble cationic polypeptides are influenced by sex hormones. In cervicovaginal lavage (CVL) samples, levels of SLPI and HNP 1-3 transiently decreased at the time of ovulation and thereafter increased during the secretory phase (Keller et al., 2007). In the uterus, Elafin mRNA expression peak during menstrual bleeding and the peptide is produced mainly by infiltrating neutrophils (King et al., 2003b). Our findings (Paper IV) show significantly lower levels of LL-37 and Trappin-2 in cervicovaginal secretions (CVS) from women currently using combined oral contraceptives (OC) as well as decreased Trappin-2 mRNA expression in OC users.

1.6 HIV EXPOSED SERONEGATIVE INDIVIDUALS

Some individuals remain uninfected despite repeated exposure to HIV and thus seem to be resistant to the infection. These HIV exposed seronegative (HESN) individuals have been found in a variety of cohorts including discordant couples (one partner HIV-positive and the other HIV-negative), commercial sex workers, exposed health care workers and infants of HIV-infected mothers (Kulkarni et al., 2003). Several investigators have sought for the correlates of protection in these individuals and multiple mechanisms including systemic and mucosal cellular and humoral immune responses, innate immune responses, co-receptor mutations and genetic variations have been described. Here, a brief summary of some of these protective mechanisms will be presented. The majority of data has been obtained on uninfected individuals exposed by the sexual route. Of note, the already described mutation of the co-receptor CCR5 (CCR5delta32) is the only mechanism that alone provide resistance to HIV infection (providing homozygous genotype and R5 strain infection). All other mechanisms described here most probably act together to provide an HIV-protective immune response.

1.6.1 Innate immune responses in HESN

Many innate immune factors may provide anti-HIV activity including the previously described cationic polypeptides as well as chemokines and cytokines. For example, three β -chemokines are natural ligands to the HIV co-receptor CCR5; Regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein 1 α (MIP-1 α) and macrophage inflammatory protein 1 β (MIP-1 β), and are believed to reduce HIV-binding in a competitive manner. Elevated levels of RANTES have been found in the genital mucosa of HESN sex workers (Hirbod et al., 2006, Iqbal et al., 2005). MIP-1 β and RANTES levels were elevated in the saliva of HESN MSM and the chemokine levels correlated with salivary HIV neutralization capacity (Hasselrot et al., 2010). Using a proteomics approach, the expression of 17 proteins in genital fluids was found to differ between HESN sex workers versus HIV-uninfected and HIV-infected sex workers in Kenya (Burgener et al., 2008). Most of the proteins that were up-regulated in the HESN individuals were anti-proteases. Further investigation is needed to explore if these anti-proteases protect against mucosal HIV acquisition, and in that case, whether they function similarly to other known anti-proteases with anti-HIV activity (i.e. SLPI and Elafin).

1.6.2 Adaptive immune responses in HESN

HIV specific CD4⁺ T cells and CTLs as well as HIV neutralizing IgA antibodies have been found both systemically and in the FGT mucosa of HESN individuals (Miyazawa

et al., 2009). Proposed explanations for the induction of adaptive immune responses in HESN include: a) extraordinarily low levels of virus replication in the submucosal tissue without further dissemination to the lymphoid organs and/or, b) immune processing of viral fragments presented to the immune system without productive infection (Hirbod and Broliden, 2007). Furthermore, a continuous exposure to HIV is believed to be necessary to maintain an HIV-specific immunity both at the systemic and mucosal level in HESN subjects. Examples of adaptive immune responses in HESN women include HIV-specific CTLs in the systemic and mucosal compartment of sex workers (Kaul et al., 2000). Our group and others have found HIV-neutralizing IgA antibodies in saliva of HESN MSM (Hasselrot et al., 2009) and genital fluids of HESN sex workers (Devito et al., 2000, Hirbod et al., 2008). The neutralizing IgA antibodies may target antigens such as the receptors CCR5 and CD4 (Barassi et al., 2004), HLA-molecules (Lopalco et al., 2000), carbohydrates on the HIV-envelope or epitopes displayed during the viral fusion (Ditzel et al., 1995). Other protective effects of soluble antibodies include opsonization, which may result in activation of the complement system and complement-mediated lysis or uptake and destruction by phagocytes (Stoiber, 2009).

2 AIM OF THESIS

Toward the long-term goal of protecting women against STIs including HIV, the general aim of this thesis is to enhance understanding of HIV transmission events in the female genital tract. Specifically, the present work aims to:

1. Ascertain and characterize the distribution of potential HIV target cells and cellular receptors in different regions (ectocervix, endometrium and endocervix) of the human female genital tract (Papers I-III respectively).
2. Determine whether sexual behavior associated with a high-risk of acquiring HIV changes the distribution and abundance of HIV target cells (Paper I).
3. Establish whether or not innate peptide levels are hormonally regulated (Paper IV).
4. Evaluate the content of innate immune peptides with suggested anti-HIV activity in genital secretions and ectocervical tissue to seek a correlation, if any, between soluble levels and tissue expression at the individual level (Paper IV).

3 MATERIAL AND METHODS

3.1 STUDY SUBJECTS AND SAMPLE COLLECTION

Prior to start of all studies, ethical approvals were obtained.

3.1.1 Paper I

Cervical biopsies from the superior extocervix (avoiding the transformation zone) were collected from:

1) Eight African HIV-seronegative female sex workers that due to their occupation and reported lack of condom use were considered to be at high-risk for acquiring STIs including HIV.

2) Ten African and Caucasian low-risk HIV-seronegative volunteers.

The biopsies were immediately snap frozen in liquid nitrogen and stored at -80°C . All participating women were screened for STIs and answered a questionnaire regarding menstrual cycle stage, medical history and active medications including current use of contraceptives.

3.1.2 Papers II and III

Eight HIV-seronegative women with planned hysterectomy were recruited from the Department of Gynecology and Obstetrics at the Karolinska University Hospital Huddinge, Stockholm. Inclusion criteria were: HIV seronegative status, no clinical symptoms of STIs three months prior to the surgery and no use of hormonal therapy. All tissues were free from malignant and inflammatory diseases and pathology diagnosis was either leiomyomata or adenomyomata. Endometrial (n=8) and endocervical (n=6) tissue biopsies were collected by a gynecological pathology specialist. The biopsies were snap frozen in liquid nitrogen within 30 minutes of surgical removal and stored at -80°C . Menstrual cycle stage was determined by endometrial dating.

3.1.3 Paper IV

Twenty-five healthy HIV-seronegative female volunteers were enrolled from March 2007 through June 2009 at the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge, Sweden. Each woman underwent a pelvic examination followed by collection of a cervicovaginal secretion (CVS) sample and two biopsies taken from the superior portion of the ectocervix. Of the 25 women, 20 underwent a

second sampling of CVS and two ectocervical biopsies within nine to 26 months (median 23.8 months). The study also included samples from nine HIV-seronegative female sex workers from Nairobi, Kenya. At recruitment, these women had been active sex workers for a median of six years.

Each CVS was collected using first a cotton-tipped swab that was rotated 360° in the ectocervical os and with a second swab that was used to collect secretions from the posterial vaginal fornix. Both swabs were transferred into a single vial containing 5 ml of phosphate-buffered saline (PBS) and transported on ice within 1h to the laboratory. To remove cellular debris the samples were centrifuged at 1500 rpm for 5 minutes, thereafter the supernatant was transferred to a new vial which was cryopreserved at -80°C.

One of the collected ectocervical biopsies was immediately transferred to RNAlater buffer and stored at -80°C; the second biopsy was immediately snap frozen in liquid nitrogen and thereafter stored at -80°C.

3.2 METHODS

3.2.1 In situ detection of cellular markers, receptors and innate immune factors by immunostaining – Papers I - IV

Eight μm thick sections from cryopreserved ectocervical (papers I and IV), endometrial (paper II) and endocervical (paper III) biopsies were fixed in 2% formaldehyde, permeabilized, blocked for endogenous biotin and immunohistochemically stained as previously described (Lore and Andersson, 2004). Anti-human CD3, CD4, CD8, HLA-DR, CD68, CD1a, CD11c, CD123, CD56, CD19, BDCA-2, Langerin, DC-SIGN, MR, IgD, CCR5 antibodies (papers I-III) as well as anti-human HNP 1-3, Trappin-2, SLPI, and LL-37 antibodies (paper IV) were used to detect the immune molecules of interest. Biotinylated secondary antibodies in combination with diaminobenzidine tetrahydrochloride (DAB) (which creates a brown color) were used to visualize the positively stained markers of interest. Nuclear counterstaining was performed with hematoxylin. Negative control staining consisted of irrelevant anti-human IgG. Digital images were transferred from a DMR-X microscope (Leica, Wetzlar, Germany) into a computerized image analysis system, Quantimet, Q 550 IW (Leica Imaging Systems, Cambridge, UK). For one and double - fluorescent staining (papers I, II and III), the tissue sections were prepared as described above except that Alexa Fluor 488 streptavidin or Alexa Fluor 594 labeled streptavidin were used to visualize the cells of interest.

3.2.2 HNP 1-3, SLPI, Trappin-2 and LL-37 quantification – Paper IV

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used, according to the manufacturers protocols, to quantify the innate immune molecules of interest (additional CVS dilution in brackets): the α -defensins HNP-1-3 (1 : 100- 1 : 500), LL-37 (1 : 5 - 1 : 10) Trappin-2 (1 : 50) and SLPI (1 : 100).

3.2.3 Quantification of HNP 1-3, SLPI, Trappin-2 and LL-37 mRNA by real-time RT-PCR – Paper IV

Detection of mRNA expression of interest was performed as previously described (Brismar Wendel et al., 2010). The RNAlater buffer stored biopsies were thawed and disrupted in lysis buffer with a mechanical rotor. RNA was extracted according to the manufacturers' protocol (RNeasy kit) and converted in equal dilutions to cDNA in a single reverse transcriptase reaction using superscript II reverse transcriptase and random hexanucleotide primers. Amplification of ubiquitin C (UBC), Trappin-2, HNP1-3, SLPI and LL-37 was performed using the ABI PRISM 7500 sequence detection system and commercial FAMTM dye-labeled TaqMan MGB probes and primers. Each sample and control was run in duplicates. Ct values for target cDNA were normalized to UBC by using the $2^{-\text{dCT}}$ method.

3.3 STATISTICAL ANALYSES

3.3.1 Paper I

Data were not normally distributed, thus all analyses were performed with non-parametric tests. Intergroup variations of the markers of interest were analyzed by using the non-parametric Mann-Whitney U test. All calculations were performed with SPSS version 15.0 software (Chicago, IL, USA).

3.3.2 Papers II and III

No statistical analyses were performed in studies II and III due to the small number of study participants.

3.3.3 Paper IV

CVS samples with undetectable levels of innate factors were assigned a value at the assay cut-off. Tissue samples with undetectable mRNA levels of innate factors were assigned a value above Ct 40. Also the data in study IV were analyzed with the non-parametric Mann-Whitney U test to compare two groups and the Spearman rank-order correlation test to assess correlations. All analyses were performed with the software GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA).

3.4 TECHNICAL CONSIDERATIONS

When collecting human mucosal samples several factors have to be taken into consideration. Furthermore, all laboratory methods have their pros and cons. Here, I discuss some aspects of these limitations that are relevant for this thesis. My focus will be on immunostaining since this is the major laboratory method used for this work.

3.4.1 Collection of human mucosal samples

As discussed previously, several factors may influence the immune milieu in the FGT including sex hormones and genital infections and, as such, constitute possible confounders. However, collection of a homogeneous study population with regard to menstrual cycle stage, use of hormonal contraception and presence of STIs is a complex task for several reasons. Firstly, hormonal contraceptive use is widespread, over 100 million women worldwide currently use the combined oral contraceptive pill (OC) (Hatcher, 2008). OC contain a combination of an estrogen and a progestin (progesterone) and inhibit fertilization by blocking ovulation and by decreasing cervical mucus permeability, whereas the inhibition of ovulation by contraceptives containing only progesterone is dose-dependent. Thus, study subjects using OC do not have intact ovulation and can therefore not be divided into pre- or postovulatory menstrual cycle stages. Secondly, many women have irregular menstrual cycles, usually due to differences in the length of the preovulatory phase of the cycle. Less than 15% of 30 000 menstrual cycles reported by 650 women corresponded to a 28-day cycle (Hatcher, 2008). Thus, self-reporting of menstrual stage may be difficult and should ideally be supplemented with testing of serum estrogen and progesterone levels. Another option used in papers II and III is endometrial dating; however this requires endometrial biopsies. Screening for STIs is more straightforward, but includes the possible confounding factors that different laboratory methods may be used or diagnosis is determined clinically.

3.4.2 Immunohistochemical and immunofluorescence staining

Immunohistochemical staining enables the identification of immune markers at the cellular level within a tissue. In this thesis, both surface markers and secreted proteins have been assessed by this technique. The distribution of the markers of interest can be visualized, both in relation to other markers and to relevant anatomical structures (for example distance to the lumen). Immunofluorescence techniques facilitate multiple colors staining which can be used for phenotypical identification of for example CLR expression on a DC subset.

In my opinion, the greatest advantage of immunostaining techniques is the visual identification of markers' distribution at the cellular level. The other technique

commonly used to investigate immune cell populations in tissues; fluorescence-activated cell sorting (FACS) (Quayle et al., 2007, Prakash et al., 2004), does not provide this information. However, with FACS the number of cells can be more accurately determined and the phenotype can be thoroughly investigated by multicolor FACS staining. A disadvantage of immunostaining is that the analysis is very time consuming and the percentages calculated may not be precise due to the semi-quantitative analysis. But as long as one compares different groups that are analyzed with the same computerized analysis system one will be able to distinguish relevant differences. Furthermore, as no standard analysis system is agreed upon in the research community comparisons between studies are difficult.

In the ectocervix, DC subsets and CLR (paper I) as well as cationic polypeptides (paper IV) could be compartmentalized by tissue site; therefore the ectocervical biopsies were subdivided into epithelium and submucosa for the quantitative analysis. In an attempt to reflect the different staining patterns of cellular markers and secreted factors, the expression of cellular markers was determined as a percentage of positive staining out of total cell area, whereas expression of secreted innate immune factors was determined as a percentage of positive staining out of total tissue area. In the endocervical tissue (paper III), a distinct distribution pattern was seen for CD4⁺, CD8⁺ and CD11c⁺ cells with the majority of positive cells localized within or adjacent to the endocervical epithelium, whereas CD68⁺ cells were more evenly distributed in the endocervical tissue. Thus, the frequency of CD4⁺, CD8⁺ and CD11c⁺ expressing cells varied between different areas in the endocervical tissue, but was more uniform for CD68⁺ cells. However, we chose to quantify all markers in the same way so as to compare numbers of cells within different populations.

A separate problem concerns sampling and the limited size of the tissue biopsies. Ideally, several tissue biopsies corresponding to the whole ectocervical region; i.e., a biopsy from the part of the ectocervix bordering the vagina, a biopsy closest to the transformation zone and several biopsies in between, should be assessed to investigate possible differences in abundance and distribution of cells (or other molecules) related to anatomical localization in the ectocervix. This is perhaps doable with hysterectomy specimens but, for ethical and clinical reasons, less feasible when taking biopsies during a gynecological examination.

4 RESULTS AND DISCUSSION

4.1 PAPER I

If HIV overcomes the physical and immunological barriers in the FGT mucosa, DC subsets in the epithelium or in the subjacent submucosa are among the first cells to encounter the virus. In paper I, we therefore characterized the distribution of DCs in the human ectocervix, determined their expression of HIV-binding CLRs and investigated whether the frequencies of immune cells and/or receptors differed between women in two groups: HIV-uninfected low-risk women and HIV-uninfected high-risk sex workers. At the time of biopsy collection, three women used hormonal contraception (one in the HIV high-risk group and two in the HIV low-risk group), and all but six women were in their postovulatory menstrual cycle stage (Errata in paper I where three participants using hormonal contraception are divided into postovulatory stage). Furthermore, all women were *C trachomatis*-, *N gonorrhoea*-, syphilis- and candida-negative, but two of the subjects in the HIV high-risk group had clinical signs of bacterial vaginosis (BV).

HLA-DR⁺, CD3⁺, CD4⁺, CD8⁺, CD11c⁺, CD1a⁺, CD123⁺ and CD68⁺, MR⁺, DC-SIGN⁺ and Langerin⁺ cells were identified at the single cell level by immunohistochemistry and quantified by computerized image analysis. As MHC class II (HLA-DR) is expressed by all professional APCs to some extent, this marker was used to identify APCs including DCs. Cells expressing CD11c⁺ were defined as mDCs, whereas pDCs were identified by CD123⁺ expression. However, CD123 may also be expressed by other cell types including basophils (Agis et al., 1996, Janeway, 2005); therefore, a pDC-specific CLR; BDCA-2 (Dzionek et al., 2001) was included in papers II and III. LCs were identified by CD1a expression and macrophages defined as CD68⁺ cells.

By two-color staining, we characterized the phenotype of the DCs. The different subsets were found to be differentially distributed in the ectocervical tissue. The CD123⁻ CD68⁻ CD11c⁺ mDCs were present both within the epithelium and subjacent in the submucosal compartment, whereas CD11c⁻ CD123⁺ pDCs and CD1a⁻ CD11c⁻ CD123⁻ CD68⁺ macrophages were only found in the submucosa. In all study individuals CD11c⁺ cells were more abundant than CD123⁺ cells. All CD1a⁺ LCs, which were located mainly in the epithelium, expressed CD11c. We further investigated CLR expression. Langerin was present on CD1a⁺ LCs and on rare CD1a⁻ CD11c⁺ cells in the epithelium. In contrast, MR and DC-SIGN were exclusively expressed on cells in the submucosa: CD11c⁺ DC-SIGN⁺ mDCs, DC-SIGN⁺ MR⁺ CD68⁺ mDCs or macrophages and MR⁺ CD68⁺ macrophages. The CD123⁺ cells did not express any of the investigated CLRs. Several of the ectocervices examined had submucosa that formed finger-like projections (papillae) into the squamous epithelium resulting in a reduced distance between the submucosa and the luminal surface. In several of the subjects, CD11c⁺, CD123⁺, MR⁺ and DC-SIGN⁺ cells were found within these papillae.

Although the frequency of HLA-DR⁺, CD4⁺, and CD8⁺ cells was similar in the two study groups, the HIV high-risk women had a significantly higher expression of Langerin, MR and DC-SIGN as compared to the low-risk women (Figure 4). Although we could not perform intra-group statistical analysis, due to the small number of study subjects in each group, we did not note any obvious differences in the expression of the analyzed receptors in the two high-risk women with BV compared to the other women.

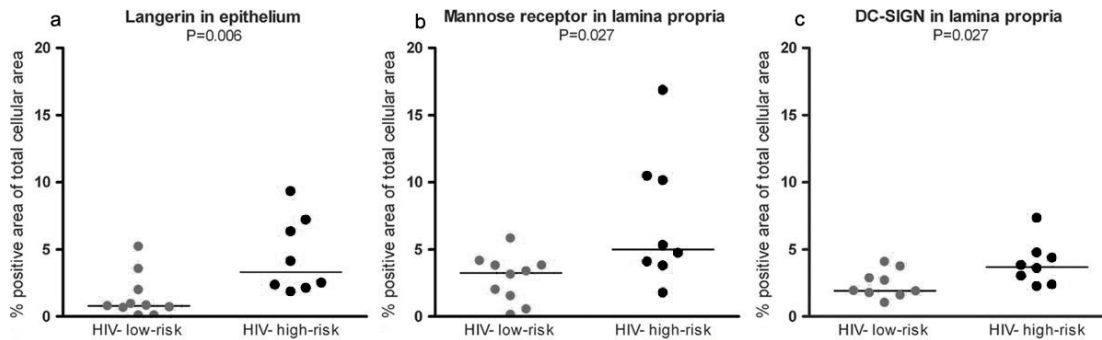


Figure 4.

Langerin a), Mannose Receptor b) and DC-SIGN c) expression in the ectocervix of HIV low-risk and high-risk women. Y axis indicates the percentage of positive area as compared to the total cellular area. X axis indicates the study groups. Bar indicates the medians

In conclusion, paper I describes the distribution of four cell populations expressing APC markers in the human ectocervix and further characterizes these cells with regard to their expression of HIV-binding CLRs. The findings contribute to previous reports on immune cell populations in the ectocervix (Geijtenbeek et al., 2000, Poppe et al., 1998, Pudney et al., 2005). Whether the altered expression of CLRs in HIV high-risk women is caused by environmental factors such as exposure to HIV/other STIs or results from other factors such as genotype requires further investigation. Although the two study groups displayed similar numbers of APCs and T cells, the HIV high-risk women may have an up-regulated turn-over of their DCs as a result of mucosal disruptions from frequent sexual behaviors and possibly episodes of STIs. Tests of biopsies taken at several occasions would resolve this issue. Furthermore, a higher density of any CLR does not necessarily signify protection. As described previously, LCs may play dual roles in sexual transmission of HIV; immature LCs may protect against HIV acquisition via Langerin binding and degradation (de Witte et al., 2007b), whereas mature LCs may mediate HIV spread (Fahrbach et al., 2007). Thus, a high density of immature LCs (expressing high levels of Langerin) may contribute to protection against HIV in the context of low viral inoculum, whereas this protective mechanism may be lost if the LCs mature and down-regulate their Langerin expression (for example, during inflammatory conditions) or if Langerin binding is saturated due to a high viral load (de Jong et al., 2008, de Witte et al., 2007b). In support of this, we recently found up-regulated Langerin expression in the ectocervix of HESN sex workers as compared with lower risk individuals (Hirbod, unpublished data). In contrast, DC-SIGN interaction with HIV is considered to contribute to enhanced infectivity via cis- and trans-infection (Geijtenbeek et al., 2000, Burleigh et al., 2006). Furthermore, in addition to viral dose and maturation stage of the cell, the relative contribution of CLRs versus CD4 and CCR5 in HIV transmission events may vary in

the context of Langerin polymorphisms (Ward et al., 2006) and possibly in the presence of soluble HIV receptor ligands.

4.2 PAPER II

The lower FGT is often the main focus in research articles on sexual HIV transmission but initial HIV transmission events might also take place in the upper FGT (Wira and Fahey, 2008). In paper II, we therefore set out to investigate the distribution of potential HIV target cells and cellular receptors in the human endometrium. Previous work had showed that immune cell populations including CD4⁺ T cells, DCs and macrophages were present in the endometrium (Kamat and Isaacson, 1987, Laguens et al., 1990, Schulke et al., 2008, Starkey et al., 1991, Vassiliadou and Bulmer, 1996), and our aim was to further characterize these cells with regard to their expression of CCR5 and HIV-binding CLRs. Endometrial tissue biopsies were therefore collected from eight women undergoing hysterectomy due to benign bleeding disorders. Based on endometrial dating, two women had proliferative endometrium, three had secretory endometrium and three had inactive endometrium. In addition to the immune markers characterized in paper I, BDCA-2⁺ and CCR5⁺ cells were also identified at the single cell level by immunohistochemistry and quantified by computerized image analysis. The myometrium was excluded from the quantitative analysis.

CD4⁺ cells were found within the columnar epithelium and in the endometrial stroma. CD4⁺ cells were either scattered in the stroma or aggregated within lymphoid formations. Endometrial lymphoid aggregates have been described previously, and they varied in size during the menstrual cycle (Kamat and Isaacson, 1987, Yeaman et al., 1997). The phenotype of the cells was further characterized by two-color staining (Figure 5). The CD4⁺ cells represented T cells (defined as CD3⁺ CD4⁺ T cells) and DC subsets including mDCs (CD4⁺ CD11c⁺ cells) and LCs (CD4⁺ CD1a⁺ Langerin⁺ cells). Furthermore, both the intraepithelial and stromal CD4⁺ cells as well as CD3⁺ cells expressed the main co-receptor for HIV; CCR5. In addition to mDCs and LCs, CD68⁺ macrophages and CD123⁺ BDCA-2⁺ pDCs were found in the endometrium. Similarly to the findings in ectocervix, the APC subsets showed a compartmentalized distribution (Figure 6). Thus, the rare pDCs were localized solely in the endometrial stroma. In contrast, mDCs and macrophages were detected within the epithelium as well as scattered in the stroma and in lymphoid aggregates. LCs were localized mainly within the epithelium; however, Langerin expression was also detected in lymphoid aggregates. MR- and DC-SIGN-expressing cells were detected only in the stroma, albeit in close proximity to the uterine lumen, and represented both CD11c⁺ and CD68⁺ cells (Figure 6).

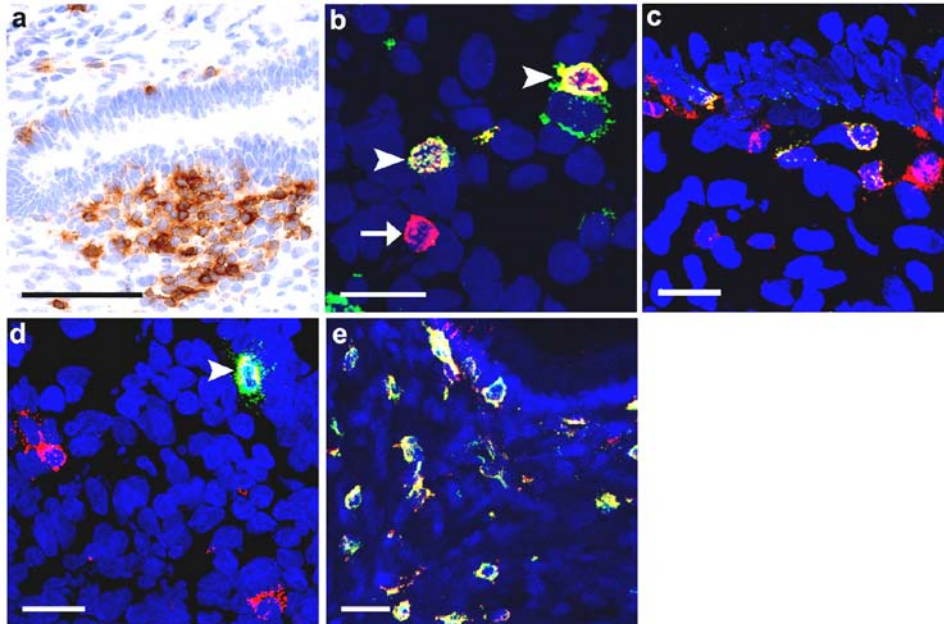


Figure 5.

a) Aggregate of $CD4^+$ cells (stained brown) adjacent to glandular epithelium. Confocal images illustrating that $CD4^+$ cells consisted of *b*) $CD3^+$ T cells, cells co-expressing $CD4^+$ (red) and $CD3^+$ (green) are yellow, *c*) $CD11c^+$ myeloid DCs, double positive cells (yellow) co-expressing $CD11c^+$ (red) and $CD4^+$ cells (green) and *d*) $CD1a^+$ Langerhans cells, $CD4^+$ cells (red) and $CD1a^+$ cells (green) double positive cells are yellow. *e*) Double-positive cells co-expressing (yellow) $CCR5^+$ (red) and $CD4^+$ (green) were frequently observed within the luminal epithelium and underlying stroma. Scalebar $100\mu\text{m}$ (*a*) $25\mu\text{m}$ (*b, d, e*) $20\mu\text{m}$ (*c*).

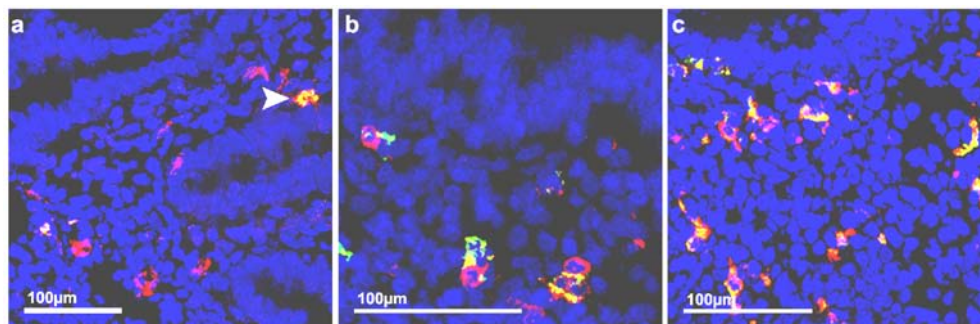


Figure 6.

Confocal images illustrating *a*) that some but not all stromal $CD123^+$ cells (red) co-expressed (yellow) $BDCA-2^+$ (green). Both *b*) $DC-SIGN^+$ (green) and *c*) MR^+ expressing cells (green) were present in the endometrial stroma and were co-expressed (yellow) on *b*) $CD11c^+$ (red) and *c*) $CD68^+$ cells (red).

To summarize paper II, we have described the presence of intraepithelial Langerin-, CD4- and CCR5-expressing cells in the human endometrium. Additional receptors with HIV-binding capacity such as MR and DC-SIGN were localized in the endometrial stroma. The role of all these potential HIV target cells as well as the occasional pDCs in HIV transmission events at this site remains to be established, for example in animal models or in human explant models. In theory, intraepithelial cells in single layer columnar epithelium are in direct contact with the lumen and could thus bind to HIV. Furthermore, as exposure of HIV gp120 can reduce the epithelial integrity through up-regulation of inflammatory cytokines (Nazli et al., 2010), stromal cells may also be exposed to the virus. Owing the small size of the study group, the question whether

menstrual cycle stage influenced immune cell numbers was not addressed in paper II (nor in paper III). However, the distribution pattern of all investigated cell populations and receptors was similar in these women. Previously, the impact of sex hormones on endometrial cell populations was described as relatively stable numbers of CD4⁺ and CD8⁺ T cells throughout the menstrual cycle (Starkey et al., 1991, Vassiliadou and Bulmer, 1996), but an increase of macrophages, CD1a⁺ cells and CD56⁺ CD16⁻ lymphocytes in the secretory stage (Kamat and Isaacson, 1987, Schulke et al., 2008, Starkey et al., 1991). CCR5 expression has been reported to be the highest during the proliferative stage (Yeaman et al., 2003), although another study showed no significant variations in CCR5 expression across the cycle (Mulayim et al., 2003). The conflicting results may result from different staining techniques or antibodies used and type of study subjects.

4.3 PAPER III

Owing to its anatomical localization and the fragile single layer epithelium, the endocervix has been considered a likely site for primary transmission of HIV. In support of this, endocervical foci of SIV RNA⁺ cells consisting primarily of CD4⁺ T cells were found in all infected monkeys in the rhesus macaque model, (n=9) (Li et al., 2009). However, with respect to sexual transmission, the animal model differs from HIV in humans in several ways. A sexual act constitutes a mechanical trauma to the genital tissue, which may result in microabrasions (Norvell et al., 1984) as well as influx of leukocytes (Pandya and Cohen, 1985, Prakash et al., 2004). This contrasts with the atraumatic viral deposition in the animal model. Semen from an HIV-infected individual contains free virions and infected leukocytes, and both forms may establish HIV infection (Hladik and Hope, 2009), however to date virions are most commonly used in the animal model (Anderson, 2010). Thus, whether the human endocervix plays the same role in initial HIV transmission requires further studies. Ectopy has been associated with an increased risk of HIV (Moss et al., 1991), suggesting that the exposure of single-layered epithelium on the outside of cervix increases the risk of HIV acquisition. Furthermore, several immune cell populations including CD4⁺ T cells, DCs and macrophages are present in the human endocervix, particularly in the transformation zone (Pudney et al., 2005).

In paper III, we characterized potential HIV target cells and receptors as well as described the distribution of immune effector cells in the human endocervix. Study participants were as in paper II, but endocervical biopsies were obtained from only six women. Based on endometrial dating, two participants were found in each group; proliferative, secretory and inactive endometrium. In addition to the immune markers used in papers I and II, CD8⁺, CD19⁺, CD56⁺ and IgD⁺ cells were identified at the single cell level by immunohistochemistry in paper III. Cells expressing CD8 were defined as CTLs, CD19⁺ cells were defined as B cells and CD56⁺ cells as a subset of NK cells.

In a distinct distribution pattern, most CD4⁺ cells, CD11c⁺ mDCs and CD8⁺ CTLs, but not the CD68⁺ macrophages, were located within or subjacent to the columnar epithelium (Figure 7). CD68⁺ cells were the most frequent of the investigated cells in the endocervix, yet CD1a⁺ LCs were the least abundant. LCs were located mainly within the epithelium, but were also found in the submucosa.

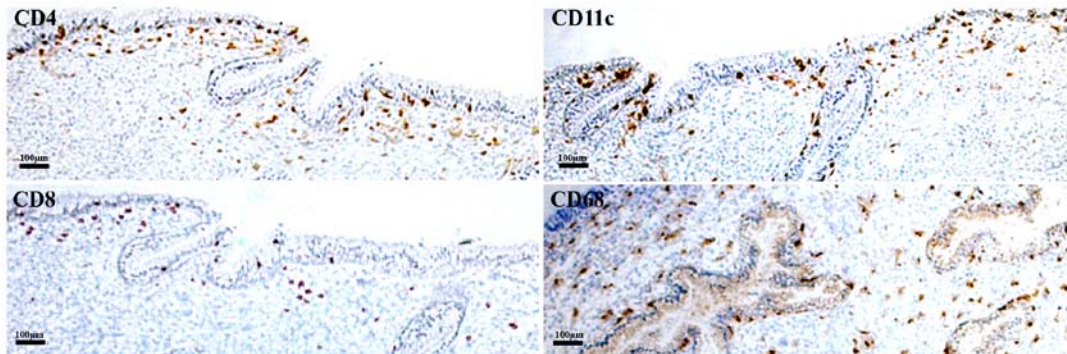


Figure 7.

Immunohistochemically stained sections of endocervical tissue (positive cells are stained brown) illustrating the distribution of CD4⁺, CD11c⁺, CD8⁺ and CD68⁺ cells.

The phenotype of the cells was further characterized by two-color staining (Figure 8).

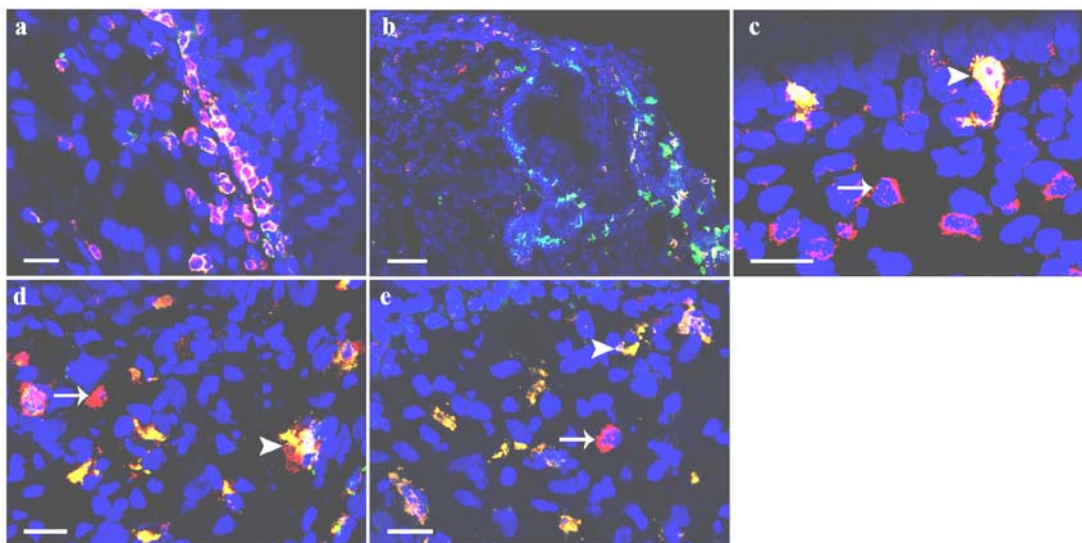


Figure 8.

Confocal images of endocervical tissue sections illustrating a positive co-localization (yellow) of a) CD4⁺ (green) and CCR5⁺ (red) cells and of b) CD11c⁺ (green) and CCR5⁺ (red) cells. c) Langerin⁺ (green) LCs were located mainly in the epithelium and co-expressed (yellow) CD4⁺ (red). MR- and DC-SIGN-expressing cells were present in the submucosa and consisted of both CD11c⁺ and CD68⁺ cells; d) DC-SIGN⁺ (red) and CD68⁺ (green) cells, double-positive cells (yellow) e) MR⁺ (red) and CD11c⁺ (green) cells.

Similarly to the endometrium, endocervical CD4⁺ cells consisted of CD3⁺ T cells as well as DC subsets including mDCs, LCs and pDCs. Furthermore, CCR5 expression was detected on CD11c⁺ and CD4⁺ cells. The HIV-binding CLRs were expressed by different APC subsets. Thus, Langerin was expressed by CD1a⁺ LCs, which also expressed CD4 and CD11c, but not CCR5. MR- and DC-SIGN-expressing cells were

exclusively present in the submucosa, albeit in close proximity to the lumen. MR and DC-SIGN were expressed by macrophages and mDCs, but not by LCs. In accordance with our previous findings in the ectocervix and endometrium, the rare CD123⁺ cells detected were present only in the submucosa. A subpopulation of the CD123⁺ cells (17%) co-expressed BDCA-2 and was thus defined as immature pDCs. The CD123⁺ cells did not express Langerin, MR or DC-SIGN. CD56⁺ NK cells were located both within the epithelium and in the submucosa, whereas CD19⁺ B cells and IgD⁺ cells were present in the submucosa alone.

In conclusion, our findings in paper III suggest that several potential HIV target cells are present within or in close proximity to the endocervical epithelium under non-inflammatory conditions. However, their role in HIV transmission events *in vivo* remains to be established. The distribution of HIV-binding CLR in the endocervix followed a similar pattern as in the ectocervix and endometrium; thus, Langerin⁺ cells were localized mainly in the epithelium, whereas MR⁺ and DC-SIGN⁺ cells were present in the submucosa. This compartmentalization of MR- and DC-SIGN-expressing cells is also seen in human skin (Angel et al., 2007). Although CD1a⁺ cells have been described in the endocervices of rhesus macaques (Miller et al., 1992), and CD1a⁺ and Langerin⁺ cells have been detected in cytobrush-obtained human cervical samples (Prakash et al., 2004), ano-genital columnar epithelium has been proposed to lack LCs (Kawamura et al., 2005). Our finding of Langerin⁺ CD4⁺ CD11c⁺ CD1a⁺ LCs within and subjacent to the endocervical epithelium suggests that LCs indeed are present at this location. Based on the dual role of LCs in HIV acquisition, the function of the endocervical LCs may be either beneficial or detrimental depending on the circumstance. In addition, we observed occasional BDCA-2⁺ CD123⁺ pDCs in the endocervical submucosa. In contrast to human HSV-2 infection, where pDCs are proposed to play a role in the immune control of recurrent infection (Donaghy et al., 2009) pDCs are part of the inflammatory innate immune response following SIV exposure that results in an influx of susceptible target cells in the endocervices of rhesus macaques (Li et al., 2009). The role of human pDCs during sexual HIV transmission remains to be established.

4.4 PAPER IV

In paper IV, we focused on the endocrine regulation of innate immune proteins with anti-HIV activity. We assessed several innate immune proteins in the ectocervix and in corresponding cervicovaginal fluids of both HIV-uninfected low-risk women and HIV-uninfected high-risk female sex workers and characterized their distribution in ectocervical tissue sections. We elucidated the association with hormonal contraception use and further examined if the soluble levels correlated with the tissue expression at the individual level. Entry characteristics of the participating women were determined by evaluation of the questionnaires and the results from STI screening. In Table 1, properties of the HIV low-risk women are summarized. As expected, the group of Kenyan female sex workers at high-risk of acquiring HIV differed with regard to several demographic variables (see Paper IV) as compared to the HIV low-risk women.

However, our aim when adding a HIV high-risk study group was to ascertain if the levels of innate peptides in genital fluids and ectocervical tissue correlated at the individual level in the context of different variables. Intergroup comparisons between HIV low-risk and HIV high-risk women were thus not performed.

Table 1. Entry characteristics of HIV low-risk women

	Median or number	Range or %
Age, (years)	32	23-50
Oral contraception, N (%)	7	28%
Progesterone IUD, N (%)	6	24%
Condom use \geq 50% (%)	7	28%
Candidiasis (%) ^a	3	12%
Bacterial vaginosis (%) ^a	2	8%
HSV-2 seropositive, N (%)	5	20%
HPV-positive, N (%)	11	44%
Preovulatory menstrual phase, N (%)	3	12%
Postovulatory menstrual phase, N (%)	7	28%

^aYeast infection or bacterial vaginosis in the pap smear

All women were Chlamydia, Gonorrhea and HIV uninfected.

A discrepancy between soluble levels and tissue expression of innate immune peptides was observed in the low-risk women. In CVS, levels of HNP 1-3, SLPI and Trappin-2 were plentiful, whereas LL-37 levels were lower. HNP 1-3 was the most abundant factor in CVS, whereas SLPI had the highest protein and mRNA expression in ectocervical tissue.

To determine whether the innate immune peptides were hormonally regulated data from low-risk women were stratified according to current use of hormonal contraception. Significant associations were found between current use of OC and lower Trappin-2 mRNA expression as well as OC use and lower levels of soluble Trappin-2 and LL-37 (Figure 9). For progesterone IUD users the relationship was the opposite, i.e., levels of soluble LL-37 were significantly higher (Figure 9). The significant association for Trappin-2 and OC use persisted in a second sample taken within two years from sample one, which strengthens this observation. In a previous study performed on CVL samples from HIV-uninfected Kenyan sex workers, no correlation was found between hormonal contraceptive use and soluble levels of Trappin-2 and Elafin (Iqbal et al., 2009). Plausible explanations for this discrepancy are differing epidemiological factors between the study groups and disparate quantitative and sampling techniques. In addition to their anti-HIV activity in vitro (Ghosh et al., 2010), elevated levels of Elafin and Trappin-2 have been associated with HIV non-acquisition in an epidemiological study (Iqbal et al., 2009). Consequently, if the use of OC decreases the expression of Trappin-2, HIV susceptibility could increase. However, several factors may influence HIV susceptibility making the impact of lower Trappin-2 levels alone difficult to interpret. Little is known about hormonal regulation of LL-37, but our findings indicate hormonal control of LL-37. In contrast, neither SLPI nor HNP 1-3 differed in relation to hormonal contraception use. We recognize that the sample size of 25 women, of whom just over half were using hormonal contraception, is a limitation of the study. Nevertheless, significant differences were observed for certain

peptides. Most importantly, the present study will serve as a pilot for future evaluations of hormonal regulation of innate immune peptides and as a baseline for innate peptide levels in a low-risk setting.

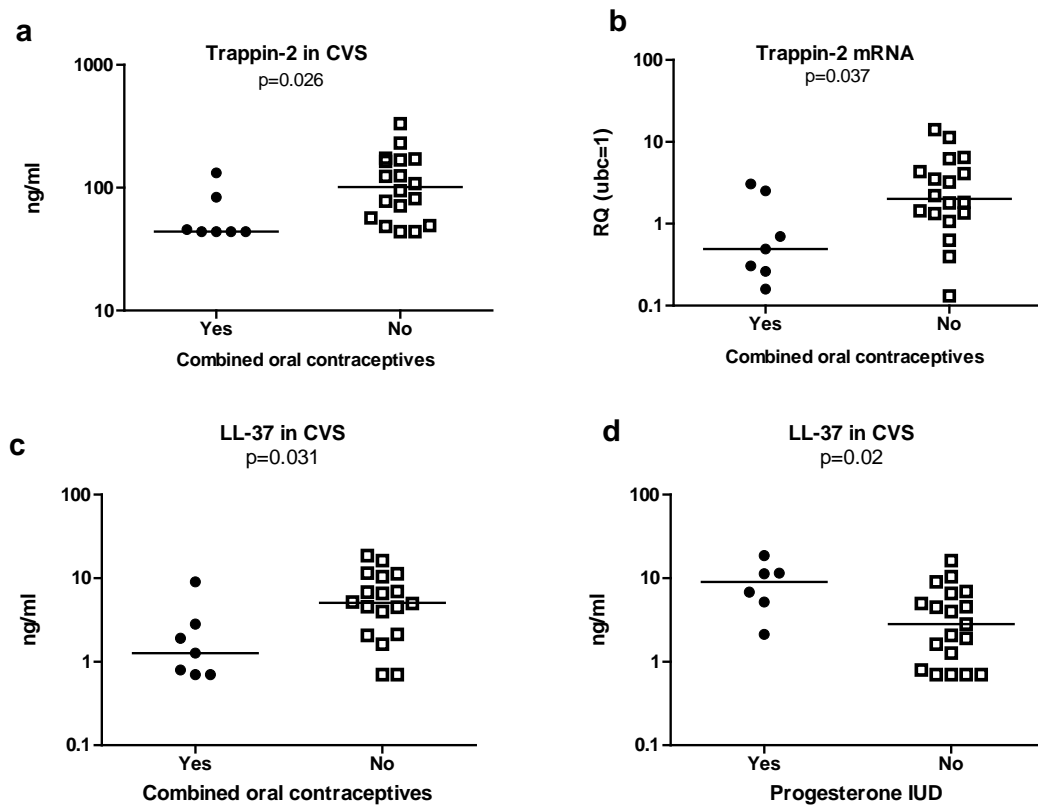


Figure 9.

a) CVS levels of Trappin-2 b) Trappin-2 mRNA expression and c) soluble levels of LL-37 in women with (filled circles) and without (boxes) current use of combined oral contraceptives. d) CVS levels of LL-37 in women with (filled circles) and without (boxes) progesterone IUD. Lines depict median.

We further investigated the distribution of SLPI⁺, HNP 1-3⁺, Trappin-2⁺ and LL-37⁺ cells in the ectocervix of HIV low-risk and high-risk woman by immunohistochemistry. The distribution pattern differed among the innate immune peptides; SLPI⁺, HNP 1-3⁺ and LL-37⁺ cells were located both in the submucosa and in the epithelium, whereas Trappin-2⁺ cells were observed mainly at the epithelial level. The compartmentalization of Trappin-2 expression to the ectocervical epithelium has been reported previously in the ectocervix as well as in other squamous epithelia (Pfundt et al., 1996). Our findings suggest that epithelial cells are the main producers of Trappin-2 in the ectocervix under non-inflammatory conditions. Two different staining patterns were observed for SLPI⁺- and HNP 1-3⁺-expressing cells. In the case of SLPI, the morphological appearance of positive cells in the epithelium differed from those in the submucosa. The cause may be different cellular sources of SLPI in these two compartments, which further investigation with double-staining techniques should clarify.

At the individual level in both HIV low-risk and high-risk women, no correlation between soluble levels and tissue expression (including mRNA and protein expression)

of the innate immune peptides was observed. These findings thus suggest that CVS levels of the investigated peptides do not reflect their expression in the ectocervical tissue. As discussed previously in this thesis, genital fluids stem from various sources, and peptide levels measured in cervicovaginal fluid thus reflect the production by epithelial cells and immune cells at several locations in the FGT. With this in mind, the lack of association between individual levels of soluble peptide and tissue expression is comprehensible. Nevertheless, it emphasizes the importance of an evaluation at the tissue level in addition to cervicovaginal fluid, since we may miss important information about the mucosal immune response if only fluid samples are assessed. Moreover, the observed compartmentalization between ectocervical tissue and genital secretions may have implications for the evaluating of these factors' impact on HIV susceptibility. Potentially, high levels of these molecules have a proinflammatory effect including target cell recruitment at the tissue level, whereas the same molecules could inhibit HIV entry when present in genital secretions.

5 CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the work presented here shows the presence of several likely HIV target cells and receptors at three anatomical sites in the FGT. Although immune cell populations have been investigated previously in the FGT, our results in papers I – III add considerable information about the phenotypes of those cells and the expression of HIV-binding receptors including CLRs and CCR5. In the ectocervices of HIV high-risk women, the density of CLRs was significantly higher than in low-risk individuals. Further investigation is needed to understand the underlying mechanisms and function of this up-regulation and is currently on-going in our laboratory (Hirbod et al, unpublished data). As genital inflammation may increase HIV susceptibility by several mechanisms including recruitment of potential target cells, future studies must include women with inflammatory conditions. By increasing the number of study participants, potential changes in abundance and distribution of immune cell populations during the menstrual cycle could be statistically verified. Since the diverse collection techniques used to sample genital tract fluids often generate different quantitative results, possibly due to dilution effects or different amount of mucus collected, a standardization of sampling procedures would facilitate interpretation between studies. This also holds true for other mucosal samples including biopsies and cytobrush-derived fluids.

A goal of HIV/AIDS research is the development of products designed to protect women against STIs including HIV. A microbicide is a topically applied (on the vaginal or rectal mucosa) compound designed to prevent the sexual transmission of HIV. In 2010, a randomized controlled trial of women in South Africa showed that sexual acquisition of HIV decreased in women using a vaginal gel of Tenofovir (an antiretroviral drug) as compared to placebo gel (Abdool Karim et al., 2010). However, when viral exposure is high, it is likely that more than one biomedical approach is needed to prevent primary HIV transmission (Shattock et al., 2011). Microbicide candidates could be based on hindering the interaction of HIV with its receptors including CCR5 (Kelly and Shattock, 2011) and CLRs (de Jong et al., 2010), thus knowledge of the presence and anatomical distribution of HIV-binding receptors and innate peptides in both low-risk and high-risk settings may be important when designing HIV-receptor blocking compounds. The work in this thesis was performed mainly on low-risk individuals, but our research group is currently investigating these molecules in women epidemiologically defined as HIV-resistant. Furthermore, in microbicide trials, it is important to monitor the elicited immune response, which may include production of chemokines, cytokines and/or innate immune peptides as well as potential changes in numbers of immune cells. Therefore, to assess the effect as well as safety of any such compound, we must have basic knowledge about the “normal” expression of these factors in both low- and high-risk individuals as a standard for comparison. Based on our findings in paper IV, which documented a compartmentalized distribution of innate immune peptides between ectocervical tissue and genital fluids, we suggest that mucosal immune responses should be monitored at the tissue level in addition to those in genital fluids. Moreover, menstrual cycle stage

and hormonal contraception use should be taken into account, since hormone levels may influence several aspects of mucosal immune responses.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Sommaren 1981 beskrevs de första fallen av förvärvat immunbristsyndrom (AIDS) i USA. Två år senare identifierade en fransk forskargrupp viruset som orsakat sjukdomen, humant immunbristvirus (HIV). Sedan starten av den globala HIV-epidemin på 1980-talet, har över 25 miljoner människor dött av sjukdomens slutstadium AIDS. Över 33 miljoner människor lever med HIV idag, de flesta i södra Afrika. Trots intensiv forskning finns det ingen bot eller vaccin mot HIV, men sedan i mitten av 1990-talet kan man med effektiva mediciner bromsa AIDS-utveckling. Dessvärre har bara ungefär en tredjedel av alla människor i behov av bromsmedicin tillgång till detta.

Oskyddat samlag är den vanligaste smittovägen för HIV, men viruset kan även smitta från en infekterad moder till hennes barn under graviditet, förlossning och amning eller via infekterat blod. Vid sexuell smitta måste viruset ta sig igenom slemhinnorna i anus, vagina eller munnen. Förutom vid oskyddat analsex, är den uppskattade risken för HIV-infektion vid oskyddat vaginalt och oralt samlag inte större än 1:200. Orsaken till detta är sannolikt att slemhinnans naturliga immunförsvar hindrar HIV-smitta vid de flesta oskyddade samlag. Naturligt förekommande skydd mot HIV i kvinnliga genitalia utgörs av både fysiska och immunologiska barriärer. Exempel på fysiska barriärer är den tjockflytande vätskan (sekretet) som täcker slemhinnan och en intakt yttre del (epitel) på slemhinnan. Sekretet innehåller dessutom flera olika lösliga molekyler som verkar skyddande mot HIV (anti-HIV-effekt). Om viruset trots allt tar sig igenom skyddsbarriärerna så kan det binda till och infektera flera olika typer av celler (HIV-målceller); $CD4^+$ T lymfocyter, dendritiska celler (DCs) och makrofager. För att HIV ska infektera en målcell måste den ta sig in i cellen genom att först binda till ett ytprotein på målcellen, $CD4$, och därefter till ytterligare ett protein (de så kallade co-receptorerna) $CCR5$ eller $CXCR4$. Förutom via $CD4$ och $CCR5/CXCR4$ så kan DC och makrofager även binda HIV med hjälp av andra ytmolekyler; till exempel via C-type lectin receptorer (CLRs). Tre stycken olika CLRs; Langerin, DC-SIGN och Mannose Receptor (MR), kan binda till HIV men resultatet av denna binding varierar. Om DC-SIGN binder till viruset ökar smittsamheten eftersom detta resulterar i att även $CD4^+$ T cells blir infekterade. Langerin däremot kan i vissa fall skydda mot HIV-smitta, då viruset efter Langerin bindning förstörs inuti cellen. Langerin, DC-SIGN och MR finns uttryckt på olika typer av DCs och makrofager. Langerin finns endast på en DC subtyp som kallas Langerhanska celler (LCs) som framförallt är lokaliserade i slemhinnans epitel. MR och DC-SIGN finns på makrofager och DCs. De celler som uttrycker MR och DC-SIGN är belägna djupare i slemhinnan nedanför epitelet i den så kallade submukosan.

I denna avhandling har vi undersökt distributionen av potentiella målceller för HIV på tre ställen i kvinnliga genitalia; ektocervix (yttre delen av livmodertappen), endocervix (inre delen av livmodertappen) och endometriet (livmoderns slemhinna) samt förekomsten av lösliga faktorer med anti-HIV-effekt i genitalt sekret och på vävnadsnivå i ektocervix. Resultaten från studie I-III visar att flera potentiella målceller

för HIV fanns i ektocervix, endocervix och endometriet. Till exempel fann vi både CD4⁺ T celler och Langerin⁺ LC i det enkelradiga epitelet som är ytterst på slemhinnan i endocervix och endometriet. Dessa målceller är i princip i direkt kontakt med utsidan på slemhinnan och skulle därför kunna binda till virus i HIV-infekterad sperma. Enkelradigt epitel är därtill mer känsligt för HIV-smitta jämfört med flerradigt epitel eftersom det lättare går sönder. Vidare bekräftar våra resultat tidigare fynd att de olika CLR:s uttrycks av olika typer av DCs och makrofager. På samtliga undersökta lokalisationer fanns Langerin på LCs, medan MR och DC-SIGN fanns på makrofager och DC i submukosan. Vidare såg vi att HIV-negativa prostituerade kvinnor hade mer Langerin, MR och DC-SIGN i ektocervix jämfört med HIV-negativa friska kvinnor. Huruvida den högre produktionen av CLR ledde till ett skydd mot sexuell HIV-smitta går inte att fastställa av vår studie, men vår grupp undersöker detta vidare hos bland annat prostituerade som trots att de upprepade gånger exponerats för HIV inte blivit HIV-infekterade (så kallade exponerade oinfekterade individer). I studie IV observerade vi att förekomsten av faktorer med anti-HIV-effekt skiljde sig mellan genitalt sekret och i ektocervikal vävnad. Vi föreslår därför att man bör undersöka både sekret och vävnad vid studier av slemhinnans immunförsvar, för om man bara undersöker det i en lokal så är risken att man missar viktig information. Vidare såg vi att användning av p-piller minskade nivåerna av några av de undersökta faktorerna. Då dessa faktorer verkar skyddande mot HIV skulle p-pilleranvändning därmed eventuellt kunna öka risken för sexuell HIV-smitta. Viktigt att påpeka i sammanhanget är att ett flertal faktorer påverkar risken för sexuell HIV-smitta och därför är det svårt att uttala sig precis om betydelsen av enskilda faktorer.

Sammanfattningsvis önskar jag att studierna i denna avhandling ska bidra till en ökad förståelse för mekanismerna bakom HIV-infektion i kvinnliga genitalia samt att de kan vara betydelsefulla när man ska designa skyddande läkemedel mot sexuell HIV-smitta.

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