Dendritic cell-mediated HIV-1 transmission
Groot, F.

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DENDRITIC CELL-MEDIATED HIV-1 TRANSMISSION

FEDDE GROOT
DENDRITIC CELL-MEDIATED HIV-1 TRANSMISSION


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DENDRITIC CELL-MEDIATED HIV-1 TRANSMISSION

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Faculteit der Geneeskunde
By destiny compell'd, and in despair,
The Greeks grew weary of the tedious war,
And by Minerva's aid a fabric rear'd,
Which like a steed of monstrous height appear'd:
The sides were plank'd with pine; they feign'd it made
For their return, and this the vow they paid.
Thus they pretend, but in the hollow side
Selected numbers of their soldiers hide:
With inward arms the dire machine they load,
And iron bowels stuff the dark abode.

[...]
The Trojans, coop'd within their walls so long,
Unbar their gates, and issue in a throng,
Like swarming bees, and with delight survey
The camp deserted, where the Grecians lay.
Part on the pile their wond'ring eyes employ:
The pile by Pallas rais'd to ruin Troy.

[...]
A spacious breach is made; the town lies bare;
Some hoisting-levers, some the wheels prepare
And fasten to the horse's feet; the rest
With cables haul along th' unwieldly beast.
Each on his fellow for assistance calls;
At length the fatal fabric mounts the walls,
Big with destruction. Boys with chaplets crown'd,
And choirs of virgins, sing and dance around.
Thus rais'd aloft, and then descending down,
It enters o'er our heads, and threats the town.
O sacred city, built by hands divine!
O valiant heroes of the Trojan line!

Virgil's Aeneid, Book II (19 B.C.)
Translation John Dryden (1697 A.D.)
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General introduction

Dendritic cell-mediated HIV-1 transmission
CHAPTER ONE

1 HUMAN IMMUNODEFICIENCY VIRUS TYPE I

Acquired immunodeficiency syndrome (AIDS) is one of the major death causes in the world. In 2006, over 40 million people are living with HIV-1, the virus that is causing this syndrome. The most-affected countries are located in Sub-Saharan Africa, which is home to 60% of all people infected with HIV (http://www.unaids.org). HIV-1 was identified in 1983 as the causative agent of AIDS\(^1\), and in 1986 a related but less virulent virus was isolated from AIDS patients in West-Africa, now known as HIV-2\(^2,3\). HIV-1 and HIV-2 evolved independently from each other, and originate from respectively a chimpanzee virus (SIV\(_{cpz}\)) and an SIV variant in sooty mangabeys. Both viruses may have crossed the monkey-human species barrier at several independent occasions\(^4,5\).

HIV-1 belongs to the lentivirus genus, a subfamily of the Retroviridae. Lentiviruses generally cause a slow, degenerative disease consisting of 3 phases: acute infection, latency and disease progression\(^6\). The retroviral life cycle is characterized by reverse transcription of the single stranded RNA genome into DNA by the viral reverse transcriptase enzyme. A schematic representation of the HIV-1 replication cycle is depicted in Fig. 1. HIV-1 attaches to the target cell via its envelope protein gp120, which binds to CD4 and a co-receptor. These interactions induce fusion of the viral and cellular membranes, followed by release of the viral core into the cytoplasm. In the core, viral RNA is reverse transcribed into DNA, which subsequently is inserted into the cellular genome by the viral integrase enzyme. The inserted viral DNA serves as a template for the production of viral mRNAs and new viral RNA genomes. Viral structural proteins and two RNA genome copies assemble into a virion particle that leaves the cell by budding. A detailed description of the HIV-1 replication cycle is given in references 7 and 8.

By infecting cells of the immune system, HIV-1 causes a gradual collapse of this defense system, leading to opportunistic infections, various forms of cancer, and finally death\(^8,10\). HIV-1 can infect cells of the immune system that bear the appropriate receptors: CD4 and an additional co-receptor\(^8,11\). Cells that are susceptible to HIV-1 infection are CD4\(^+\) T cells, monocytes, macrophages, dendritic cells (DC), thymocytes and microglia\(^12,17\).
CCR5 is the major co-receptor used by HIV-1, and in some patients the virus evolves to use CXCR4 as co-receptor\cite{9,11,18}. The CCR5 co-receptor is primarily expressed on the memory T cell subset and macrophages\cite{19-22}. Within 3 weeks after transmission, the virus becomes established in a lymphatic tissue reservoir, which is the principal site of virus production, storage, persistence and pathology\cite{23-29}. When HIV-1 starts using CXCR4, the virus expands its target cell repertoire to naïve T cells, which coincides with faster disease progression\cite{30,31,32}.
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Sexual transmission of HIV-1 is complicated by a mucosal barrier that has to be crossed, a process that is not yet entirely understood\textsuperscript{33,35}. DC are thought to play an important role in this process\textsuperscript{33,36-42}, which is the topic of this thesis. I will therefore review the role of DC in immunity and in particular their role in sexual HIV-1 transmission below.

2 THE ROLE OF DENDRITIC CELLS IN IMMUNITY

2.1 Initiation of an immune response

DC are professional antigen-presenting cells that sample the environment at sites of pathogen entry and they play an essential role in the induction of adaptive immune responses. Upon activation by micro-organisms or inflammatory signals, immature DC migrate to the draining lymph nodes while upregulating co-stimulatory molecules and developing into mature effector DC\textsuperscript{43,44} (Fig. 2). There, DC present pathogen-derived peptides in association with major histocompatibility class II (MHC-II) molecules to naïve T helper (Th) cells, which recognize the MHC-II/peptide complex via the T cell receptor (TCR)\textsuperscript{43-45}. With the appropriate additional interactions mediated by co-stimulatory molecules on the DC, naïve Th cells will become effector Th cells\textsuperscript{46,47}. Strong adhesion between the cells is important for this crosstalk between DC and T cell, which is provided by integrin molecules. The whole complex of molecules interacting between DC and T cell is referred to as ‘the immunological synapse’\textsuperscript{48-50}.

Depending on the type of pathogen and the micro-environment of the immature DC, different subsets of effector DC develop, which promote the development of protective effector Th type-1 (Th1) or type-2 (Th2) cells from naïve Th cells. In this way, the type of T cell response is adapted to the type of invading pathogen and the infected tissue\textsuperscript{46,51}. The distinct subsets of effector DC that bias the polarization of naïve Th cells into Th1 cells, Th2 cells or both, are designated DC1, DC2 and DC0, respectively\textsuperscript{51-53}. 
Figure 2. The role of DC in adaptive immunity. DC sample the environment at sites of pathogen entry (1). After recognition of a pathogen (-derived molecule) and potentially receiving additional inflammatory signals from the surrounding tissue, DC are activated and will migrate to the draining lymph node (2), where naïve Th cells are being primed (3). The resulting effector Th cells will migrate to the site of pathogen entry and take appropriate actions to eradicate the pathogen (4). See section 2.1 and 2.2 for more details.

2.2 Pathogen recognition

DC can identify pathogens through evolutionary conserved pattern recognition receptors (PRR)\textsuperscript{54}. PRR recognize pathogen-derived molecules that are also conserved, so-called pathogen-associated molecular patterns (PAMP). Binding of PAMP to PRR can lead to opsonization, endocytosis, DC activation or apoptosis\textsuperscript{55}. Toll-like receptors (TLRs) and C-type lectins are two of the major PRR families\textsuperscript{56-58}. So far, 11 different TLRs have been identified, which bind to a wide variety of microbial compounds, such as RNA or DNA sequences from several pathogens, bacterial cell wall components (LPS and peptidoglycan), bacterial flagella proteins and carbohydrates from yeast cell wall\textsuperscript{45,56,59,60}. TLR binding by PAMPs leads to DC activation through NF-κB signaling\textsuperscript{56}.

In contrast to TLRs, binding to C-type lectins can lead to internalization and presentation of the antigen\textsuperscript{57,61,62}. C-type lectins include amongst others the mannose receptor (MR), BDCA-2, langerin, DEC-205 and DC-specific ICAM-3-
grabbing nonintegrin (DC-SIGN) (reviewed in 63). DC-SIGN recognizes a variety of micro-organisms, including bacteria\textsuperscript{64,65}, fungi\textsuperscript{66}, viruses including HIV-1\textsuperscript{42,67,73} and several parasites\textsuperscript{74,75}. Although DC-SIGN binding can lead to internalization and lysosomal degradation, followed by antigen presentation to T cells\textsuperscript{61}, most pathogens that bind to DC-SIGN are causing long-lasting and chronic infections. This suggests that pathogen binding to DC-SIGN induces tolerance or immune evasion (reviewed in 76 and 77). Besides playing an important role as PRR, C-type lectins have been shown to act as adhesion receptors\textsuperscript{58,78}. For instance, DC-SIGN mediates the contact between DC and T cells by binding to ICAM-3, and mediates rolling of DC on endothelium by interacting with ICAM-2\textsuperscript{79,80}.

### 2.3 Different DC subsets

In humans, two main DC subsets have been identified: CD11c\textsuperscript{+} myeloid DC (MDC) and CD11c\textsuperscript{−} CD123\textsuperscript{+} plasmacytoid DC (PDC)\textsuperscript{43,45,81-83}. MDC include Langerhans cells, dermal DC and interstitial DC, and are widely distributed throughout the body. In blood, at least 4 different types of MDC can be distinguished on the basis of surface markers. PDC are primarily located in blood and secondary lymphoid organs, but they can be recruited to sites of inflammation\textsuperscript{82-86}. Besides location, the difference between MDC and PDC is manifested by TLR expression and cytokine secretion. MDC express all TLRs except TLR7 and TLR9, which are selectively expressed by PDC\textsuperscript{56,86-90}. MDC secrete high levels of interleukin-12 (IL-12), whereas PDC are thought to play an important role in the innate immune response to different viruses by producing interferon alpha (IFN\alpha)\textsuperscript{86,87,89,91}.

DC are a heterogeneous group of cells, which is manifested by differences in location within the body, phenotype and behavior. Although all currently known DC populations are now well described on the basis of their surface phenotype, the lineage and developmental relationship of the DC types is not yet entirely clear\textsuperscript{45,81,83,92}. 
3 DC-MEDIATED HIV-1 TRANSMISSION

3.1 Sexual HIV-1 transmission and the role of MDC
The probability of sexual HIV-1 transmission for each encounter with the virus is rather small (0.001-0.009), and varies widely with the partner’s stage of infection and viral load\(^{93-96}\). A barrier for virus transmission is the genital mucosal epithelium that has to be crossed\(^{33-35,97}\). HIV-1 can cross this barrier in several ways (Fig. 3, upper panel). The virus can enter through capture by intraepithelial MDC, which extend processes into the lumenal surface to sample contents and to trap pathogens\(^{40,97-100}\). Alternatively, HIV-1 crosses at places of thinning, breaches, or lesions caused by hormones or other (sexually transmitted) diseases\(^{33,100-106}\). The virus can also be captured by epithelial cells that subsequently transfer the virus to target cells underneath the epithelia\(^{107,108}\). In the latter two cases, the virus has access to subepithelial dermal MDC\(^{40,100}\).

Several in vivo studies indicate that intraepithelial and submucosal MDC are the first cell types that are the target of HIV/SIV after intravaginal infection\(^{36,39,100,109,110}\). Combined with the findings that MDC dramatically enhance HIV-1 replication in T cells\(^{42,111-113}\) and that MDC migrate to lymph nodes where they interact tightly with T cells\(^{43,44}\), a generally accepted hypothesis is that MDC play a crucial role in sexual HIV-1 transmission through viral capture in the mucosa and subsequent transfer to T cells in the lymph nodes\(^{38,40}\). In addition to HIV-1 transmission in the lymph nodes, MDC may also facilitate HIV-1 replication in mucosal T cells at the portal of viral entry\(^{9,40,114}\) (Fig. 3).

3.2 PDC and HIV-1 transmission
PDC are not located in high amounts at sites of pathogen entry, they do not capture, endocytose and process antigens as effectively as MDC, and their role in T cell proliferation is less pronounced\(^{87,115}\). PDC are involved in the innate immune response against many viruses, including HIV-1\(^{87}\). It is therefore not very likely that PDC are involved in HIV-1 capture and HIV-1 transmission in mucosal tissues as described for MDC. They may have an opposite role by controlling the virus\(^{116}\). Since some in vitro studies have shown that PDC are susceptible to HIV-1
infection and that they are able to transmit HIV-1 to T cells\textsuperscript{117-120}, their role in HIV-1 pathogenesis remains to be elucidated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hiv_transmission_model.png}
\caption{HIV-1 transmission model. In sexual transmission, HIV-1 has to cross a mucosal barrier. The virus can enter (1, upper panel) through capture by intraepithelial MDC that extend processes into the luminal surface to sample contents and to trap pathogens; (2) through breaches or lesions caused by hormones, microbicides or other (sexually transmitted) diseases; or (3) through capture by epithelial cells that transfer the virus to target cells underneath the epithelia. In (2) and (3), HIV-1 has access to subepithelial dermal MDC. DC can capture HIV-1 through C-type lectin receptors, of which DC-SIGN is the best studied example (box A). After capture, the virus dissociates from DC-SIGN and resides in an unidentified non-lysosomal compartment. After T cell encounter, HIV-1 is recruited to the site of T cell interaction (box B). This so-called ‘infectious synapse’ between DC and T cell depends on DC-SIGN expression and strong cell-cell adhesion mediated by ICAM-1—LFA-1 interaction. The precise composition of this synapse is largely unknown. Transmission can either take place to T cells in the lymph node or to mucosal T cells at the site of viral entry. See section 3.1-3.4 for more details.}
\end{figure}
### 3.3 HIV-1 capture by MDC

Although DC express the appropriate receptors for productive HIV-1 infection\(^{119,121}\), viral binding and uptake primarily occurs via other surface molecules, like C-type lectin receptors, and (to a lesser extent) via adhesion molecules, complement receptors and Fc-receptors\(^ {122,123}\). Since C-type lectins play an important role in sexual transmission of HIV-1, they will be discussed in greater detail.

DC-SIGN\(^ {80}\) is the best studied example of a C-type lectin receptor on DC that mediates capture and infection by HIV-1 and SIV\(^ {42,124,125}\) (Fig. 3, box A). HIV-1 attachment to DC-SIGN is mediated by the highly glycosylated HIV-1 envelope protein gp120. The binding site of DC-SIGN for gp120 involves both protein and carbohydrate interactions, and differs from the binding site for ICAM-3\(^ {126}\). DC-SIGN has been detected on MDC in the lamina propria of mucosal tissues (rectum, uterus, colon, vagina and cervix), on CD1a\(^ {low}\) dermal MDC, on a rare CD14\(^ +\) blood DC precursor and on MDC in lymph nodes and tonsils\(^ {110,127-132}\).

Other C-type lectins may also be involved in HIV-1 capture and transmission. CD1a\(^ {high}\) dermal MDC and MDC in vaginal epithelium (Langerhans cells) lack DC-SIGN and bind HIV-1 via the MR and langerin, respectively\(^ {99,132}\). The majority of the blood MDC and PDC subsets lack DC-SIGN and bind HIV-1 in a C-type lectin independent manner through CD4\(^ {132}\). Moreover, additional HIV-1 binding C-type lectins remain to be identified on monocyte-derived MDC and Langerhans cells\(^ {132,133}\). These studies demonstrate that not a single receptor is responsible for HIV-1 binding to all DC subsets\(^ {132,133}\). The relative contribution of these receptors and the different DC subsets in HIV-1 transmission remains to be determined\(^ {40,99,134-136}\).

### 3.4 DC-SIGN-mediated HIV-1 transmission

After binding of HIV-1 to DC-SIGN and internalization, the virus dissociates from DC-SIGN and ends up in distinct intracellular locations\(^ {137,138}\). Significant viral degradation occurs after viral capture\(^ {138}\), but a proportion of the virus survives for a prolonged period of time by residing in an acidic, non-lysosomal compartment.
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(Fig. 3, box A), thus evading the degradation process that can be mediated by DC-SIGN binding\textsuperscript{42,61,139-141}. How HIV-1 accomplishes this escape is largely unknown.

After an HIV-loaded DC encounters a T cell, the DC concentrates the virus to the site of cell-cell contact. Furthermore, CD4 and co-receptors on the T cell are recruited to the same region, the so-called ‘infectious synapse’\textsuperscript{140,142,143}. Formation of an infectious synapse and efficient HIV-1 transmission requires DC-SIGN expression\textsuperscript{143}, and depends on strong cell-cell contact through ICAM-1—LFA-1 interaction\textsuperscript{144,145} (Fig. 3, box B). Additionally, HIV-1 Nef can affect the transmission process by interfering with intracellular trafficking of DC-SIGN. As a result, DC-SIGN expression on the surface is increased, thus enhancing DC-T cell clustering\textsuperscript{146}.

The transfer of HIV-1 from DC to T cells after DC-SIGN-mediated capture, without the DC itself being infected, is designated trans-infection\textsuperscript{42,134,139}. In addition to promoting infection of T cells, DC-SIGN can also facilitate infection of the DC itself (designated cis-infection). In that case, HIV-1 attachment to DC-SIGN is followed by transfer of the virus to CD4 and a co-receptor on the same membrane\textsuperscript{124,134}. Both pathways can take place simultaneously, and DC can transmit both captured as newly produced virus to T cells\textsuperscript{138}.

3.5 DC as selectors of CCR5-using HIV?

An increasing number of studies on HIV-1 and SIV demonstrate that the initial burst of viral replication takes place in CCR5\textsuperscript{+} memory T cells in the lamina propria of the mucosa\textsuperscript{147-153}. Within a couple of weeks, CCR5\textsuperscript{+} T cells in lymphatic tissue become the principal site of virus production\textsuperscript{23,25,26}. It is only later in infection that HIV-1 proviral DNA can be isolated from CXCR4\textsuperscript{+} (naïve) T cells\textsuperscript{156,157}. The mechanism responsible for CCR5-using (R5) HIV-1 predominance early in infection is not known. One proposed mechanism is the exclusive transport of R5 viruses over the mucosal barrier by epithelial CCR5\textsuperscript{+} cells\textsuperscript{107,108}. Likewise, MDC (Langerhans cells and/or subepithelial dermal MDC) were proposed to be responsible due to the exclusive expression of CCR5, assuming that HIV-1 has to replicate in MDC in order to be transmitted\textsuperscript{37,99,121,158,159}. However, the exclusive replication of R5 HIV-1 in MDC has not been consistently
reported\textsuperscript{122,160-162,162-164}. Furthermore, DC do not necessarily need to be productively infected to transmit HIV-1. Only after 1-2 days after viral capture by DC, \textit{de novo} HIV-1 production is required for transmission to T cells\textsuperscript{113,138,164,165}. In addition, we and others have shown that DC can transmit both X4 as R5 HIV-1 to T cells\textsuperscript{120,121,166,167}. Thus, no consensus has yet been reached about the role of DC in R5 selection.

4. OUTLINE OF THIS THESIS

In the first part of the thesis, DC-mediated HIV-1 transmission is described from a mechanistic point of view, thereby focusing on the ICAM-1—LFA-1 interaction. We have demonstrated before that DC subsets expressing higher levels of ICAM-1 are better HIV-1 transmitters\textsuperscript{144}. ICAM-1 binds LFA-1 on T cells, an integrin responsible for adhesion and signaling at the immunological synapse\textsuperscript{48}. In Chapter 2, we describe our corroborations on this research by using LFA-1 negative leukocytes from Leukocyte Adhesion Deficiency type 1 (LAD-1) patients. Due to a mutation in one of its subunits, LFA-1 is not expressed on the cells of these patients\textsuperscript{167-169}. In addition, we used T cells from a unique patient with mild LAD-1 symptoms that express a mutant LFA-1 that cannot be induced into an active conformation\textsuperscript{170}. DC-mediated HIV-1 transmission to T cells from both types of patients was severely impaired, demonstrating the necessity of active LFA-1 on T cells for HIV-1 transmission.

In the second part of the thesis, we make an inventory of the different cell types that may play a role in DC-mediated HIV-1 transmission. In Chapter 3 we investigated whether HIV-1 transmission to different T cell subsets is taking place with different efficiencies. We used naïve T cells (T\textsubscript{N}), central memory T cells (T\textsubscript{CM}), and effector memory T cells (T\textsubscript{EM}). T\textsubscript{N} and T\textsubscript{CM} cells are primarily found in blood and lymphoid tissue, and play a role in long-term protection against respectively new or previously encountered pathogens. T\textsubscript{EM} cells are enriched in gut, liver and lung and provide immediate protection against pathogens\textsuperscript{171,172}. We determined the contribution of DC in HIV-1 infection of these different T cell
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... subsets, and found that co-receptor expression is a decisive factor for transmission
diated HIV-1 transmission, of which bovine lactoferrin (bLF) turned out to be
its impact on HIV-1 infection. Candida albicans is an opportunistic fungus causing
oropharyngeal candidiasis in a majority of HIV-1 infected patients. To obtain a
more powerful drug against this fungus, a naturally occurring anti-Candida peptide from saliva (Histatin 5) was improved by replacing several amino acids in the active domain. Surprisingly, 3 out of 4 derivatives stimulated HIV-1 infection, whereas the original peptide inhibited HIV-1 infection. This study demonstrates that the effect of peptide modification is hard to predict, and may have unwanted and potential dangerous side effects on other pathogens like HIV-1.

In Chapter 7, the main findings of the thesis are put in perspective with recent
literature, against a background of preventing HIV-1 transmission with microbicides.
REFERENCES


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CHAPTER TWO

Dendritic cell-mediated HIV-1 transmission to T cells of LAD-1 patients is impaired due to the defect in LFA-1

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Retrovirology (2006)
CHAPTER TWO

ABSTRACT

Dendritic cells (DC) have been proposed to mediate sexual HIV-1 transmission by capturing the virus in the mucosa and transporting it to the lymph nodes where HIV-1 is presented to CD4+ T cells. We have demonstrated before that DC subsets expressing higher levels of intercellular adhesion molecule-1 (ICAM-1) are better HIV-1 transmitters. ICAM-1 binds leukocyte function-associated molecule-1 (LFA-1) on T cells, an integrin responsible for adhesion and signaling at the immunological synapse. To corroborate the importance of the ICAM-1–LFA-1 interaction, we made use of LFA-1 negative leukocytes from Leukocyte Adhesion Deficiency type 1 (LAD-1) patients. We clearly show that DC-mediated HIV-1 transmission to LAD-1 T cells is impaired in comparison to healthy controls. Furthermore, HIV-1 transmission to T cells from a unique LAD-1 patient with a well characterized LFA-1 activation defect was impaired as well, demonstrating that activation of LFA-1 is crucial for efficient transmission. Decreased cell adhesion between DC and LAD-1 T cells is furthermore illustrated by significantly smaller DC-T cell clusters after HIV-1 transmission. By making use of cells from unique patients, this study provides more insight into the mechanism of HIV-1 transmission by DC. This may offer new treatment options to reduce sexual transmission of HIV-1.

INTRODUCTION

One of the first cell types encountered by HIV-1 during sexual transmission are intraepithelial and submucosal dendritic cells (DC)\textsuperscript{1–3}. DC are professional antigen presenting cells that sample the environment at sites of pathogen entry. Sentinel immature DC (iDC) develop into mature effector DC (mDC) upon activation by micro-organisms or inflammatory signals, and migrate to the draining lymph nodes where they encounter and stimulate naïve Th cells\textsuperscript{4,5}. HIV-1 has been proposed to make use of this migratory process, being captured by DC and delivered to the lymph node where the virus is transmitted to CD4+ T cells. In
addition to this, DC can facilitate local HIV-1 replication in mucosal T cells\textsuperscript{6,7}. DC are able to capture HIV-1 by a range of receptors, of which the best studied example is DC-SIGN, which mediates HIV-1 internalization such that the virus remains infectious for several days\textsuperscript{8,9}. Subsequent transmission to T cells takes place via cell-cell contact through an ‘infectious synapse’\textsuperscript{10,11}.

We have shown before that intercellular adhesion molecule-1 (ICAM-1) expression on DC is crucial for HIV-1 transmission to T cells: monocyte-derived DC subsets that express higher levels of ICAM-1 show higher HIV-1 transmission efficiencies to T cells\textsuperscript{10}, and transmission by both monocyte-derived DC and DC isolated from blood can be inhibited with blocking antibodies against ICAM-1\textsuperscript{10,12}. During antigen presentation, ICAM-1 expressed by DC binds to T cells via leukocyte function-associated molecule-1 (LFA-1). This interaction plays a key role in the initiation of immune responses by strengthening the adhesion between DC and T cells at the immunological synapse\textsuperscript{13-15}. LFA-1 is an integrin composed of the non-covalently bound \(\alpha\)L-subunit CD11a and \(\beta2\)-subunit CD18\textsuperscript{16}. Lack of proper \(\beta2\) expression due to a deletion or mutation in the CD18 gene leads to Leukocyte Adhesion Deficiency type-1 (LAD-1). Patients with this rare recessive disorder suffer from impaired wound healing without pus formation and recurring necrotic soft tissue infections. As CD11/CD18 heterodimers pair intracellularly, LFA-1 is not expressed at the cell surface of leukocytes from LAD-1 patients. The migration of leukocytes from the bloodstream into inflamed tissue is consequently hampered. In healthy individuals, stimulation of rolling leukocytes along endothelial cell lining induces a conformational change of CD11/CD18 heterodimers from a low to a high ligand-binding state, bringing cells to a halt. As expected, this adhesive process is impaired in LAD-1 patients\textsuperscript{17-21}. A unique variant of the LAD-1 disorder has been described (LAD-1/variant syndrome)\textsuperscript{22}. Cells of this patient with clinical features of a mild LAD-1 disorder do express LFA-1, but cellular activation does not result in activation of LFA-1, i.e. the ‘inside-out signaling’ that is necessary for increased ICAM-1 binding is impaired\textsuperscript{14,22-24}.

To further corroborate the importance of LFA-1 in HIV-1 transmission, we made use of T cells from LAD-1 patients. We found that DC-mediated HIV-1 transmission to LFA-1 negative T cells is impaired in comparison to healthy
controls. Furthermore, HIV-1 transmission to T cells isolated from the unique LAD-1/variant patient is impaired too, meaning that not only recognition of ICAM-1 but also high-activity binding is important for efficient transmission. Finally, we show that one day after HIV-1 transmission, DC-T cell clusters of LAD-1 and LAD-1/variant cells are significantly smaller than control clusters, which is illustrative for the reduced cell-cell adhesion in LAD-1 patients. By making use of cells isolated from unique patients, this study provides more insight into DC-mediated HIV-1 transmission, which may offer new options to inhibit HIV-1 transmission.

MATERIALS AND METHODS

Generation of monocyte-derived dendritic cells and peripheral blood leukocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Subsequently, PBMC were layered on a Percoll gradient (Pharmacia, Uppsala, Sweden) with three density layers (1.076, 1.059, and 1.045 g/ml). The light fraction with predominantly monocytes was collected, washed, and seeded in 24-well or 6-well culture plates (Costar, Cambridge, MA, USA) at a density of 5×10⁵ cells or 2.5×10⁶ per well, respectively. After 60 min at 37°C, non-adherent cells were removed, and adherent cells were cultured to obtain immature DC in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies Ltd., Paisley, United Kingdom) with gentamicin (86 µg/ml; Duchefa, Haarlem, The Netherlands) and 10% fetal calf serum (HyClone, Logan, UT, USA) and supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml; Strathmann Biotec AG, Hannover, Germany). At day 3, the culture medium with supplements was refreshed. At day 6, maturation was induced by culturing the cells with poly (I:C) (20 µg/ml; Sigma-Aldrich, St. Louis, MO, USA). After two days, mature CD14⁺ CD1b⁺ CD83⁺ DC were obtained. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors. Mature DC
were analyzed for the expression of cell surface molecules by FACS. Mouse anti-human mAbs were used against the following molecules: CD14 (BD Biosciences, San Jose, CA, USA), CD1b (Dialclone, Besançon, France), CD83 (ImmuneTech, Marseille, France) and ICAM-1 (CD54) (Pelicluster, Sanquin, Amsterdam, The Netherlands). All mAb incubations were followed by incubation with FITC-conjugated goat F(ab')2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Samples were analyzed on a FACScan (BD Biosciences).

Peripheral Blood Leukocytes (PBL) were isolated by layering PBMC on a Percoll gradient. The heavy fraction with predominantly PBL was collected and stored at −150°C. During co-culture with DC, PBL were cultured in IMDM with 10% FCS, gentamycin, 10 U/ml IL-2 (Cetus, Emeryville, CA, USA) and Staphylococcus enterotoxin B (SEB; Sigma-Aldrich; final concentration, 10 pg/ml). Mouse mAb to human CD28 (CLB-CD28/1) and human CD3 (CLB-T3/4E-1XE) were obtained from Sanquin (Amsterdam, The Netherlands).

Virus stocks
C33A cervix carcinoma cells or PM1 T cells were transfected using calcium phosphate or electroporation respectively with 5 μg of the molecular clone of T-tropic HIV-1 LAI. The virus containing supernatant was harvested 3 to 5 days post transfection, filtered and stored at −80°C. The concentration of virus was determined by CA-p24 ELISA. C33A and PM1 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) or Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies) respectively, both supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) (Sigma-Aldrich) and streptomycin (100 μg/ml) (Invitrogen, Breda, The Netherlands).

HIV transmission assay and CA-p24 measurement
Fully matured DC were incubated in a 96-well-plate (40-50×10³ DC/50 μl/well) with PM1 produced virus (10 ng CA-p24/well) or C33A produced virus (20 ng) for 2 hr at 37°C. The DC were washed with PBS after centrifugation at 400×g to
remove unbound virus. Washing was repeated, followed by addition of 50×10³ PBL. Prior to addition to DC, the PBL were analyzed by FACS with the following mouse anti-human antibodies: FITC-labeled CD11a (Pelicluster, Sanquin), APC-labeled CD4 (BD Biosciences) and PE-labeled CXCR4 (BD Biosciences). CD18 (Pelicluster, Sanquin) incubation was followed by incubation with FITC-conjugated goat F(ab’)₂ anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories). Viral replication after transmission was followed by measuring CA-p24 in the culture supernatant by ELISA. To determine intracellular CA-p24 in the single-cycle transmission assay, saquinavir (SQV, Roche, London, United Kingdom at 0.2 μM) was added to prevent cell-to-cell spread of newly produced virions. After 48 hr, the cells were harvested and stained with FITC-labeled CD3 (BD Biosciences) and APC-labeled DC-SIGN (R7D Systems, MN, USA), followed by fixation with 4% PFA and washing with washing buffer (PBS with 2 mM EDTA and 0.5% BSA). Fixated cells were then washed with perm/wash buffer (BD Biosciences), and incubated with PE-labeled CA-p24 (KC57-RD1, Coulter, Hialeah, FL, USA) followed by washing with successively perm/wash- and washing buffer. Cells were then analyzed by FACS.

**Statistical analysis**

Data were analyzed for statistical significance (GraphPad InStat, Inc, San Diego, CA, USA) using ANOVA. A p value <0.05 was considered to be significant.
LFA-1 IS CRUCIAL FOR EFFICIENT DC-MEDIATED HIV TRANSMISSION

Expression of CD11a/CD18 on leukocytes was determined by FACS before addition to DC. MFI: mean fluorescence intensity. n.d.: not determined. BM: bone marrow

<table>
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<th>Table I: Characteristics of LAD-1 patients</th>
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Expression of CD11a/CD18 on leukocytes was determined by FACS before addition to DC. MFI: mean fluorescence intensity. n.d.: not determined. BM: bone marrow

RESULTS

HIV-1 replication in LFA-1 negative T cells after DC-mediated transmission is delayed

To investigate the importance of the ICAM-1–LFA-1 interaction in DC-mediated HIV-1 transmission, we performed transmission experiments with DC obtained from healthy donors and peripheral blood leukocytes (PBL) from LAD-1 patients or healthy controls. We isolated leukocytes from three different LAD-1 patients, whose characteristics are given in Table 1. To confirm the negative LFA-1 status of LAD-1 leukocytes, we performed FACS analysis on CD11a and CD18, of which one representative patient and control are depicted in Fig. 1, upper two panels. We further determined by FACS that the expression of CD4 and CXCR4 was comparable to healthy controls (results not shown). In order to test the transmission efficiency to LAD-1 T cells, we used DC stimulated by poly (I:C) since this subset expresses the highest level of ICAM-1 and is the most efficient HIV-1 transmitter.
We incubated the DC with HIV-1 for 2 hr, followed by washing steps to remove unbound virus. After addition of LAD-1 or control PBL we followed HIV-1 replication by measuring the accumulation of CA-p24 in the supernatant. The replication of HIV-1 after transmission to LAD-1 PBL is delayed with several days in comparison to healthy controls (Fig. 2). Since CA-p24 levels eventually reach a similar plateau, we conclude that LAD-1 cells are susceptible to HIV-1, but that the transmission is taking place at a lower efficiency.

![Figure 1. Phenotype of LAD-1 and control PBL. Representative FACS staining for CD11a (LFA-1) and CD18 of PBL from one healthy donor (upper panel), a LAD-1 donor (# 1 from Table I) (middle panel) and a unique patient with a LFA-1 activation defect (LAD-1/variant) (lower panel). The open histograms represent the isotype controls. The mean fluorescence intensity (MFI) is indicated.](image1)

![Figure 2. HIV-1 replication in LFA-1 negative T cells after DC-mediated transmission. DC were incubated with HIV-1, followed by washing to remove unbound virus. After addition of LAD-1 and control PBL, viral replication was followed by measuring CA-p24 production in the supernatant by ELISA. We used cells of three LAD-1 and three healthy donors (n=3). Error bars represent SEM.](image2)
DC-mediated transmission to LAD-1 T cells is impaired

A quantifying and more direct way of determining the transmission efficiency is measuring intracellular CA-p24 levels. We therefore added saquinavir (SQV) to prevent subsequent rounds of viral replication after transmission. This inhibitor of the HIV-1 protease prevents proper virion assembly and maturation. By FACS we analyzed intracellular CA-p24 in T cells. To distinguish virus transmitted to T cells from HIV in DC, we co-stained with CD3 and DC-SIGN. DC and T cells tend to cluster, which would hamper an accurate estimation of transmission efficiency by FACS. We therefore added EDTA to our FACS buffer to reduce the amount of cell clustering. Indeed, the majority (95%) of the CD3 positive cells was negative for DC-SIGN, showing that only a few DC were attached to T cells during the FACS analysis (Fig. 3A). In uninfected controls of LAD-1 and healthy PBL only background percentages of CA-p24 positive T cells were scored (0.06%, Fig. 3B).

Figure 3. DC-mediated HIV-1 transmission to LFA-1 negative T cells is impaired. After DC-HIV incubation and washing, LAD-1 or control PBL were added. Cells were co-cultured in the presence of SQV to prevent production of new virions (single-cycle transmission assay). Two days after transmission, PBL were harvested and stained for CD3, DC-SIGN and intracellular CA-p24 to determine the transmission efficiency. (A) CD3 and DC-SIGN staining. (B) CA-p24⁺ CD3⁻ T cells of an uninfected sample. (C) and (D) Representative FACS staining of a healthy control and a LAD-1 patient, respectively. The percentage CA-p24⁺ CD3⁻ cells is indicated. (E) Kinetics of intracellular CA-p24 levels for one representative healthy and LAD-1 donor (n=1). Error bars represent SD. (F) Summary of HIV-1 transmission to T cells of three healthy controls and three LAD-1 patients (n=3), two days post transmission. Error bars represent SEM. One DC donor was used for all transmissions, to reduce variation. *p<0.05; **p<0.01; ***p<0.001.
Addition of HIV-1 resulted in an increase in CA-p24 positive T cells when using PBL from healthy controls (1.09%, Fig. 3C), whereas only a slight increase was observed in PBL from LAD-1 donors (0.14%, Fig. 3D). The intracellular CA-p24 levels reached a maximum two days after DC-mediated HIV-1 transmission for both control and LAD-1 PBL, which is depicted in Fig. 3E for one representative control and LAD-1 patient. On average, DC-mediated HIV-1 transmission to control T cells was nine times more efficient (Fig. 3F, n=3). These results demonstrate the transmission to LFA-1 negative cells is severely impaired, explaining the delayed replication of Fig. 2.

**Activation of LFA-1 is crucial for efficient HIV-1 transmission**

In order to efficiently bind ICAM-1, LFA-1 needs to be activated. Cellular activation by chemokines from endothelial cells or by TCR/CD3 cross-linking results in a conformational change of LFA-1 from a low to a high ligand-binding state\(^{14,23,27}\). We questioned whether LFA-1 expression by T cells is sufficient for efficient HIV-1 transmission by DC, or that additional activation of LFA-1 is necessary. To investigate this, we used cells from a unique patient with mild LAD-1 symptoms (LAD-1/variant). The leukocytes from this patient express LFA-1 (Fig. 1 and Table 1), but the integrin cannot be induced into an active conformation\(^{22}\). In comparison to the experiments with LFA-1 negative cells, we obtained similar results: HIV-1 replication in LAD-1/variant T cells after transmission was delayed (Fig. 4A) due to a lower transmission efficiency as determined in the single-cycle transmission assay (Fig. 4B) This demonstrates that LFA-1 has to be activated to a high ligand-binding state in order to mediate HIV-1 transmission. As an additional control, we infected LAD-1/variant PBL with HIV-1 in the absence of DC (Fig 4C). HIV-1 replication in CD3/CD28 stimulated PBL from the LAD-1/variant patient was comparable to healthy controls, showing that not replication but efficient transmission by DC depends on LFA-1 activation.
**Figure 4. Activation of LFA-1 is crucial for efficient HIV-1 transmission.** (A) Viral replication in T cells after DC-mediated transmission to healthy and LAD-1/variant PBL was followed by CA-p24 ELISA of the supernatant. (B) Single-cycle transmission assay. Two days after DC-mediated transmission in the presence of SQV, PBL were harvested and stained for CD3, DC-SIGN and intracellular CA-p24 to determine the percentage of HIV-1 positive T cells. Experiments were performed twice with cells from the same patient isolated on two separate occasions. Cells of two different healthy controls were used. *p<0.02 (C) LAD-1/variant PBL or control cells were stimulated with anti-CD3/CD28 antibodies and were infected with HIV-1. Viral replication was followed by CA-p24 ELISA. Error bars represent SD.

**LAD-1 and LAD-1/variant T cells form smaller clusters with DC**

DC attract T cells and form large clusters *in vivo* and *in vitro*, a process that is dependent on cell-cell adhesion\(^{28,29}\). We studied the clusters of DC and leukocytes 24 hr after DC-mediated HIV-1 transmission. The cluster size of DC with T cells from healthy individuals was clearly larger than the clusters with T cells from LAD-1 and LAD-1/variant T cells, as is shown in the photographs (Fig 5A). Quantitative determination of the amount and diameter of clusters showed that although the number of clusters was only slightly reduced (30, 26 and 25 clusters...
on average for control, LAD-1 and LAD-1/variant respectively), the mean cluster diameter of control cells was significantly larger (9.1 versus 5.6 and 6.2 in arbitrary units for LAD-1 and the variant respectively; \( p<0.001 \) and \( <0.002 \)). We subsequently grouped the clusters according to diameter (Fig. 5B), and clearly demonstrate that their actual number is not reduced for the LAD-1 and LAD-1/variant T cells, but that they are significantly smaller in size.

![Figure 5: LAD-1 and LAD-1/variant T cells form smaller clusters with DC.](image)

(A) Representative light microscopic images of DC-T cell clusters with healthy, LAD-1 and LAD-1/variant T cells, one day after HIV-1 transmission. (B) The number and diameter of DC-T cell clusters of cells from LAD-1 patients \( (n=3) \), the LAD-1/variant patient \( (n=1) \), and healthy controls \( (n=3) \) was determined one day after HIV-1 transmission, and the clusters were subsequently grouped according to size. Error bars represent standard deviations. *\( p<0.05 \); **\( p<0.01 \), compared to corresponding cluster group from ‘control’.
DISCUSSION

In the present study, we demonstrate the importance of LFA-1 for efficient HIV-1 transmission by DC. Previously we have shown that ICAM-1 expression on both monocyte-derived DC and DC from blood is critical for HIV-1 transmission\textsuperscript{10,12}. In accordance with this, we now show that DC-mediated transmission to LFA-1-negative T cells from LAD-1 patients is severely impaired. Normally, LFA-1 is activated by different kinds of stimuli, and binding to ICAM-1 is subsequently up-regulated (inside-out signaling)\textsuperscript{14,15,23,30}. Currently, it is assumed that activation of LFA-1 may be regulated via changes in affinity (active conformation), avidity (clustering) or both\textsuperscript{24,31-34}. The fact that transmission to T cells of a unique patient (LAD-1/variant syndrome)\textsuperscript{22}, with an inside-out signaling deficient LFA-1, was impaired as well, demonstrates for the first time that LFA-1 activation is crucial for DC-mediated HIV-1 transmission. Although LFA-1 of this patient is able to recognize its ligand, no high avidity/affinity binding to ICAM-1 is taking place. Since there is no strong binding to ICAM-1, signaling through LFA-1 into the T cell (outside-in signaling) is probably not taking place either. In healthy individuals, signaling through LFA-1 after ICAM-1 binding leads to actin polymerization and remodeling, which is important for enhanced cell adhesion\textsuperscript{35}. Impaired cell adhesion in LAD-1 (and variant) patients can also be illustrated by the significantly smaller clusters of DC with T cells (Fig. 5). A smaller number of T cells that is tightly attached to DC will result in a decrease of the window of opportunity for HIV-1 transmission. Furthermore, it is likely that the creation of an ‘infectious synapse’ is disturbed in LAD-1 and LAD-1/variant patients. Others have shown that DC-SIGN is an important component of the infectious synapse\textsuperscript{11,36}. Our results strongly indicate that LFA-1 is also important for infectious synapse formation, possibly through cytoskeletal rearrangements that are induced by ICAM-1 binding.

The infectivity and subsequent replication of HIV-1 in T cells can be influenced by T cell activation and proliferation. Due to the young age of the patients and severity of the disease, no more cells could be obtained from these patients to perform a separate mixed lymphocyte reaction (MLR). However, we
found no lower cellular proliferation of LAD-1 and LAD-1/variant T cells after co-culture with DC during FACS analysis, nor did we find higher percentages of dead cells. In addition, the leukocytes of the LAD-1/variant patient have been shown to proliferate normally, and have normal calcium influx, actin metabolism and protein kinase activity. Another factor influencing HIV-1 infectivity is the incorporation of host ICAM-1 in budding virions and expression of LFA-1 on target cells. To critically test this hypothesis, we performed transmission experiments with HIV-1 produced both in C33A cells and in PM1 T cells. C33A cells do not express ICAM-1 (or LFA-1), yielding virions without ICAM-1. With both virus stocks, we found impaired DC-mediated transmission to T cells of LAD-1/variant and LAD-1 patients, ruling out that the virus-producer cell is of influence. This observation is in concordance with our previous work and the work of Bounou and co-workers, who showed that in DC-mediated HIV-1 transmission, virion-associated ICAM-1 is of no influence. Furthermore, we have shown that LAD-1/variant and control T cells are equally susceptible to HIV-1 in the absence of DC, demonstrating that the DC-mediated transmission itself is impaired, instead of the ability of HIV-1 to infect these cells.

Although LAD-1 patients suffer from severe illness and usually die at a young age, they should be less susceptible to HIV-1 infection. This phenotype resembles that of a CCR5 mutant, the co-receptor of which a homozygous deletion leads to protection against sexual acquisition of HIV-1. The question remains whether there are more LFA-1 variants in the human population that are less efficient in ICAM-1 binding, yet causing even milder symptoms than the patient with LAD-1/variant syndrome whose cells we used. These persons may have a lower chance of being infected with HIV-1, so a further screen for LFA-1 polymorphisms seems of interest.

The importance of the ICAM-1–LFA-1 interaction for DC-T cell contact and HIV-1 transmission suggests a new therapeutic target for the development of transmission-blockers. Interestingly, the fungal metabolite lovastatin, which belongs to the statin compounds used in the treatment of hypercholesterolemia, was shown to bind LFA-1 and inhibit the interaction with ICAM-1. Furthermore, lovastatin was recently shown to block entry of ICAM-1-containing
HIV-1 virion particles into T cells\textsuperscript{46}. We therefore tested whether lovastatin could block DC-mediated HIV-1 transmission. Although we measured a significant decrease in HIV-1 transmission, inhibition was due to toxicity of the compound at the micromolar range that is required for blocking the ICAM-1–LFA-1 interaction (results not shown). Given the importance of LFA-1 in HIV-1 transmission by DC, future research should focus on the development of less toxic derivatives or other small molecule inhibitors of the ICAM-1–LFA-1 interaction\textsuperscript{47,48}. Now that there is proof that compounds can be generated that potently inhibit and target integrins like LFA-1\textsuperscript{49} the use of such selective oral compounds may prove very useful in preventing or treating various diseases. With respect to HIV-1 transmission, these compounds can be used in combination with other drugs in a microbicide mixture that will help slowing down the ongoing HIV-1 pandemic.

ACKNOWLEDGEMENTS

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REFERENCES

CHAPTER TWO


CHAPTER THREE

Differential susceptibility of naïve, central memory, and effector memory T cells to dendritic cell-mediated HIV-1 transmission

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CHAPTER THREE

ABSTRACT

Dendritic cells (DC) have been proposed to facilitate sexual transmission of HIV-1 by capture of the virus in the mucosa and subsequent transmission to CD4+ T cells. Several T cell subsets can be identified in humans: naïve T cells (T_N) that initiate an immune response to new antigens, and memory T cells that respond to previously encountered pathogens. The memory T cell pool comprises central memory (T_{CM}) and effector memory cells (T_{EM}), which are characterized by distinct homing and effector functions. The T_{EM} cell subset, which can be further divided into effector Th1 and Th2 cells, has been shown to be the prime target for viral replication after HIV-1 infection, and is abundantly present in mucosal tissues. In this study, we determined the susceptibility of T_N, T_{CM} and T_{EM} cells to DC-mediated HIV-1 transmission and found that co-receptor expression on the respective T cell subsets is a decisive factor for transmission. Accordingly, CCR5-using (R5) HIV-1 was most efficiently transmitted to T_{EM} cells, suggesting that mucosal T cells are an important target for DC-mediated transmission. This may contribute to the initial burst of virus replication that is observed in these cells. In contrast, CXCR4-using (X4) HIV-1 was preferentially transmitted to T_N cells, which are considered to inefficiently support HIV-1 replication. These results indicate that DC may play a decisive role in the susceptibility of T_N cells to X4 tropic HIV-1.

INTRODUCTION

Several CD4+ T cell subsets can be identified in humans: naïve T cells (T_N) to mount an immune response to a variety of new antigens, and memory T cells to respond to previously encountered pathogens. T_N cells preferentially circulate between blood and secondary lymphoid tissues, using high endothelial venules to enter lymph nodes\(^1\). The memory T cell pool comprises distinct populations of central memory (T_{CM}) and effector memory T cells (T_{EM}), characterized by distinct homing and effector function\(^2\). Like T_N cells, T_{CM} cells express CCR7 and CD62L,
two receptors required for migration to T cell areas of secondary lymphoid tissue. They furthermore have limited effector function, but can proliferate and become TEM cells upon secondary stimulation with antigen, and therefore play a role in long term protection. TEM cells have lost CCR7 expression, and home to peripheral tissues and sites of inflammation to provide immediate protection against pathogens2,3. Consequently, TN and TCM cells are primarily found in blood and lymphoid tissue, whereas TEM cells are enriched in gut, liver and lung. Within the TEM cell subset, effector Th1 and Th2 cells are recognized, which are classified by different functional properties based on unique cytokine profiles. Th1 cells produce high levels of IFNγ and TNFβ, which is instrumental in cell-mediated immunity against intracellular pathogens like viruses. Th2 cells secrete a large variety of cytokines (IL-4, IL-5, IL-9 and IL-13) that are crucial for the clearance of parasites, like helminths. Both types of effector cells play a role in the induction of a humoral (antibody) response against different extracellular pathogens4.

Sexual transmission of HIV-1 involves the crossing of mucosal tissue by the virus, and several studies have shown that one of the very first cell types encountered are intraepithelial and submucosal dendritic cells (DC). Consequently, they have been proposed to facilitate HIV-1 transmission and infection5–8. DC are professional antigen presenting cells that sample the environment at sites of pathogen entry. Sentinel immature DC (iDC) develop into mature effector DC (mDC) upon activation by micro-organisms or inflammatory signals, and migrate to the draining lymph nodes where they encounter and stimulate naïve Th cells9,10. DC are able to capture HIV-1 by a range of receptors, of which the best studied example is DC-SIGN11. Subsequent transmission to T cells takes place in lymph nodes via cell–cell contact through an ‘infectious synapse’12. Additionally, DC can support local virus replication in T cells present in the mucosal tissue7,8.

An increasing number of studies on HIV-1 and SIV demonstrate that the initial burst of viral replication takes place in CCR5+ CD4+ (effector) memory T cells in the lamina propria of mucosal tissues13–18. CCR5 and CXCR4 are the major co-receptors used by HIV-1, with CCR5 being the initial co-receptor used by the virus after transmission. This receptor is primarily expressed on the memory T cell
subset and macrophages\textsuperscript{19}. Over time, HIV-1 starts to use CXCR4 in some patients, thereby expanding its target cell repertoire to \( T_N \) cells. This coincides with faster disease progression\textsuperscript{20,21}.

Because DC play an important role in HIV-1 pathogenesis, and \( T_N \), \( T_{CM} \) and \( T_{EM} \) cells have distinct functions and locations in the body, we set out to investigate the contribution of DC in infection of these T cell subsets. We found that CCR5-using (R5) HIV-1 is efficiently transmitted to \( T_{EM} \) cells but not to \( T_N \) cells. Transmission to \( T_{CM} \) cells was of intermediate efficiency. Transmission to pure populations of Th1 or Th2 cells, or to an unbiased population containing both types (Th0) was equally efficient. The highly efficient R5 transfer to \( T_{EM} \) cells suggests that mucosal (\( T_{EM} \)) cells are an important target for DC-mediated transmission, which may contribute to the observed initial burst of virus replication in these cells. CXCR4-using (X4) HIV-1 could be transmitted to all T cell subsets, due to expression of CXCR4 on all subsets. Surprisingly, X4 HIV-1 was preferentially transmitted to \( T_N \) cells, which are considered to inefficiently replicate X4 HIV-1\textsuperscript{22-24}. This study shows that co-receptor expression is a decisive factor for DC-mediated HIV-1 transmission, and more importantly, that DC may play a crucial role in making \( T_N \) cells susceptible to X4 HIV-1 replication later in infection.

**MATERIALS AND METHODS**

**Generation of monocyte-derived dendritic cells**

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nycamed, Torshov, Norway). Subsequently, PBMC were layered on a Percoll gradient (Pharmacia, Uppsala, Sweden) with three density layers (1.076, 1.059, and 1.045 g/ml). The light fraction with predominantly monocytes was collected, washed, and seeded in 24-well culture plates (Costar, Cambridge, MA, USA) at a density of \( 5 \times 10^5 \) cells per well. After 60 min at 37\(^\circ\)C, non-adherent cells were removed, and adherent cells were cultured to obtain immature DC in Iscove's modified Dulbecco's medium (IMDM; Life
Technologies Ltd., Paisley, United Kingdom) with gentamicin (86 μg/ml; Duchefa, Haarlem, The Netherlands) and 10% fetal calf serum (HyClone, Logan, UT, USA), supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml; Strathmann Biotec AG, Hannover, Germany). At day 3, the culture medium with supplements was refreshed. At day 6, maturation was induced by culturing the DC with maturation factors only (MF; IL-1β (10 ng/ml) and TNFα (50 ng/ml); Strathmann Biotec AG), or MF with either IFNγ (1000 U/ml; Strathmann Biotec AG), or prostaglandin E2 (10−6 M; Sigma-Aldrich, St. Louis, MO), see results for more details23. After two days, mature CD14+ CD1b+ CD83+ DC were obtained. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors. Mature DC were analysed for the expression of cell surface molecules on a FACSscan (BD Biosciences, San Jose, CA, USA). Mouse anti-human mAbs were used against the following molecules: CD14 (BD Biosciences), CD1b (Diaclone, Besançon, France), CD83 (Immunotech, Marseille, France) and ICAM-1 (CD54) (Pelcluster, Sanquin, Amsterdam, The Netherlands). All mAb incubations were followed by incubation with FITC-conjugated goat F(ab′)2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

CD4+ T cells

Naïve and memory T cells were live sorted from pure CD4+ T cells on a FACS ARIA (BD Biosciences). The following mouse-anti-human antibodies were used: CD45RA-FITC (Coulter, Hialeah, FL, USA), CD45RO-APC (BD Biosciences), CD4-PE-Cy7 (BD Biosciences). Rat-anti-human CCR7 (BD Biosciences) incubation was followed by biotin-rabbit-anti-rat (Zymed Laboratories Inc., San Francisco, CA, USA) and streptavidin-PerCp-Cy5.5 (BD Biosciences) incubation. CD4+ CD45RA+ CD45R0− cells were considered naïve T cells (Tn). CD4+ CD45RA− CD45R0+ cells (the memory population) was separated into central memory (Tcm) (CCR7+) and effector memory (Tem) (CCR7−) cells, according to the classification described by Sallusto et a.25. Polarized Th1 and Th2 cells, and an unpolarized population containing both types (Th0 cells) were generated from purified Tn cells as previously described26. In short, Tn cells (10⁵/200 μl) were stimulated with
immobilized α-CD3 (CLB-T3/3; 1 µg/ml) and α-CD28 (CLB-CD28/1; 2 µg/ml) (both from Sanquin, Amsterdam, The Netherlands) and cultured for 10 days in the absence (Th0) or presence of IL-12 (100 U/ml; a gift from Dr. M. K. Gately, Hoffma-La Roche) or IL-4 (1000 U/ml) for Th1 and Th2 cells respectively. To generate fully polarized Th cells, the cells were restimulated with PHA (10 µg/ml; Difco, Detroit, MI, USA) and 3000 rad-irradiated feeder cells (PBMC of two unrelated donors and EBV-B cells (JY cells)) in the presence of IL-4 for Th0 cells; IL-4 neutralizing antibodies (CLB_IL-4/6, Sanquin) plus IL-12 for Th1 cells; and IL-12 neutralizing antibodies (U-CyTech, Utrecht, the Netherlands) plus IL-4 for Th2 cells. All T cells were cultured in IMDM with 10% FCS, gentamycin and IL-2 (Cetus, Emeryville, CA, USA). During co-culture with DC, Staphylococcus enterotoxin B (SEB; Sigma-Aldrich; final concentration, 10 pg/ml) was added. α-CD3/CD28 stimulation of T cells for viral replication experiments was done with mouse mAb to human CD28 (CLB-CD28/1) and human CD3 (CLB-T3/4E-1XE, Sanquin).

Cytokine production by polarized Th cells
12 days after the second stimulation round, resting T cells were restimulated with PMA (10 ng/ml) and ionomycin (1 µg/ml) for 6 hr, the last 4.5 hr in the presence of Brefeldin A (10 µg/ml) (all Sigma-Aldrich). Cells were fixed in 2% PFA, permeabilized with 0.5% saponin (Sigma-Aldrich), and stained with anti-IFNγ-FITC and anti-IL4-PE (both BD Biosciences). Cells were then analysed by FACS.

Virus stocks
C33A cervix carcinoma cells were transfected using calcium phosphate with 5 µg of the molecular clone of CXCR4-using HIV-1 LAI or CCR5-using HIV-1 JR-CSF. The virus containing supernatant was harvested 3 days post transfection, filtered and stored at -80°C. The concentration of virus was determined by CA-p24 ELISA. C33A cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Breda, the Netherlands), supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) (Sigma-Aldrich) and streptomycin (100 µg/ml; Invitrogen).
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HIV transmission assay and CA-p24 measurement
Fully matured DC (IFNγ/MF if indicated otherwise) were incubated in a 96-well-plate (45×10⁴ DC/100 µl/well) with HIV-1 (15 ng CA-p24/well) for 2 hr at 37°C. The DC were washed with PBS after centrifugation at 400×g to remove unbound virus. Washing was repeated 2 times, followed by addition of 50×10⁴ Tₙ, T_CM or T_EM cells. In some experiments, T1249 (250 ng/ml; Trimeris, Durham, NC, USA), RANTES (500 ng/ml, R&D Systems, Abingdon, UK) or AM3100 (10 µg/ml, Sigma-Aldrich) was added. The latter two were pre-incubated with the T cells for 30 min at 37°C. Prior to addition to DC, the T cells were analyzed by FACS with the following mouse anti-human antibodies: CD4-PE, CCR5-PE and CXCR4-PE (all BD Biosciences). Viral replication after transmission was followed by measuring CA-p24 in the culture supernatant by ELISA. To determine intracellular CA-p24 in the single-cycle transmission assay, saquinavir (Roche, London, UK at 0.2 µM) was added to prevent cell-to-cell spread of newly produced virions. After 48 hr, the T cells were harvested and stained with FITC-labeled CD3 (BD Biosciences), followed by fixation with 4% PFA and washing with washing buffer (PBS with 2 mM EDTA and 0.5% BSA). Fixated cells were then washed with perm/wash buffer (BD Biosciences), and incubated with PE-labeled CA-p24 (KC57-RD1, Coulter) followed by washing with successively perm/wash- and washing buffer. Cells were then analysed by FACS.

T cell proliferation
Fully matured DC (45×10⁴ DC/well) were incubated in a 96-well-plate with Tₙ, T_CM, T_EM cells, or polarized Th cells (50×10⁴ T cells/well) in a final volume of 200 µl. After 4 days, cell proliferation was assessed by the incorporation of [³H]-TdR after a pulse with 13 KBq/well during the last 16 hr of the co-culture, as measured by scintillation spectroscopy. Alternatively, Tₙ, T_CM or T_EM cells were stimulated with α-CD3/CD28 antibodies, followed by the [³H]-TdR pulse 4 days later.

Statistical analysis
Data were analysed for statistical significance (GraphPad InStat, Inc, San Diego, CA, USA) using ANOVA. A p value <0.05 was considered to be significant.
RESULTS

T cell subsets differ in susceptibility to DC-mediated transmission of R5 and X4 HIV-1

To investigate whether different CD4+ T cell subsets differ in their susceptibility to DC-mediated HIV-1 transmission, we isolated by live sorting highly purified populations of CD45RA+ CD45RO naïve T cells (Tn) and CD45RA- CD45RO- memory T cells from pure CD4+ T cells. Based on the expression of CCR7, a homing receptor for secondary lymphoid tissue, the memory pool was further divided in CCR7+ central memory T cells (TCM) and CCR7- effector memory T cells (TEM)2,3. We subsequently incubated DC with the R5 virus JR-CSF isolate or the X4 virus LAI isolate for 2 hr, followed by washing steps to remove unbound virus. After addition of the respective T cell subsets, we determined the transmission efficiency by measuring the accumulation of HIV-1 capsid protein p24 (CA-p24) in T cells by FACS. To prevent subsequent rounds of HIV-1 replication after transmission in this single-cycle transmission assay, we added an inhibitor of the viral protease (saquinavir27).

In a control experiment without HIV-1, no CA-p24 positive CD3+ T cells were scored (Fig. 1A). Addition of R5 HIV-1 resulted in high percentages of CA-p24+ TEM cells, and hardly any CA-p24+ Tn cells (2.9 and 0.1 %, respectively). The transmission to TCM cells was of intermediate efficiency (1.9%). With X4 HIV-1, the pattern was reversed: X4 HIV-1 was preferentially transmitted to Tn cells (4%), then to TCM cells (2.2%), and the transmission to TEM cells was least efficient (1.4%) (Fig. 1A). Overall, X4 transmission was more efficient than R5 transmission, and could take place to all subsets. For both viruses, the percentage CA-p24+ T cells reached a maximum value 2 days post transmission, and these data are quantified in Fig. 1B. This experiment demonstrates that there is not one exclusive T cell subset that is the preferred target of DC-mediated HIV-1 transmission, but that the efficiency depends on the tropism of the transmitted virus.
Figure 1. T cell subsets differ in susceptibility to DC-mediated transmission of R5 and X4 HIV-1. (A) DC were incubated with R5 or X4 HIV-1, or mock treated, followed by extensive washing to remove unbound virus. DC were subsequently co-cultured with CD4+ naïve T cells (T_N), central memory T cells (T_CM) or effector memory T cells (T_EM) in the presence of saquinavir to prevent spreading infection (single-cycle transmission assay). Two days after transmission, T cells were harvested and stained for CD3 and intracellular CA-p24 to determine the percentage HIV+ T cells. Representative FACS plots are shown. (B) Summary of one representative experiment. Error bars represent standard deviations. *p<0.05; **p<0.01; ***p<0.001.

DC-mediated HIV-1 transmission is co-receptor dependent
The different transmission patterns for R5 and X4 HIV-1 prompted us to investigate the co-receptor expression on each T cell subset (Fig. 2A). We found that the level of co-receptor expression for both CCR5 and CXCR4 correlates with
the transmission efficiencies depicted in Fig. 1B: CCR5 expression is most pronounced on T<sub>EM</sub> cells, and is undetectable on T<sub>N</sub> cells; CXCR4 is detectable on all subsets, but its expression declines from T<sub>N</sub> cells via T<sub>CM</sub> to T<sub>EM</sub> cells.

To investigate the role of co-receptor expression in DC-mediated HIV-1 transmission, we added RANTES or AMD3100 in the single-cycle transmission assay. These compounds inhibit HIV-1 infection of T cells by blocking the co-receptors CCR5 and CXCR4, respectively<sup>28,29</sup>. Transmission of HIV-1 was completely blocked through the addition of these compounds (Fig. 2B, grey bars). We furthermore could block transmission completely with inhibitor T1249 (Fig. 2B, black bars). This peptide prevents fusion of viral and cellular membranes<sup>30</sup>. Our results thus demonstrate that DC-mediated HIV-1 transmission requires ‘regular’ infection through CD4 and a co-receptor.

**Figure 2. DC-mediated HIV-1 transmission is co-receptor dependent.** (A) FACS analysis of T<sub>N</sub>, T<sub>CM</sub> and T<sub>EM</sub> cells for CD4 and co-receptors CCR5 and CXCR4. Open histograms represent isotype controls. (B) Transmission inhibition by co-receptor ligands and a fusion inhibitor. A single-cycle transmission assay to T<sub>N</sub>, T<sub>CM</sub> and T<sub>EM</sub> cells was performed with R5 and X4 HIV-1 loaded DC. Prior to co-culture with DC, the T cells were pre-incubated with ligands for CCR5 (RANTES) or CXCR4 (AMD3100) (grey bars) or alternatively, with fusion inhibitor T1249 (black bars). After 2 days, the percentage CA-p24<sup>+</sup> T cells was determined by FACS. The percentage inhibition of transmission relative to transmission without inhibitors is indicated on the y-axis. Error bars represent standard deviations.
Method of T cell stimulation determines HIV-1 susceptibility

In addition to quantification of the transmission efficiency in a single-cycle transmission assay (Fig. 1 and 2), we followed viral replication after transmission (Fig. 3). In this spreading infection assay, we did not add saquinavir to allow cell-cell spread of newly produced virus. Replication of R5 and X4 HIV-1 in T_N, T_CM and T_EM cells following DC-mediated transmission reflects the results of the single-cycle transmission assay: R5 HIV-1 preferentially replicates in memory T cells, whereas X4 HIV-1 prefers T_N cells over the memory subsets (Fig. 3A and B).

Figure 3. Spreading infection assay. Replication of R5 (A) and X4 (B) virus in T_N, T_CM and T_EM cells after DC-mediated HIV-1 transmission. Alternatively, the T cell subsets were stimulated by crosslinking CD3/CD28 with antibodies and infected with R5 (C) or X4 (D) virus. Viral replication was followed by CA-p24 ELISA on the supernatant. Error bars represent standard deviations.
CHAPTER THREE

Since this spreading infection assay involves two different steps, i.e. transmission and subsequent replication, we also studied R5 and X4 HIV-1 replication in TN, TCM and TEM cells in a DC-independent system. Therefore, cellular proliferation was induced by cross linking of CD3 and CD28 on the T cells with antibodies (Fig. 3C and D). As expected, the susceptibility of all T cell subsets to R5 HIV-1 replication was low after CD3/CD28 stimulation. This phenomenon was previously described for CD4+ T cells in general, and is the consequence of CCR5 down regulation and production of natural CCR5 ligands that compete for co-receptor binding\cite{31,32}. But despite this low replication capacity, the pattern of R5 replication was comparable to the replication after DC-mediated transmission of R5 HIV-1: replication was lower in TN cells. Surprisingly, X4 replication in Tn cells was significantly delayed in comparison to TCM and TEM cells (Fig. 3D), which does not reflect the enhanced transmission and replication in TN cells in the transmission experiments (Fig. 1 and 3B).

This discrepancy prompted us to compare HIV-1 replication in T cells stimulated by either DC or α-CD3/CD28 antibodies, without any complicating factors like transmission steps. We therefore stimulated all T cell subsets with DC, or alternatively, with α-CD3/CD28 antibodies and harvested the T cells after 4 days of proliferation. The cells were subsequently infected with X4 HIV-1. DC-stimulated TN cells were more susceptible to X4 HIV-1 replication than the memory subsets (Fig. 4A), which reflects the replication after transmission (Fig. 3B). The reverse was observed with α-CD3/CD28 stimulated T cells (Fig. 4A), which is in concordance with the results of Fig. 3D in which the cells were infected immediately after stimulation. This indicates that the enhanced replication of X4 HIV-1 in TN cells following DC-mediated transmission, is due to a higher HIV-1 susceptibility. It further demonstrates that crosslinking of CD3 and CD28 by antibodies is not comparable to DC-T cell stimulation, although this crosslinking is considered to mimic DC encounter. The difference between both stimulation methods is further manifested by the proliferative capacity of the T cells, as determined by ³H-thymidine incorporation (Fig 4B). The proliferation pattern of the different T cell subsets after DC or α-CD3/CD28 stimulation is clearly not the same.
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Figure 4. Method of T cell stimulation determines HIV-1 susceptibility. (A) Comparison of viral replication in \( T_N \), \( T_{CM} \) and \( T_{EM} \) cells that were stimulated by DC or by CD3/CD28 crosslinking with antibodies. The T cells were stimulated for 4 days, harvested and re-plated before infection with X4 HIV-1. Viral spread was followed by CA-p24 ELISA, of which the results of day 6 are shown. (B) To measure T cell proliferation, \( T_N \), \( T_{CM} \) or \( T_{EM} \) cells were incubated with DC or \( \alpha \)-CD3/CD28 antibodies and after 4 days, cellular proliferation was determined by \(^3\)H-thymidine incorporation. Error bars represent standard deviations. *\( p<0.05 \); **\( p<0.01 \); ***\( p<0.001 \).

DC transmit HIV-1 with equal efficiency to Th1 and Th2 cells, or to an unpolarized population

The \( T_{EM} \) cell subset can be further divided into effector Th1 and Th2 cells\(^4\). We generated \textit{in vitro} polarized populations of pure Th1 and Th2 cells, or an unbiased population containing both types (Th0 cells), by culturing purified \( T_N \) cells with or without IL-12 or IL-4, as previously described\(^26\). We next investigated whether HIV-1 is differentially transmitted to these subsets of effector Th1, Th2 or Th0 cells. In addition, we tested different mature DC subsets. Depending on the type of pathogen and tissue factors, immature DC develop into mature effector DC that are specialized to stimulate naïve T cells to develop into IFN\(\gamma\)-producing Th1 cells or IL-4-producing Th2 cells, designated DC1 and DC2 respectively\(^33\). DC0 induce an unpolarized response (Th0). DC0, DC1 and DC2 were generated by culturing immature DC with maturation factors (MF, IL-1\(\beta\) and TNF\(\alpha\)) only (DC0), or MF with either IFN\(\gamma\) (DC1) or prostaglandin E\(_2\) (DC2)\(^25\).

The intracellular cytokine profiles of the effector Th cell populations were analyzed by FACS (Fig 5A). The Th1 population consists primarily of IFN\(\gamma\) producers, whereas the Th2 population contains mostly IL-4 producers. The
unpolarized Th0 population is composed of both cell types. All T cell subsets expressed similar levels of CCR5 and CXCR4, and proliferated to a comparable extent, as determined by ³H incorporation (results not shown). DC0, DC1 and DC2 were subsequently incubated with R5 and X4 HIV-1, followed by washing and addition of Th0, Th1 and Th2 cells. Two days later, the transmission efficiency was determined in the single-cycle transmission assay (Fig. 5B).

**Figure 5. DC transmit HIV-1 with equal efficiency to Th0, Th1 and Th2 cells.** (A) In vitro generated polarized populations of Th1 and Th2 cells, or an unbiased population (Th0), were analyzed for intracellular cytokines IFNγ and IL-4 by FACS. The percentage single and double positive cells is indicated. (B) Th0, Th1 and Th2 cells were co-cultured with R5 or X4 virus-loaded DC in a single-cycle transmission assay to determine the transmission efficiency. Different DC subsets were used: DC1 that stimulate Tn cells to develop into Th1 cells, DC2 that induce Th2 cells, or DC0 that induce an unpolarized response (Th0). The percentage CA-p24⁺ T cells was determined by FACS 2 days post transmission. Error bars represent standard deviations. *p<0.05; **p<0.01; ***p<0.001.
Consistent with Fig. 1B, R5 virus was a bit more efficiently transmitted to these polarized T<sub>EM</sub> cells than X4 HIV-1. More importantly, we found no significant differences in HIV-1 transmission efficiency to Th0, Th1 or Th2 cells within one DC subset, <i>i.e.</i> a particular DC subset transmits HIV-1 with equal efficiency to Th0, Th1 or Th2 cells. We also did not find a preference of HIV-1 transmission by a DC subset and its corresponding Th type: DC1 was the most efficient HIV-1 transmitter in all cases. The latter was previously demonstrated by us, using unpolarized peripheral blood leukocytes (PBL) and T cell lines<sup>24</sup>. We now show that this also applies to polarized Th subsets.

**DISCUSSION**

T<sub>N</sub>, T<sub>CM</sub> and T<sub>EM</sub> cells have distinct functions and locations in the body<sup>1,2</sup>, which may have, combined with the differential expression of HIV-1 co-receptors, an impact on HIV-1 transmission and infection. Since DC play an important role in HIV-1 pathogenesis, we studied DC-mediated transmission of R5 and X4 virus to the different T cell subsets, and found that R5 HIV-1 is preferentially transmitted to T<sub>EM</sub> cells, whereas DC transmit X4 HIV-1 most efficiently to the T<sub>N</sub> subset.

It is known that R5 viruses are primarily transmitted between individuals and that X4 viruses emerge only later in infection<sup>19,35</sup>. An increasing number of studies on HIV-1 and SIV demonstrate that the initial burst of viral replication takes place in CCR5<sup>+</sup> CD4<sup>+</sup> (effector) memory T cells in the lamina propria of the mucosa<sup>13-18</sup>. Later in infection, proviral DNA can be isolated from both naïve and memory CD4<sup>+</sup> T cells<sup>36,37</sup>. The mechanism responsible for R5 predominance early in infection is not known. One proposed mechanism is the exclusive transport of R5 viruses over the epithelial barrier by epithelial CCR5<sup>+</sup> cells<sup>38</sup>. Moreover, DC were proposed to be responsible due to the preferential replication of R5 HIV-1<sup>39-41</sup>, although this R5 replication is not entirely exclusive<sup>42-45</sup>. In addition, DC do not need to be productively infected to transmit HIV-1 to T cells<sup>46,47</sup>, and DC can transmit both X4 as R5 HIV-1 to T cells<sup>41</sup>. In fact, we demonstrate in this study that X4 virus is generally transmitted more efficiently than R5 virus. Therefore,
DC are probably not the ‘gatekeepers’ that select R5 viruses, although their role in sexual transmission is a crucial one.\textsuperscript{7,8}

One of the remaining questions is whether DC either facilitate local HIV-1 replication, or transport the virus to the lymph nodes, or both\textsuperscript{7,8,19}. R5 HIV-1 is efficiently transmitted to T\textsubscript{CM} cells (Fig. 1), which are primarily present in lymphoid tissue, and even more efficiently to T\textsubscript{EM} cells, which are abundantly present at sites of viral entry in the mucosa. This suggests that transmission can take place at both locations.

Although X4 HIV-1 is very efficiently transmitted to T\textsubscript{S} cells, X4 virus does not emerge in recently infected HIV patients. Thus, DC-mediated X4 HIV-1 transmission to T cells may not take place following sexual transmission, or may not be a factor of relevance. DC may nonetheless play an important role later in infection (when X4 virus emerges), e.g. by making T\textsubscript{S} cells susceptible to X4 HIV-1 as we have shown in this study.

We furthermore subdivided T\textsubscript{EM} cells into Th1 and Th2 cells, which did not reveal more differences. DC transmit HIV-1 with equal efficiency to Th1 or Th2 cells, or to an unbiased population containing both types (Th0). Reports on the ability of R5 and X4 virus to replicate in Th0, Th1 or Th2 cells are not univocal\textsuperscript{48-51}. Based on our results, the type of T\textsubscript{EM} cell (Th0, 1 or 2) is not of importance for susceptibility to DC-mediated HIV-1 transmission, although the state of activation is an important (though not decisive) factor\textsuperscript{52-54}. Furthermore, antigen specific T cells may be preferred\textsuperscript{55}.

We have shown here that the decisive factor for efficient HIV-1 transmission to the different T cell subsets is co-receptor expression. These HIV-1 transmission results with DC are in concordance with other studies that have shown \textit{in vivo} and \textit{ex vivo} the correlation between differential expression of CCR5 and CXCR4 on naïve and memory T cells and HIV-1 susceptibility\textsuperscript{56-58}. We are the first to further divide the memory T cell pool into populations of effector and central memory T cells. We furthermore found that the presence of DC seems to enhance HIV-1 infection and replication, but does not change the pattern of susceptibility. Under certain conditions, no correlation was found between co-receptor expression and HIV-1 susceptibility. When the T cells were stimulated with \textalpha-CD3/CD28
antibodies, replication of X4 HIV-1 in T<sub>N</sub> cells was restricted in comparison to the memory subsets. We therefore compared stimulation of T cells by α-CD3/CD28 with stimulation by DC, and found differences in T cell proliferation and X4 susceptibility.

Crosslinking CD3 and CD28 by antibodies is a commonly used laboratory method for T cell stimulation, and mimics T cell activation through triggering of these molecules by DC-bound MHC-II and CD80/86, respectively. However, many more interactions play a role in DC-T cell interaction and stimulation, e.g. CD30L-CD30; OX40L-OX40; 41BBL-41BB; CD70-CD27; ICOSL-ICOS; CD40-CD40L and ICAM-1-LFA-1<sup>10,33,59,60</sup>. Each of these interactions could have an influence on the replication capacity of HIV-1 in T cells, and some of these interactions therefore are the subject of further study. Our results demonstrate that DC play a vital role in priming T<sub>N</sub> cells to become susceptible to HIV-1, and that α-CD3/CD28 stimulation is not a very good model for DC stimulation in the context of HIV-1 studies.

In conclusion, we have shown that DC transmit R5 and X4 HIV-1 with different efficiencies to T<sub>N</sub>, T<sub>CM</sub> and T<sub>EM</sub> cells, and that this correlates with co-receptor expression of the different T cell subsets. The highly efficient transmission of R5 HIV-1 to T<sub>EM</sub> cells, which are abundantly present at sites of viral entry, may contribute to the observed burst of viral replication in these cells after HIV-1 infection. Later on in infection, DC may play an important role in the replication of X4 HIV-1 in T<sub>N</sub> cells.

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Opposing roles of blood myeloid and plasmacytoid dendritic cells in HIV-1 infection of T cells: transmission facilitation versus replication inhibition

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CHAPTER FOUR

ABSTRACT

CD11c⁺ myeloid dendritic cells (MDC) and CD11c⁻ CD123⁺ plasmacytoid DC (PDC) have been identified as main human DC subsets. MDC are professional antigen-presenting cells for T cells, and include Langerhans cells, dermal DC and interstitial DC. They have been associated with HIV-1 capture and sexual transmission, whereas PDC play an important role in the innate immune responses to different types of viruses, including HIV-1. To compare the influence of MDC and PDC on HIV-1 infection of T cells, we isolated donor-matched MDC and PDC from peripheral blood, activated them by adding different maturation-inducing compounds, and co-cultured them with T cells and HIV-1. We found that MDC enhance HIV-1 infection through capture of the virus and subsequent transmission to T cells, and that differently matured MDC subsets have different HIV-1 transmission efficiencies. These differences were not due to soluble factors, viral capture differences or the expression of integrins ICAM-1, -2, -3 or LFA-1. In contrast, regardless of their state of maturation, PDC inhibit HIV-1 replication in T cells through the secretion of IFNα and an additional, unidentified small molecule. This study shows that the two main types of DC have opposing roles in HIV-1 infection of T cells.

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells that play an important role in the initiation of immune responses. Immature DC develop into mature effector DC upon activation by micro-organisms or inflammatory signals, and migrate to the draining lymph nodes where they encounter and stimulate naive Th cells. HIV-1 has been proposed to make use of this process, being captured by DC in mucosal tissues and delivered to the lymph node, which then becomes the principal site of virus replication. In humans, two main DC subsets are found: CD11c⁺ myeloid DC (MDC) and CD11c⁻ CD123⁺ plasmacytoid DC (PDC). MDC include Langerhans cells, dermal DC and interstitial DC, and are
found in blood, skin and mucosal tissues and have been associated with HIV-1 capture and sexual transmission. PDC are located in blood and secondary lymphoid organs but they can be recruited to sites of inflammation. The difference between MDC and PDC is furthermore manifested by differential expression of Toll-like receptors (TLRs) and secretion of different cytokines: MDC are known to secrete high levels of interleukin-12 (IL-12) whereas PDC are thought to play an important role in innate immune responses to different types of viruses by producing IFNα. Several studies have shown that the numbers of both MDC and PDC are substantially reduced in the blood of patients infected with HIV-1 and that the DC are functionally impaired with respect to T cell proliferation and cytokine production. In addition, both types of DC have been shown to be susceptible to HIV-1 infection, and to be able to transmit the virus to CD4+ T cells in vitro.

In this study, we set out to compare the influence of differently matured MDC and PDC on HIV-1 infection of T cells. A large proportion of DC research is being performed with monocyte-derived DC, which represent the MDC subset. However, several differences have been described for these in vitro generated DC and MDC isolated from blood or skin. In addition, there is no in vitro PDC model available. Therefore, we isolated donor-matched MDC and PDC directly from blood and investigated their influence on HIV-1 infection by co-culturing them with T cells. We found remarkable differences between MDC and PDC. MDC enhance HIV-1 infection through capture of the virus and subsequent transmission to T cells. Differently matured MDC subsets exhibit distinct HIV-1 transmission efficiencies, and secreted factors by MDC did not modulate the transmission of HIV-1 to T cells. In contrast, irrespective of their maturation status, PDC inhibit HIV-1 replication in T cells by secretion of IFNα and an additional, heat-sensitive small molecule of smaller than 3 kDa. This study shows that the two main DC subsets found in blood have opposing roles in respect to HIV-T cell infection.
CHAPTER FOUR

MATERIALS AND METHODS

Isolation and culturing of DC
Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nycoderm, Torshov, Norway). Subsequently, MDC and PDC were isolated using magnetic bead isolation and AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). For MDC isolation, PBMC were depleted from B cells using anti-CD19 microbeads (Miltenyi Biotec) followed by positive selection with mouse-anti-human BDCA-1-PE and anti-PE-microbeads (Miltenyi Biotec). PDC isolation was performed with mouse-anti-human BDCA-4-PE and anti-PE microbeads (Miltenyi Biotec). Prior to incubation with CD19/BDCA-1/BDCA-4 antibodies the cells were pre-incubated with FcR-block for 5 min (Miltenyi Biotec). Culturing was done in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies Ltd., Paisley, United Kingdom) with gentamicin (86 µg/ml; Duchefa, Haarlem, The Netherlands) and 10% fetal calf serum (HyClone, Logan, Utah) at a density of 35×10³ DC/well. The medium was supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) or IL-3 (10 ng/ml; Strathmann Biotech AG, Hannover, Germany) for MDC and PDC respectively. Maturation of the DC was induced by culturing the cells for 24 hr with the following factors, provided alone or in combination as indicated in the text: maturation factors (MF; LPS (100 ng/ml, Difco, Detroit, MI, USA), IL-1β (10 ng/ml; Strathmann Biotech AG) and TNFα (50 ng/ml; Strathmann Biotech AG)), poly (I:C) (20 µg/ml; Sigma-Aldrich, St. Louis, MO), IFNγ (1000 U/ml; Strathmann Biotech AG), R-848 (2 µg/ml, Invivogen, San Diego, CA, USA) or fixed Staphylococcus Aureus Cowan strain 1 bacteria (SAC, 25 µM; Calbiochem, La Jolla, CA, USA). Each experiment in this study is a representative of 4-8 different donors.

Flow cytometry
All MDC and PDC fractions were tested for purity (>90%) with CD11c-APC (BD Biosciences, San Jose, CA, USA), lineage-markers (Lin-1-FITC, BD Biosciences) and CD19-FITC (Dakopatts, Glostrup, Denmark). After maturation, DC were analyzed for the expression of cell surface molecules by FACS. Mouse anti-human
mAbs were used against the following molecules: BDCA-1-PE, BDCA-4-PE, CD11c-APC, CD11a-FITC (LFA-1) (Pelicluster, Sanquin, Amsterdam, The Netherlands), CD83-APC, CD86-FITC, CD4-APC, ICAM-3-FITC (all BD Biosciences) or biotinylated ICAM-1 or ICAM-2 (R&D Systems, Abingdon, United Kingdom) followed by Streptavidin-PerCP-Cy5.5 (BD Biosciences). Samples were analyzed on a FACScan (BD Biosciences).

**Virus stocks and T cells**

C33A cervix carcinoma cells were transfected using calcium phosphate with 5 ng of the molecular clone of CXCR4-using HIV-1 LAI and CCR5-using JR-CSF. The virus containing supernatant was harvested 3 to 5 days post transfection, filtered and stored at −80°C. The concentration of virus was determined by CA-p24 ELISA. PM1 cells, which are susceptible to CXCR4- and CCR5-using HIV-1²⁴, were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies), supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) (Sigma-Aldrich) and streptomycin (100 µg/ml) (Invitrogen, Breda, The Netherlands). The reporter cell line LuSIV was kindly donated by Janice E. Clements (Johns Hopkins University School of Medicine, Baltimore, MD, USA). This CEMx174 derived cell line contains the firefly luciferase reporter gene downstream of the SIVmac239 LTR. Infection by HIV-1 results in Tat-mediated expression of luciferase which can be measured 24 hr later²⁵. Cells were maintained in the same medium as PM1 cells, but with 300 µg/ml hygromycin B to maintain the luciferase construct. For experiments with DC, hygromycin B-free medium was used.

**Single-cycle replication assay with LuSIV cells**

35×10³ MDC and PDC were stimulated with maturation-inducing compounds for 24 hr as indicated in the text. DC were subsequently incubated with HIV-1 LAI (3 ng CA-p24/well) and 40×10³ LuSIV cells for 24 hr. Alternatively, only the supernatant of 24 hr stimulated MDC or PDC was incubated with LuSIV cells and HIV-1. After 24 hr of co-culture, luciferase was measured as described.
previously\textsuperscript{26}, 40×10\textsuperscript{3} LuSIV cells grown without DC or HIV-1 were used to obtain the background luciferase value, which was subtracted from all data.

**MDC transmission assay**
35×10\textsuperscript{3} matured MDC were incubated for 2 hr with 5 ng CA-p24 HIV-1 LAI, followed by two washing steps in the plate to remove unbound virus. DC were then co-cultured with LuSIV cells for 24 hr, after which luciferase was measured. Control or blocking antibodies against ICAM-1 or -3 (20 μg/ml, Immunotech, Marseille, France) were pre-incubated with DC and LuSIV cells for 30 min at 37°C before co-culture. Background luciferase level of LuSIV cells grown without HIV-1 was subtracted from all data.

**MDC CD40-ligation**
35×10\textsuperscript{3} MDC that were stimulated as indicated in the text were washed twice to remove all soluble factors. DC were then co-cultured with 35×10\textsuperscript{3} CD40 ligand (CD40L)-expressing mouse plasmacytoma cells (J558 cells; a gift from Dr. P. Lane, University of Birmingham, Birmingham, UK). Supernatants were harvested after 24 hr and added to 40×10\textsuperscript{3} LuSIV cells and 3 ng CA-p24 of HIV-1 LAI. Luciferase levels were measured after 24 hr. Background luciferase level of LuSIV cells grown without HIV-1 was subtracted from all data.

**HIV-1 capture by MDC**
MDC that were stimulated with different maturation-inducing compounds for 24 hr as indicated in the text were incubated in a sterile FACS-tube (100×10\textsuperscript{3} DC/100 μl) with 5 ng CA-p24 HIV-1 LAI for 4 hr at 37°C. After centrifugation at 400×g, the DC were washed with PBS to remove unbound virus. This step was performed three times, followed by lysis of the cells and CA-p24 ELISA to determine the amount of HIV-1 capture.

**HIV-1 replication with PDC supernatant**
60×10\textsuperscript{3} PM1 T cells were infected with 1 ng of CA-p24 HIV-1 LAI or 2 ng JR-CSF with or without supernatant of SAC-stimulated PDC in a final volume of 250 μl.
We used 80 µl PDC supernatant/well, or made dilutions as indicated in the text. In some cases, supernatant and T cells were pre-incubated for 30 min with blocking antibodies against type I interferons27 (a kind gift of Dr Julkunen, National Public Health Institute, Helsinki, Finland) as indicated in the text. Viral replication was followed by CA-p24 ELISA of the supernatant.

**Size fractionation of PDC supernatant**

To separate supernatant of SAC-stimulated PDC into fractions of 0-3 kDa, 3-10 kDa, 10-30 kDa, 30-100 kDa and >100 kDa, Microcon Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, USA) were used. 80 µl of each fraction was incubated with 150 µl LuSIV cells and 3 ng CA-p24 HIV-1 LAI, followed by luciferase measurement after 24 hr. Background luciferase level of LuSIV cells grown without HIV-1 was subtracted from all data. The obtained fractions were analyzed for the presence of IFNα by ELISA with a detection limit of 10 pg/ml (coating antibody, detection antibody and standard were obtained from R&D systems, Endogen (Etten-leur, The Netherlands) and Roche (Nutley, NJ, USA) respectively). Blocking antibodies against type I IFNs were used as described above in a 1:100 dilution. Heat inactivation of the different fractions was done at 50, 75 or 100°C for 20 min.

**Statistical analysis**

Data were analyzed for statistical significance (GraphPad InStat, Inc, San Diego, CA, USA) using ANOVA. A p value <0.05 was considered to be significant.

**RESULTS**

**MDC enhance, whereas PDC inhibit, HIV-1 infection of T cells**

To compare the influence of MDC and PDC on HIV-1 infection of T cells, donor-matched MDC and PDC were isolated from peripheral blood and matured by addition of several stimulating compounds. MDC were cultured in medium only (immature DC), or matured with poly(I:C), R-848, fixed Staphylococcus aureus
Cowan strain I bacteria (SAC) or IFNγ plus maturation factors (MF; LPS, IL-1β and TNFα). PDC were cultured in medium only, or with poly(I:C), R-848 and SAC. After 24 hr, the DC were co-cultured with HIV-1 LAI and the reporter T cell line LuSIV, which has an HIV-1 inducible promoter cloned upstream of the luciferase gene. HIV-1 infection of these cells results in luciferase production, which can be measured after 24 hr. Since no cell-to-cell spread of newly produced virus occurs, this single-cycle replication assay represents an effective way of determining HIV-1 infectivity.

To determine the basal amount of luciferase production induced by HIV-1 in the absence of DC, LuSIV cells were infected with HIV-1 with the different culture media. The luciferase levels were comparable for all different maturation-inducing compounds, meaning that they did not influence the induction of luciferase production by HIV-1 (Fig. 1A and B, white bars). Please note that the scales of Fig 1A and B are different. The presence of DC had opposite effects on HIV-1 infectivity: MDC strongly stimulated HIV-1 infection, whereas PDC reduced HIV-1 infection to below the basal levels (Fig. 1A and B, black bars). Furthermore, MDC matured by R-848 were significantly more efficient in HIV-1 stimulation than MDC matured by other compounds (p<0.01). To test whether the stimulating and inhibitory effects of MDC and PDC were mediated by secreted factors, LuSIV cells were infected with HIV-1 in the presence of the supernatant of matured DC. Interestingly, the stimulating effect of MDC was not reproduced with the supernatant of MDC, but supernatant from PDC was sufficient to reduce luciferase levels (Fig. 1A and B, grey bars). These results demonstrate that both DC subsets have opposing effects on HIV-1 infection of T cells, mediated through different mechanisms.

**MDC do not stimulate HIV-1 infection through secreted factors**

The results shown in Fig. 1A demonstrate that the stimulating effect of MDC is not mediated through a factor that is secreted during DC maturation. However, T cells can activate DC via CD40 ligand (CD40-L)-CD40 signaling, leading to increased expression of co-stimulatory molecules CD80/CD86 and cytokine release. It is therefore still possible that the nature of the enhancing effect of MDC is a factor...
Figure 1. MDC enhance, whereas PDC inhibit, HIV-1 infection of T cells. (A and B) Donor-matched MDC (A) and PDC (B) from peripheral blood were differently stimulated with maturation-inducing compounds, as indicated on the x-axis. After 24 hr, the DC were co-cultured with HIV-1 and reporter LuSIV cells. HIV-1 infection of these cells results in luciferase production, which was measured after 24 hr (black bars). LuSIV cells were also infected in the presence of only supernatant of stimulated DC (grey bars). Please notice the different scales of the graphs. To determine basal luciferase induction by HIV-1 in the absence of DC or DC supernatant, LuSIV cells were infected with the respective culturing media only (white bars). *p<0.05; **p<0.01 compared to culturing medium. (C) Factors secreted by MDC following T cell encounter are not responsible for the stimulation of HIV-1 infection. MDC were cultured in medium only (left), or were matured either with R-848 (middle) or IFNγ plus MF (right), and were subsequently cocultured with CD40L-expressing J558 cells to mimic T cell encounter. After 24 hr, the supernatant was collected and incubated with LuSIV cells and HIV-1, followed by luciferase measurement one day later. *p<0.01; **p<0.001 compared to ‘no DC’. Background luciferase levels of LuSIV cells grown without HIV-1 was subtracted from all data. RLU, relative light units. Error bars represent standard deviations.
that is secreted after T cell engagement. To critically test this alternative explanation, we co-cultured differently matured MDC with a CD40L-expressing cell line to mimic T cell encounter. After 24 hr, the supernatant was collected and incubated with LuSIV cells and HIV-1, followed by luciferase measurement one day later. We found that MDC supernatant after CD40 ligation did not stimulate HIV-1 infection (Fig. 1C). This demonstrates that in order to enhance HIV-1 infection, MDC must be present and that secreted factors do not play a role.

**Differences in HIV-1 stimulation are not due to differences in HIV-1 capture**

Interestingly, differently matured MDC vary in their ability to stimulate HIV-1 infection: MDC matured with R-848, a stimulator of TLR7 and TLR8\(^8\), are most efficient in stimulation of HIV-1 infection in co-culture experiments without washing steps (Fig. 1A/C). All mature MDC expressed comparable levels of maturation/activation markers CD83/86 (data not shown). Also in a transmission assay, in which the MDC were washed after HIV-1 incubation to remove unbound virus and other soluble compounds, the R-848-matured MDC are more efficient in

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**Figure 2.** MDC matured with R-848 demonstrate enhanced HIV-1 transmission capacity, which is not due to increased viral capture. (A) MDC were cultured in medium only, or with R-848 or IFN\(\gamma\)/MF for 24 hr, followed by HIV-1 incubation and extensive washing to remove all factors. DC were subsequently co-cultured with LuSIV cells to allow HIV-1 transmission. Luciferase was measured after 24 hr. *p<0.01, compared to medium and IFN\(\gamma\)/MF maturation. RLU, relative light units. (B) Differently matured MDC as indicated on the x-axis were incubated for 4 hr with HIV-1, followed by extensive washing to remove unbound virus. Viral capture was subsequently determined by lysis of the cells and CA-p24 ELISA. Error bars represent standard deviations. N.S. not significant.
HIV-1 stimulation (Fig. 2A). This shows that MDC stimulate HIV-1 infection of T cells through capture and transmission of the virus. To investigate whether differences in HIV-1 capture are responsible for this effect, differently matured MDC were incubated for 4 hr with HIV-1, followed by extensive washing to remove unbound virus. Viral capture was subsequently determined by lysis of the cells and CA-p24 ELISA. We found no differences in the amount of HIV-1 capture (Fig. 2B), showing that the increased transmission by R-848-matured MDC must act at a later step.

ICAM-1 expression is required for MDC-mediated transmission

We have previously shown that the interaction between the integrins ICAM-1 on DC and LFA-1 on T cells is crucial for HIV-1 transmission. Mature monocyte-derived DC that express higher levels of ICAM-1 have an enhanced capacity to transmit HIV-1\(^{27}\), and transmission to LFA-1 negative T cells is impaired\(^{30}\). The ICAM-1–LFA-1 interaction plays a key role in the initiation of immune responses by strengthening the adhesion between DC and T cells at the immunological synapse\(^{31,32}\). To explore the possibility of increased integrin expression on R-848-matured MDC, differently matured MDC were analyzed by FACS (Fig. 3A-D). MDC matured by R-848 do indeed express more ICAM-1, but MDC matured with IFN\(\gamma\)/MF express even higher levels (Fig. 3A). These data imply that there must be additional reasons for the superiority of R-848-matured MDC. LFA-1 can also interact with ICAM-2 or -3\(^{33,34}\), but no expression of ICAM-2 was found on MDC, whereas ICAM-3 was not differently expressed on matured MDC, suggesting that the latter is not responsible for the enhanced transmission by R-848 MDC (Fig. 3B and C). LFA-1 is expressed on T cells, but can also be found on DC\(^{35}\). However, we found no differences in LFA-1 expression either (Fig. 3D). To confirm our previous work on the importance of the ICAM-1–LFA-1 interaction, blocking antibodies were used to test the importance of this molecule in HIV-1 transmission by blood MDC. We pre-incubated HIV-1 loaded DC with antibodies against ICAM-1 or -3 and co-cultured them with LuSIV cells. Addition of blocking antibodies against ICAM-1 severely decreased transmission, whereas blocking antibodies against ICAM-3 did not (Fig. 3E). These results show that ICAM-1 is still a prerequisite for DC-mediated HIV-1 transmission to T cells, but that there must be another factor responsible for the increased transmission by R-848-matured MDC.
PDC supernatant inhibits replication of both CXCR4- and CCR5-using HIV-1

PDC supernatant inhibits HIV-1 in the single-cycle replication assay (Fig. 1B). We next investigated whether this supernatant could inhibit HIV-1 over an extended period of time during a spreading infection in T cells. We therefore infected the PM1 T cell line with HIV-1 in the presence of supernatant from SAC-stimulated PDC. HIV-1 replication was followed by measuring CA-p24 accumulation in the supernatant by ELISA. Both CXCR4- and CCR5-using HIV-1 were significantly inhibited 10-fold by PDC supernatant in this spreading assay (Fig. 4A; p<0.01), and the supernatant could be diluted 10 times before it lost its inhibitory properties (Fig. 4B). This spreading assay however could underestimate the actual inhibitory capacity of the supernatant, because the inhibitor is gradually lost due to possible degradation and necessary refreshment of the medium. We therefore tested the supernatant dilutions also in the single-cycle replication assay, and found that the supernatant could be diluted 100 times without losing its inhibitory properties (Fig. 4C).
Figure 4. PDC supernatant inhibits replication of both CXCR4- and CCR5-using HIV-1. (A) The PM1 T cell line was infected with CXCR4-using LAI (filled markers) or CCR5-using JR-CSF (open markers) in the presence (triangles) or absence (diamonds) of supernatant from SAC-stimulated PDC. HIV-1 replication was followed by measuring CA-p24 accumulation in the supernatant with ELISA. (B) and (C): PM1 T cells (B) or LuSIV cells (C) were infected with CXCR4-using HIV-1 LAI in the presence of PDC, or PDC supernatant dilutions. RLU, relative light units. Error bars represent standard deviations. *p<0.01; **p<0.001 compared to medium control.

Type I interferons are partially responsible for the inhibition of HIV-1 replication

A very likely candidate HIV-1 inhibitor in the supernatant of PDC is the family of type I interferons (IFNs), including IFNα. These antiviral molecules are produced in large amounts by PDC and have been shown to inhibit replication of several viruses including HIV-1. With ELISA, we found that SAC- and poly(I:C)-stimulated PDC secrete high levels of IFNα (3925 ± 75 and 4295 ± 104 pg/ml respectively). We therefore tried to counteract the inhibitory effect of PDC supernatant using well-described blocking antibodies against type I IFNs. Both PM1 T cells and PDC supernatant were pre-incubated with the antibodies before HIV-1 infection, followed by co-culture for several days during which HIV-1 replication was monitored by measuring CA-p24 accumulation in the supernatant. Neutralization of type I IFNs partially restored virus replication, as shown for the
day 5 sample of the replication curve (Fig. 5). Even at twice the optimal antibody concentration, we found no further restoration of HIV-1 replication, suggesting that type I IFNs are only partially responsible for the inhibitory effect of PDC supernatant on HIV-1 replication.

**Figure 5. Type I interferons are partially responsible for the inhibition of HIV-1 replication by PDC supernatant.** PM1 T cells and PDC supernatant were pre-incubated with type I IFN blocking antibodies (or a control antibody), followed by HIV-1 infection and subsequent replication of the virus. A control experiment was performed with mock supernatant (DC culturing medium). Viral spread was followed by CA-p24 ELISA, of which the results of day 5 are shown. Antibodies were used in a recommended 1:100 dilution, or at twice that concentration (1:50). Error bars represent standard deviations. *p<0.001; **p<0.0001 compared to respective controls with mock supernatant. # p<0.001

**PDC secrete an additional inhibitory factor of <3 kDa**
To confirm the presence of an additional inhibitory factor in PDC supernatant, a size fractionation of the supernatant was performed using centrifugal filters. The resulting fractions of <3, 3-10, 10-30, 30-100 and >100 kDa were subsequently tested for their capacity to inhibit HIV-1 infection in the single-cycle replication assay with LuSIV cells. The <3 kDa, 30-100 and >100 kDa fractions inhibited HIV-1 infection significantly, whereas the fractions between 3 and 30 kDa did not (black bars Fig. 6A). All fractions were tested for the presence of IFNα by ELISA (grey bars Fig. 6A). The 30-100 kDa fractions contained high levels of IFNα, as
well as the >100 kDa fraction, probably because this molecule (∓20 kDa) is present as dimers or complexed with other molecules or serum components. More importantly, the <3 kDa fraction contained no IFNα (Fig 6A), and addition of neutralizing antibodies against type I IFNs did not reverse the inhibitory properties of this fraction (Fig. 6B).

Figure 6. PDC secrete an additional HIV-1 inhibitory factor of <3 kDa. (A) PDC supernatant was size fractionated by filter centrifugation into fractions of <3, 3-10, 10-30, 30-100 and >100 kDa. These fractions were incubated with LuSIV cells and HIV-1 for 24 hr, followed by luciferase measurement (black bars). All fractions were furthermore tested for the presence of IFNα by ELISA (grey bars). *p<0.0001; **p<0.00001 compared to control ‘HIV-1 only’. (B) LuSIV cells and the <3 kDa and 30-100 kDa fractions were pre-incubated with type I IFN blocking antibodies, followed by co-culture and HIV-1 infection. Luciferase was measured 24 hr later. *p<0.01 compared to controls with mock supernatant; # p<0.01. N.S. not significant. RLU, relative light units. Error bars represent standard deviations.
A full restoration of HIV-1 infectivity was obtained when the antibodies were used to neutralize the type I IFNs in the 30-100 kDa fraction, confirming the functioning of the antibodies (Fig 6B). Both IFNα and the small molecule inhibitor are heat-sensitive, since incubating the respective fractions at 50, 75 or 100°C resulted in loss of inhibitory properties (Fig. 7). This suggests that the inhibitory factor is a small polypeptide.

![Figure 7](image.png)

**Figure 7. The inhibitory factor of <3 kDa is heat-sensitive.** Size fractions of PDC supernatant were incubated at 50, 75 or 100°C for 20 min, followed by incubation with LuSIV cells and HIV-1 for 24 hr. RLU, relative light units. Error bars represent standard deviations. *p<0.05; **p<0.01 compared to control ‘HIV only’

**DISCUSSION**

In the present study, we compared differently matured MDC and PDC from peripheral blood for their influence on HIV-1 infection of T cells. Opposing effects were scored: MDC strongly promote HIV-1 infection, and PDC severely inhibit HIV-1 replication. The former finding has been described before<sup>40</sup> and we extended these findings here by showing that soluble factors secreted during MDC maturation or following T cell encounter do not influence this process. To the best of our knowledge, no comparison has been made of differently matured blood
MDC and their ability to transmit HIV-1. We have previously shown that differently matured monocyte-derived DC have different transmission efficiencies, which correlates with differences in ICAM-1 expression\(^9\). Here we show that the same rule did not apply to blood MDC: MDC matured with R-848 enhance HIV-1 infection twice as efficient as MDC matured with SAC, IFN\(\gamma\)/MF or poly (I:C), which does not perfectly correlate with ICAM-1 expression. Although we could confirm that ICAM-1 is a prerequisite for MDC-mediated HIV-1 transmission (Fig 3E), an additional factor seems to contribute to the increased HIV-1 transmission by R-848-matured MDC. This factor is not another integrin like ICAM-2, ICAM-3 or LFA-1, and the amount of viral capture by R-848 MDC was comparable to that of immature MDC or IFN\(\gamma\)/MF-matured MDC. One possible explanation is that HIV-1 is degraded to a smaller extent after capture by R-848 MDC. Significant viral degradation occurs after viral capture\(^{41}\), but a proportion of the virus survives by residing in a non-lysosomal compartment followed by transmission to T cells\(^{42,43}\). Maturation of MDC with R-848 could possibly change the balance between degradation and survival of HIV-1. Consequently, R-848-matured MDC could direct HIV-1 more efficiently or in larger amounts towards the infectious synapse\(^{43,44}\). More research on the differences between MDC matured with R-848 and other compounds is necessary and may lead to the identification of novel factors that play a role in HIV-1 transmission by DC.

R-848 is a synthetic compound that stimulates TLR7/8\(^{28}\) for which the natural ligand has been proposed to be single-stranded RNA\(^{45}\). HIV-1 is a single-stranded RNA virus and our results therefore suggest that HIV-1 may enhance its own transmission through engagement with TLR7/8. However, HIV-1 by itself does not (fully) activate monocyte-derived DC or blood MDC\(^{20,46,47}\). For efficient HIV-1 transmission, MDC need additional inflammatory factors or pathogens for full maturation.

In contrast to MDC, PDC inhibit HIV-1 replication. Previously, others have shown that PDC are able to transmit HIV-1 to T cells\(^{21}\), a finding that seems to contradict our results. However, our experiments involved DC-T cell co-cultures without washing of the DC, such that all inhibitory factors remain present during the whole experiment. We identified IFN\(\alpha\) as one of these factors, which is known
to decrease the replication of several viruses including HIV-1\textsuperscript{12,36-38}. Inhibition of HIV-1 by IFNα in PDC supernatant has been described\textsuperscript{48}, but we now demonstrate that PDC secrete at least one additional inhibitory factor that is smaller than 3 kDa. This factor is heat-sensitive, but remains to be identified. Some candidate inhibitors can be excluded. The group of chemokines that block the co-receptor, thereby preventing HIV-1 entry into T cells\textsuperscript{49}, are too large. Furthermore, PDC do not secrete the SDF-1 ligand for CXCR4 and very little of the RANTES/MIP-1α/β ligands for CCR5\textsuperscript{50}. Indeed, the PDC supernatant inhibits CXCR4- and CCR5-using HIV-1 infection to the same extent (Fig. 4A).

Another group of possible candidates are antimicrobial peptides. These small peptides (1-5 kDa) are secreted by different cell types and are active against a broad range of bacteria, fungi and viruses\textsuperscript{51,52}. With respect to HIV-1, three inhibitory mechanisms of antimicrobial peptides have been described: direct virolysis, inhibition of transcription from the LTR promoter and block of entry by binding to cell surface molecules\textsuperscript{53-56}. The production of antimicrobial peptides by PDC has not been studied extensively. One study reported small amounts of intracellular human β-defensin 1 (hBD-1) in PDC from some donors\textsuperscript{57}. Another group showed that monocyte-derived MDC harbor low amounts of mRNA for hBD-1 and hBD-2\textsuperscript{58}. hBD-1, and hBD-2 in particular, have been shown to inhibit HIV-1 replication by an unidentified mechanism\textsuperscript{59}. In our study, we found that PDC of some donors express marginal amounts of mRNA for hBD-1 and hBD-2, and that no mRNA for another antimicrobial peptide, LL-37, was detected (S.J.P. Bogaards & P.H. Nibbering, unpublished results). For this reason, it is not very likely that hBD-1 or hBD-2 is the additional HIV-1 inhibitor in PDC supernatant. It cannot be excluded that other antimicrobial peptides are responsible for the inhibition. It would be of interest to perform a further search for this inhibitor and investigate whether the factor inhibits other viruses as well. Not only will this broaden our knowledge on the antiviral response of PDC, but it could also create novel therapeutic options.

The differential impact of MDC and PDC on HIV-1 infection may reflect the different location and function of these cells in the human body. MDC constantly sample the outside milieu to detect pathogens and form a direct link between
OPPOSING ROLES OF DC SUBSETS IN HIV INFECTION

innate and adaptive immunity\(^2\). It is known for several years that they stimulate HIV-1 infection\(^{40}\), and it has been proposed that they transmit the virus to T cells following migration to secondary lymphoid organs\(^{40}\). Their role in HIV-1 infection is therefore better understood than the role of PDC. It is not very likely PDC are similarly involved in HIV-1 capture in mucosal tissues. PDC are not located in high amounts at sites of pathogen entry, they do not capture, endocytose and process antigens as effectively as MDC, and their role in T cell proliferation is less pronounced\(^{11}\). In contrast, PDC are involved in the innate immune response against many viruses, including HSV-1 and -2, influenza, VSV and HIV-1\(^{11}\). Their role in HIV-1 infection may be to control the virus in order to delay disease progression. The numbers of both MDC and PDC in blood of HIV-1 infected patients are reduced\(^{13,14,16,18}\). Since both types of DC can be infected by HIV-1\(^{19,20,61}\), this reduction can be the result of depletion. Alternatively, they may have relocated to secondary lymphoid tissue as a consequence of HIV-1 infection\(^{62,63}\). Both types of DC isolated from HIV-1 patients were reported to be functionally impaired with respect to T cell stimulation and cytokine production\(^{15,17}\). It remains to be established whether this impairment is a cause or an effect of progressing HIV-1 infection, but at least the various data suggest that MDC may have a dual role in HIV-1 infection: initially MDC may help to establish HIV-1 infection, but later on, progression towards AIDS is correlated with a loss of MDC numbers and a decreased antigen presenting capacity to T cells.

In conclusion, we have shown that MDC and PDC have opposing effects on HIV-1 infection, and subsequent replication in T cells. MDC enhance HIV-1 infection by facilitating transmission, PDC inhibit infection by secreting factors that inhibit HIV-1 replication. These differences should be taken into account when studying the role of DC in HIV-1 pathogenesis.

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Lactoferrin prevents dendritic cell-mediated HIV-1 transmission by blocking the DC-SIGN—gp120 interaction

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CHAPTER FIVE

ABSTRACT

One of the cell types first encountered by HIV-1 following sexual transmission are dendritic cells (DC). DC capture HIV-1 through C-type lectin receptors, of which the best studied example is DC-SIGN, which mediates HIV-1 internalization. DC can keep the virus infectious for several days and are able to transmit HIV-1 to CD4+ T cells. We tested proteins from milk and serum for their ability to block DC-mediated HIV-1 transmission, of which bovine lactoferrin (bLF) is the most potent inhibitor. bLF binds strongly to DC-SIGN, thus preventing virus capture and subsequent transmission. Interestingly, bLF is a much more efficient inhibitor of transmission than human LF (hLF). Since bLF is non-toxic and easy to purify in large quantities, it forms an interesting candidate microbicide against HIV-1. Another advantage of bLF is its ability to block HIV-1 replication in T cells. DC-mediated capture of a bLF resistant HIV-1 variant that was selected during long-term culturing in T cells could still be blocked by bLF. This underscores the usefulness of bLF as a microbicide drug to prevent HIV-1 transmission.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infects CD4+ T cells through interaction of its envelope protein gp120 with CD4 and a chemokine co-receptor on the target cell1. However, sexual transmission of HIV-1 first requires the crossing of mucosal tissue, and the precise mechanism of HIV-1 transmission across this barrier is poorly understood. One possibility is that HIV-1 enters via lesions caused by other diseases2. However, studies with rhesus macaques show that lesions are not absolutely required for transmission and that simian immunodeficiency virus (SIV) can cross the intact epithelium3-5. One of the cell types first encountered by HIV-1/SIV are intraepithelial and submucosal dendritic cells (DC)3-9. If not via lesions, virus uptake could occur via their processes that extend into the luminal surface. DC are professional antigen presenting cells that sample the environment at sites of pathogen entry. Sentinel immature DC (iDC)
develop into mature effector DC (mDC) upon activation by micro-organisms, and migrate to the draining lymph nodes where they stimulate naïve Th cells\textsuperscript{10,11}. HIV-1 has been proposed to make use of this migratory process, being captured by the DC and delivered to the lymph node where the virus is transmitted to CD4\(^+\) T cells. The lymph node then becomes the principal site of virus production\textsuperscript{12,13}. DC capture HIV-1 through C-type lectin receptors, of which the best studied example is DC-SIGN (CD209) that mediates HIV-1 internalization such that the virus remains infectious for several days\textsuperscript{14,15}. Subsequent transmission to T cells takes place via an ‘infectious synapse’, but virus that has not been internalized can also be transmitted to T cells\textsuperscript{14,16}.

Milk proteins are known to cover a wide range of biological functions. For example, milk proteins and derivates thereof show antifungal, antibacterial and antiviral properties\textsuperscript{17-22}. Positively charged macromolecules can inhibit the binding of HIV-1 to the CD4 receptor\textsuperscript{23,24} and negatively charged macromolecules can inhibit HIV-1 by binding to the positively charged V3 loop of gp120\textsuperscript{25}. Most of these proteins need to be chemically modified in order to become inhibitory, but native lactoferrin (LF) inhibits HIV-1 replication in T cells\textsuperscript{17,19}. LF binds strongly to the V3 loop of gp120, but it may also bind to the (co)receptors on the target cell\textsuperscript{18,25}. LF is a protein of approximately 80 kDa, consisting of two symmetric lobes (N- and C-lobe) and is thought to be an important component of the innate immune system\textsuperscript{26,27}. Prior HIV-1 inhibition studies were performed in HIV-1 replication assays. In the present study, we tested proteins from milk and serum for their ability to block DC-mediated HIV-1 transmission. We found that bovine LF (bLF) is the most potent inhibitor. bLF binds to DC-SIGN, thus preventing virus capture and subsequently transmission. Interestingly, bLF is a much more efficient inhibitor of transmission than human LF (hLF). Since LF is non-toxic, available in large quantities and inexpensive, it forms an interesting candidate microbicide against HIV-1. The usefulness of bLF is underscored by the fact that DC-mediated transmission of a bLF resistant HIV-1 variant, which was selected during replication studies, can still be blocked by bLF.
CHAPTER FIVE

MATERIALS AND METHODS

Generation of monocyte-derived dendritic cells
Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Subsequently, PBMC were layered on a Percoll gradient (Pharmacia, Uppsala, Sweden) with three density layers (1.076, 1.059, and 1.045 g/ml). The light fraction with predominantly monocytes was collected, washed, and seeded in 24-well or 6-well culture plates (Costar, Cambridge, Mass.) at a density of 5x10^5 cells or 2.5x10^6 per well respectively. After 60 min at 37°C, non-adherent cells were removed, and adherent cells were cultured to obtain iDC in Iscove's modified Dulbecco's medium (IMDM; Life Technologies Ltd., Paisley, United Kingdom) with gentamicin (86 µg/ml; Duchefa, Haarlem, The Netherlands) and 10% fetal calf serum (HyClone, Logan, Utah) and supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml; Strathmann Biotec AG, Hannover, Germany). At day 3, the culture medium with supplements was refreshed. At day 6, maturation was induced by culturing the cells with the following factors alone or in combination as indicated in the text: IL-1β (10 ng/ml; Strathmann Biotec AG), TNF-α (50 ng/ml; Strathmann Biotec AG), poly (I:C) (20 µg/ml; Sigma-Aldrich, St. Louis, MO), IFN-γ (1000 U/ml; Strathmann Biotec AG), or PGE2 (10^-6 M; Sigma-Aldrich). After two days, mature CD14^+ CD10^+ CD83^+ DC were obtained. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors.

Flow cytometry
Mature DC were analysed for the expression of cell surface molecules by FACS. Mouse anti-human mAbs were used against the following molecules: CD14 (IgG2b; BD Biosciences, San Jose, CA), CD1b (B-B5, IgG1; Diaclone, Besançon, France), CD83 (HB15a, IgG2b; Immunotech, Marseille, France), CD86 (1G10, IgG2a; Innogenetics, Ghent, Belgium) and ICAM-1 (CD54) (R&D Systems, Abingdon, United Kingdom). All mAb incubations were followed by incubation with FITC-conjugated goat F(ab')2 anti-mouse IgG and IgM (Jackson Immunoresearch
Laboratories, West Grove, PA). Samples were analysed on a FACScan (BD Biosciences).

**Virus stocks and cells**
The SupT1 T cell line was transfected by electroporation with 5 μg of the molecular clone of T-tropic HIV-1 LAI. The virus containing supernatant was harvested 3 to 5 days post transfection, filtered and stored at −80°C. The concentration of virus was determined by CA-p24 ELISA. SupT1 cells were maintained in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The LuSIV cell with an integrated LTR-luciferase reporter construct has been described in reference 28. Cells were maintained in the same medium as the SupT1 cells, but with 300 μg/ml hygromycin B to maintain the luciferase genetic construct. One day before the transmission experiments, the cells were split 1:3 in fresh medium without hygromycin B.

**Milk proteins and derivates**
bLF, C-lobe bLF, HSA, Hep1-HSA, Suc-HSA, βLG, 3HP-βLG and nisin Z were prepared as described below. bLF was obtained from Borculo Domo Ingredients (Beilen, The Netherlands). Hydrolysis of 5% bLF by trypsin (TPCK-treated, obtained from Sigma Chemical Co., St. Louis) was performed at a bLF/trypsin ratio of 1/20 for 20 hr at 37°C in 0.05 M citrate-bicarbonate buffer with 5 mM CaCl₂ and 5 mM FeCl₃ at pH 8.0. The peptide mixture was further purified using reversed-phase-HPLC²⁹. The purified C-lobe (obtained as a peak eluting between 23-27 min) was analysed by means of N-terminal sequencing and by mass spectrometry (Quattro II, Micromass, Cheshire, UK). This C-lobe was identified as the fragment of bLF consisting of residue 342-689, to which iron was still bound. HSA, consisting of at least 95% monomeric protein, was obtained from the Central Laboratory of the Blood Transfusion Services (Sanquin). HSA was covalently modified with heparin and succinic acid anhydride to yield Hep1-HSA and Suc-HSA, respectively³⁰. βLG was purified as described previously³¹. 3HP-βLG was produced through modification of βLG with 3-hydroxyphthalic anhydride as
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described\textsuperscript{17}. Nisin Z was produced and purified as described\textsuperscript{32}. hLF was expressed in transgenic rice as described previously\textsuperscript{33,34}. In brief, a synthetic hLF gene with a signal sequence was linked to the rice glutelin 1 promoter and transformed to rice cells to produce recombinant hLF.

**Single-cycle transmission assay**

Fully matured DC were incubated in a 96-well-plate (35-50\times10^3 DC/50 µl/well) with virus (5 ng CA-p24/well) for 2 hr at 37°C. The DC were washed with PBS after centrifugation at 400\timesg to remove unbound virus. Washing was repeated, followed by addition of 50\times10^3 LuSIV cells. All candidate transmission inhibitors were pre-incubated either with DC, virus or LuSIV cells for 30 min at 37°C. After 24 hr, LuSIV cells were harvested for luciferase measurement. The transmission assay from DC to SupT1 cells has been described elsewhere\textsuperscript{35}. After washing of DC, SupT1 T cells were added and co-cultured. Viral replication was followed by measuring CA-p24 in the supernatant with ELISA.

LuSIV cells were collected in eppendorf tubes, spun down at 2300 rpm and resuspended in 50 µl lysisbuffer (25 mM Tris-HCl 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% TritonX100). The cells were incubated for 45 min at room temperature while shaking, followed by 10 min centrifugation at 16000\timesg. The supernatant was transferred to a white solid 96 well plate (Corning Costar), and 150 µl of luci-buffer (100 µg/ml BSA, 6.6 mM ATP, 15 mM MgSO4, 25 mM glycyglycine) was added. The Lumistar control was used to determine the amount of luciferase. 100 µl of DE(-)Luciferin (Roche Diagnostics GmbH) was injected per well (0.28 mg/ml luci-buffer excluding ATP). 50\times10^3 LuSIV cells grown without DC or HIV-1 were used to obtain the background luciferase value, which was subtracted from all data.

**HIV-1 capture by DC**

Fully matured DC were incubated in a 96-well-plate (35\times10^3 DC/50 µl/well) with hLF or medium for 30 min, followed by incubation with virus for 2 hr at 37°C. After centrifugation at 400\timesg, the DC were washed with PBS to remove unbound
virus. This step was performed twice and was followed by CA-p24 ELISA to determine the amount of HIV-1 captured by the DC.

**DC-SIGN adhesion assay**

This assay has been described elsewhere\(^3\). To test bLF, HSA, βLG, 3HP-βLG and nisin Z for their ability to inhibit the gp120-DC-SIGN interaction, gp120 (1 μg/ml) was coated onto an ELISA plate for one hr at 4°C, followed by incubation with the candidate inhibitors and soluble DC-SIGN-Fc (1.5 μg/ml) for 30 min at room temperature. After washing, binding of DC-SIGN to gp120 was measured by anti-IgG1 ELISA. To determine the DC-SIGN affinity for different forms of LF, we coated different amounts of the bLF and hLF variants (and 1 μg/ml gp120 as a positive control) onto an ELISA plate and measured the binding of soluble DC-SIGN-Fc after 30 min incubation at room temperature.

**RESULTS**

**Native bLF inhibits DC-mediated HIV-1 transmission**

We tested several modified and native proteins from human serum and bovine milk for their ability to inhibit *in vitro* HIV-1 transmission from dendritic cells (DC) to T cells. We have previously shown that DC subsets differ significantly in their ability to transmit HIV-1 to SupT1 T cells\(^5\). Based on their ability to induce a Th1 or Th2 response, mature DC (mDC) are designated DC1 and DC2 respectively\(^6\). For our experiments, we used the DC1 subset that is most efficient in HIV-1 transmission. 50×10^3 DC were incubated with a candidate inhibitor for 30 min at 37°C, followed by two hr incubation with HIV-1. The DC were washed twice to remove inhibitors and unbound virus and co-cultured with SupT1 T cells. Virus transmission and subsequent spread in the T cell culture was monitored by measuring CA-p24 in the supernatant with ELISA. First, we tested 10 μM native β-lactoglobulin (βLG), βLG modified by 3-hydroxyphthalic anhydride (3HP-βLG), native human serum albumin (HSA) and HSA modified by 13 kD heparin (Hep1-HSA) (Fig. 1A).
Figure 1. Inhibitory properties of modified and native proteins from human serum and bovine milk on DC-mediated HIV-1 transmission. (A) Mature DC were incubated with candidate inhibitors for 30 min at 37°C, followed by 2 hr incubation with HIV-1. The DC were washed twice to remove unbound virus and subsequently co-cultured with SupT1 T cells. Virus transmission and subsequent replication was measured by CA-p24 ELISA at day 6. We tested βLG and HSA as well as chemically modified forms thereof (3HP-βLG and Hep1-HSA) at 10 μM.

(B): Inhibition by native bLF and the modified proteins 3HP-βLG and Suc-HSA. Mature DC were pre-incubated with a concentration range of these proteins (0-10 μM), followed by 2 hr incubation with HIV-1, washing and co-culturing with SupT1 T cells. Virus transmission and subsequent replication was measured by CA-p24 ELISA at day 4.

Only the modified 3HP-βLG protein was able to inhibit DC-mediated HIV-1 transmission to SupT1 T cells, most likely because of its negative charge that binds to the positively charged HIV-1 envelope gp120. We next tested native bovine lactoferrin (bLF), which was compared to the negatively charged compounds 3HP-βLG and succinylated HSA (Suc-HSA) at different concentrations (Fig 1B). Suc-HSA was not as efficient in blocking transmission as 3HP-βLG. Most importantly, we measured a complete block of HIV-1 transmission and subsequently replication with 2 μM native bLF protein. We therefore decided to focus on the mechanism of bLF inhibition.

**Single-cycle transmission assay to quantify HIV transmission efficiency**

The transmission experiments shown in Fig. 1 involve DC-mediated transmission and multiple subsequent rounds of virus replication in SupT1 T cells. Thus, it cannot be formally excluded that the inhibitors target this latter process, and not
the DC-mediated transmission to T cells. We therefore set up a single-cycle transmission assay with the reporter LuSIV cells, CEMx174 derived cells that contain the firefly luciferase reporter gene downstream of the SIVmac239 LTR. This cell line is susceptible for infection by HIV/SIV, which results in Tat-mediated expression of luciferase. When the LuSIV cells are harvested within 24 hr for luciferase measurement, there is no significant T cell spread of newly produced HIV-1 virions, such that luciferase activity is a quantitative measure of the amount of virus that is transmitted by DC. We first tested this new transmission assay with three different subsets of mature DC: two types of DC1 (matured by poly I:C or interferon γ plus LPS and maturation factors IL-1β and TNF-α), and one DC2 type (matured by PgE2 and LPS plus maturation factors). Consistent with previous results, both DC1 subsets are much more efficient in transmitting HIV-1 than DC2 cells (Fig. 2). Further inhibition experiments were performed with the most efficient DC1 subset, stimulated with poly I:C.

![Figure 2. Single-cycle HIV-1 transmission assay.](image)

Figure 2. Single-cycle HIV-1 transmission assay. Immature DC were stimulated for 48 hr with poly (I:C), IFNγ plus LPS/IL-1β/TNFα and PgE2 plus LPS/IL-1β/TNFα to produce two types of DC1 and one type of DC2 respectively. The DC were incubated for 2 hr with different amounts of HIV-1 (0.3, 3 and 30 ng CA-p24), followed by washing steps to remove unbound virus. DC were co-cultured with reporter LuSIV cells and luciferase activity was measured after 24 hr. RLU: relative light units, error bars indicate SD.
bLF inhibits transmission more efficiently when pre-incubated with DC

To study the mechanism of bLF-mediated inhibition of transmission by DC, we varied the moment of bLF addition in the single-cycle transmission assay. We compared bLF pre-incubation of DC with pre-incubation of reporter LuSIV cells (Fig. 3A). DC were incubated for 30 min at 37°C at different bLF concentrations. This pre-incubation was followed by a two hr incubation with HIV-1, two times washing to remove unbound virus and bLF, and co-culturing with LuSIV cells. This resulted in severe inhibition of HIV-1 transmission. Instead, when the LuSIV cells were pre-incubated with bLF before the co-culture with DC-bound HIV-1, we observed only inhibition at the highest bLF concentration. The results indicate that bLF is a more effective inhibitor of transmission when incubated with DC rather than with LuSIV cells. This suggests that bLF interferes with the DC-HIV interaction. Next, we examined whether bLF exerts its inhibitory effect through interaction with the DC or the virus (Fig. 3B). Either DC or HIV were pre-incubated for 30 min with bLF, followed by mixing and incubation for two hr.

Figure 3. bLF blocks transmission when incubated with DC. (A) DC were incubated with HIV-1 for 2 hr, followed by washing and co-culturing with LuSIV cells. We either pre-incubated DC or the target LuSIV cells with bLF at 1, 10 or 100 µM. (B) DC or HIV-1 were pre-incubated at 1, 10 or 100 µM bLF, followed by mixing and incubation for 2 hr. After washing, DC were co-cultured with LuSIV cells. RLU: relative light units, error bars indicate SD.
After washing twice, the DC were co-cultured with LuSIV cells. The results clearly show that bLF inhibits more potently when it is pre-incubated with DC, suggesting a cellular target.

bLF prevents DC-mediated HIV-1 capture
bLF could have an indirect impact on DC function. We tested this by culturing immature DC at 10 and 100 µM bLF for two days (with or without LPS plus maturation factors (IL-1β/TNFα) or poly I:C). We measured no induction of apoptosis (annexin/PI staining), and bLF did not influence cytokine production (IL-6, IL-12 p70, TNFα), DC maturation (CD83 expression) or the expression level of the costimulatory molecule CD86. bLF did also not effect ICAM-1 expression on the DC, which is an important factor for DC-mediated HIV-1 transmission\(^{35}\). In addition, bLF did not bias naïve T cell outgrowth induced by the differentially cultured DC (results not shown). Thus, it seemed more likely that bLF interferes directly with DC-HIV interaction. To test this possibility, we measured the ability of DC to capture HIV-1 in the presence of bLF (Fig. 4). DC were pre-incubated for 30 min with 0, 1, 10 or 100 µM bLF, followed by HIV-1 incubation and washing steps. Half of the DC population was subsequently co-cultured with LuSIV reporter cells to determine the transmission efficiency. The other half was used to measure the amount of captured HIV-1 by CA-p24 ELISA. The amount of transmission and virus capture is set at 100% for the control incubation without bLF. The drop in transmission efficiency coincides with inhibition of HIV-1 capture, demonstrating that bLF blocks virus-cell contact.

![Figure 4. bLF blocks DC-mediated HIV-1 capture. DC were pre-incubated at 0, 1, 10 or 100 µM bLF for 30 min, followed by incubation with HIV-1 for 2 hr. After washing, half of the DC population was co-cultured with LuSIV cells for 24 hr. The other half was used to quantify the amount of captured HIV-1 by CA-p24 ELISA. The amount of transmission and virus capture is set at 100% for the mock-incubation without bLF. Error bars indicate SD.](image-url)
bLF blocks the DC-SIGN—gp120 interaction

Knowing that DC-SIGN plays an important role in the capture of HIV-1 by monocyte-derived DC, it is possible that bLF interacts with this surface molecule and thus interferes with HIV-1 binding. This was tested directly in a DC-SIGN—gp120 interaction assay (Fig. 5A). Gp120 was coated onto an ELISA plate and bLF or control proteins (HSA, βLG, 3HP-βLG, nisin Z) were added together with soluble DC-SIGN-Fc. After washing, the binding of DC-SIGN to gp120 was determined with anti-IgG1 ELISA. This experiment clearly shows that bLF blocks the interaction between gp120 and DC-SIGN.

**Figure 5. bLF blocks the DC-SIGN—gp120 interaction.** (A) gp120 was coated onto an ELISA plate. bLF and control proteins (HSA, βLG, 3HP-βLG, nisin Z) were added, as well as soluble DC-SIGN-Fc. After washing, the binding of DC-SIGN to gp120 was determined by anti-IgG1 ELISA. (B) Adhesion of DC-SIGN-Fc to coated gp120 was determined in the presence of bLF or DC-SIGN blocking antibody AZN-D1, or after bLF and AZN-D1 had been pre-incubated with gp120, followed by washing. Error bars indicate SD.
Next, we tested whether bLF acts by binding either DC-SIGN or gp120 (Fig. 5B). Using the same type of binding assay, we performed time-of-addition experiments with bLF and a blocking antibody against DC-SIGN (AZN-D1)\(^\text{37}\). The adhesion of soluble DC-SIGN-Fc to coated gp120 could be blocked by bLF and AZN-D1. However, we measured no inhibition when bLF or AZN-D1 were pre-incubated with the coated gp120, followed by washing and subsequent addition of DC-SIGN-Fc. These results suggest that bLF binds the DC-SIGN molecule.

**bLF binds DC-SIGN and is the most potent HIV-1 transmission inhibitor**

We next compared several LF variants for their inhibitory potential in the single-cycle transmission assay. We tested bLF, the C-lobe fragment of bLF, human LF (hLF), iron-depleted hLF (apo hLF) and iron-saturated hLF (holo hLF) (Fig. 6A). bLF is the most potent transmission inhibitor in comparison to the other variants. Partial inhibition was observed for the C-lobe fragment of bLF, which consists of the C-terminal residues 342-689 to which iron is still bound. The iron-depleted hLF showed no inhibitory capabilities, whereas the iron-saturated variant did inhibit transmission to a small extent. This suggests that iron binding and protein conformation is important for inhibition. The transmission results correlate with the ability of these proteins to inhibit HIV-1 capture by DC (results not shown). In addition, we tested these LF variants for their ability to bind DC-SIGN in a direct binding assay (Fig. 6B). We coated several amounts of the LF variants onto an ELISA plate and measured the adhesion of soluble DC-SIGN-Fc to the plate. gp120 was included as a positive control. Both bLF and the C-lobe appear to bind DC-SIGN. None of the hLF variants bind DC-SIGN. The blocking antibody AZN-D1 could prevent binding of DC-SIGN-Fc, demonstrating the specificity of the assay.
Figure 6. LF variants and inhibition of transmission. (A) DC were pre-incubated with bLF, the C-lobe fragment of bLF, human LF (hLF), iron-depleted hLF (apo hLF) and iron-saturated hLF (holo hLF). After HIV-1 incubation and washing, DC were co-cultured with LuSIV cells. The results of 8 independent experiments are combined; transmission without inhibitor was set at 100%. Error bars indicate SD. (B) Binding of LF variants to DC-SIGN. Several amounts of the LF variants were coated onto an ELISA plate and the adhesion of soluble DC-SIGN-Fc was measured by anti-IgG1 ELISA. At the highest concentration LF, blocking antibody AZN-D1 was also added. As a positive control, we coated gp120.

bLF blocks DC-mediated capture of bLF resistant HIV-1

During previous prolonged culturing of HIV-1 in the presence of bLF, a resistant HIV-1 variant was selected\(^{18}\). The envelope protein of this variant contains two mutations (T188I, G431R) that have been suggested to play a role in the virus-cell interaction. Since HIV-1 replication involves other virus-cell interactions than DC-mediated transmission, we tested whether DC-mediated capture of the bLF
resistant HIV-1 could be blocked by bLF (Fig. 7). DC were pre-incubated with bLF or mock-treated and binding of wild-type or bLF-resistant HIV-1 was determined by CA-p24 ELISA. Both viruses can be captured by DC, indicating that the resistance mutations in the envelope do not interfere with DC-SIGN binding. Most important, bLF can block DC-mediated capture of the resistant HIV-1. This indicates that different bLF-mediated inhibitory mechanisms affect the processes of HIV-1 transmission and replication.

**Figure 7. DC-mediated capture of bLF resistant HIV-1.** DC that were pre-incubated with bLF or medium were incubated with wild-type HIV-1 or bLF resistant HIV-1, which was selected during prolonged culturing in the presence of bLF. After washing twice, virus capture by DC was measured by CA-p24 ELISA. Error bars indicate SD.

**DISCUSSION**

To slow the HIV-1 pandemic, preventing HIV-1 from establishing a persistent infection after sexual intercourse may be more effective than treating HIV-1 seropositive patients with anti-retroviral medicines. In countries where women are not in the position to negotiate condom usage, alternatives like microbicides that can be applied before intercourse should be considered. Sexual transmission of HIV-1 requires the help of DC in the mucosal tissues. DC capture HIV-1 through a range of receptors, of which DC-SIGN is the best studied; it mediates HIV-1 internalization and transmission to T cells. Upon activation by microorganisms, DC migrate to the draining lymph nodes where they can transmit HIV-1 to CD4+ T cells. The lymph node then becomes the principal site of virus production.
In this study, we investigated the inhibitory capacity of several proteins from bovine milk and human serum to block HIV-1 transmission from DC to T cells. Proteins become potent inhibitors of virus replication upon chemical modification that introduces negative charges. We now demonstrate that these proteins also inhibit DC-mediated HIV-1 transmission. Interestingly, we also found that lactoferrin from bovine milk (bLF) blocks DC-mediated HIV-1 transmission in its native form. Time of addition experiments with bLF in a single-cycle transmission assay showed that the inhibitory effect of bLF is mediated through the DC, and not through the virus or the target T cells. We therefore tested whether bLF had any effects on DC physiology that could explain the loss of transmission capacity. bLF does not induce apoptosis in DC and we found no influence of bLF on the maturation status of DC or the expression of ICAM-1 and the co-stimulatory molecule CD86. We also did not find any effect of bLF on naïve T cell stimulation or cytokine production (IL-6, IL-12 p70, TNFα) (results not shown). These results are consistent with our alternative explanation that bLF inhibits attachment of HIV-1 to the DC. Indeed, DC pre-incubation with bLF could inhibit HIV-1 capture, and bLF blocked the adhesion of soluble DC-SIGN to gp120. The effect of bLF was mediated through DC-SIGN binding, as pre-incubation of coated gp120 with bLF followed by washing and subsequent addition of soluble DC-SIGN did not result in blocking. Direct binding of DC-SIGN to bLF is demonstrated in Fig. 6B, in which we coated bLF and measured DC-SIGN binding.

We tested LF variants for their ability to block HIV-1 transmission by DC. We compared bLF with the C-lobe fragment of bLF, human LF (hLF), iron-depleted hLF (apo hLF) and iron-saturated hLF (holo hLF). All LF variants could block DC-mediated HIV-1 transmission to a certain extent (except apo hLF), but bLF was the most potent inhibitor. This remarkable result is in accordance with HIV-1 replication studies, where bLF also has a higher anti-HIV-1 activity than hLF\textsuperscript{19,20}. The inhibition of transmission by the different LF variants correlates with the ability of these proteins to inhibit HIV-1 capture by DC (results not shown).

Both bLF and the C-lobe fragment bind equally well to DC-SIGN, but the C-lobe fragment is less efficient in blocking transmission of HIV-1. The complete bLF may be more efficient than C-lobe in shielding DC-SIGN due to steric
hindrance. Besides this, conformation of the LF protein might also be crucial since iron depleted hLF has no inhibitory capability. Iron binding by LF is accompanied by substantial conformational changes between the open apo form and the closed holo form\textsuperscript{40,41}. hLF and holo hLF are partially able to block DC-mediated transmission of HIV-1, but the adhesion experiment shows no binding to DC-SIGN. Native LF isolated from milk is known to interact with a range of viruses\textsuperscript{18-20,42}, and it is possible that hLF and holo hLF prevent HIV-1 binding to DC through alternative routes. Moreover, HIV-1 binding to monocyte-derived DC predominantly takes place via DC-SIGN, but other C-type lectins such as the mannose receptor and an unidentified trypsin resistant C-type lectin may also play a role\textsuperscript{43-46}.

bLF is abundantly available, easy to purify and toxicity and immunogenicity problems of this native protein may be limited. It is therefore a candidate microbicidal to prevent sexual HIV-1 transmission, possibly in combination with other compounds like entry inhibitors or neutralizing antibodies\textsuperscript{47,48}. Future research in this direction should determine the half-life of mucosally applied bLF, as well as the minimal required concentration to prevent SIV transmission \textit{in vivo} in the macaque model. Besides blocking transmission by DC, bLF has the advantage of blocking HIV-1 via interactions with the virus itself and with the target T cells\textsuperscript{17-19,25}. In case of lesions in the mucosal tissue, bLF could thus also prevent initial rounds of HIV-1 replication in submucosal CD4\textsuperscript{+} T cells. Although prolonged \textit{in vitro} culturing of HIV-1 in the presence of bLF led to the selection of escape variants\textsuperscript{18}, we could still block DC-mediated capture of this mutant with bLF. Using a microbicidal that interferes both with HIV-1 transmission and replication might be a promising protective strategy.

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LACTOFERRIN PREVENTS DC-MEDIATED HIV TRANSMISSION

Histatin 5-derived peptide with improved fungicidal properties enhances HIV-1 replication by promoting viral entry

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CHAPTER SIX

ABSTRACT

Antimicrobial peptides are found in a number of body compartments and are secreted at mucosal surfaces, where they form part of the innate immune system. Many of these small peptides have a broad spectrum of inhibitory activity against bacteria, fungi, parasites, and viruses. Generally, the peptide’s mode of action is binding and disruption of membranes due to its amphipatic properties. Histatin 5 is a salivary peptide that inhibits Candida albicans, an opportunistic fungus that causes oropharyngeal candidiasis in a majority of HIV-1 infected patients progressing towards AIDS. Previously, we increased the fungicidal properties of Histatin 5 by replacing amino acids in the active domain of Histatin 5 (Dh-5). In the current study, we tested the anti-HIV-1 activity of Dh-5 and its derivatives. Although Dh-5 inhibited HIV-1 replication, none of the peptide variants were more effective in this respect. In contrast, one of the derivatives, Dhvar2, significantly increased HIV-1 replication by promoting the envelope-mediated cell entry process. Most likely, Dhvar2 affects membranes, thereby facilitating fusion of viral and cellular membranes. This study shows that modification of antimicrobial peptides in order to improve their activity against a pathogen may have unpredictable and dangerous side effects on other pathogens.

INTRODUCTION

Antimicrobial peptides have been identified in all species investigated, where they form part of the innate and possibly adaptive immune system. Many of these peptides have a broad spectrum of activity against bacteria, fungi, parasites, and viruses. Several different types of antimicrobial peptides have been found in humans (reviewed in ref 1 and 2). Although very heterogeneous in amino acid sequence, they share some characteristics like a low molecular weight (1-5 kDa), a positively charged domain of 10-25 amino acids, and the tendency to form amphipatic structures1,3. Generally, the peptide’s mode of action is binding and
disruption of membranes, but some studies indicate that these peptides also act intracellularly⁴-six.

Three modes of action of antimicrobial peptides have been described with respect to viruses and HIV-1 in particular: direct virolysis, inhibition of transcription from the long terminal repeat (LTR) promoter, and block of cell entry by binding to cell surface receptors⁷-¹⁰. In this study, we investigated the anti-HIV-1 activity of derivatives of Histatin 5, an antimicrobial peptide present in human saliva. Histatins form a group of electrophoretically distinct histidine-rich polypeptides with microbicidal activity that are present in human parotid and submandibular gland secretions¹¹. Histatin 5 is active against the yeast Candida albicans, an opportunistic fungus that causes oropharyngeal candidiasis in a majority of HIV-1 infected patients¹². Internalization of Histatin 5 and subsequent targeting of the mitochondria leads to release of vital components and cell death of the yeast¹³. To increase the activity of this peptide, amino acids in the active domain (Dh-5) of Histatin 5 have been substituted. The resulting peptides (Dhvar2-5, Table I) have previously been shown to possess increased fungicidal activity, and all but one (Dhvar5) are more amphipatic than Dh-5¹⁴,¹⁵. Because Candida is associated with HIV-1, we tested in the present study the anti-HIV-1 activity of Dh-5 and its derivatives. Dh-5 inhibited HIV-1 replication, but none of the derivatives was a more efficient inhibitor. Surprisingly, one of the peptides (Dhvar2) dramatically increased HIV-1 replication by promoting the envelope (Env)-mediated entry process. Dhvar2 probably destabilizes membranes and thereby facilitates fusion of viral and cellular membranes. This study shows that the development of more potent antimicrobial agents against certain pathogens like Candida may have unexpected and dangerous side effects if they are used in the context of other pathogens like HIV-1.
CHAPTER SIX

MATERIALS AND METHODS

Peptide synthesis and purification
The active domain of Histatin 5 (Dh-5, KRKFHEKHSHRGY) and variants (Dhvar2, KRLFKELLFSLRKY; Dhvar3, KRLFKKLFSLRKY; Dhvar4, KRLFKKLLFSLRKY; Dhvar5, LLLFLKRRKRRKY) were synthesized by F-moc chemistry with a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, MA, USA) and purified to at least 95% by RP-HPLC (Jasco Corporation Tokyo, Japan). The authenticity of the peptides was confirmed by ion trap mass spectrometry with a LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA). During synthesis, Dh-5 and Dhvar2 were labeled with FITC for FACS analysis as described previously. Labeling was achieved by addition of 25 µl FITC (1 mg/ml) in DMSO to 1 ml of a peptide solution (0.66 mM) in water, adjusted to pH 9.7 with Na2CO3. After overnight incubation at 4°C in the dark, residual FITC was inactivated by incubation with 50 µl of 1 M NH4Cl for 2 hr. The FITC–peptide conjugates were stored in aliquots at -20°C until use. LuSIV or SupT1 T cells were incubated for 30 min at 4°C with labeled peptides at a final concentration of 40 µM. Cells were then analyzed by FACS (FACScan, BD Biosciences, San Jose, CA, USA).

Cells, viruses, and lentiviral vectors
The PM1 and SupT1 T cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies), supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) (Sigma-Aldrich, St. Louis, MO, USA) and streptomycin (100 µg/ml) (Invitrogen, Breda, The Netherlands). The LuSIV cell with an integrated LTR-luciferase reporter construct has been described. Cells were maintained in the same medium as the PM1 and SupT1 cells, but with 300 µg/ml hygromycin B to maintain the luciferase genetic construct. Human embryonic kidney (HEK) 293T adherent cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), supplemented with 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, penicillin and streptomycin. HIV-1 was produced by
electroporation of PM1 T cells with 5 μg of the molecular clone of T-tropic HIV-1 LAI. The virus containing supernatant was harvested 5 days post transfection, filtered and stored at -80°C. The concentration of virus was determined by CA-p24 ELISA. The full-length SIV-mac239 clone17 was kindly provided by Drs. Y. Guan and M.A. Wainberg (McGill University AIDS Centre, Montreal, Canada). The virus was produced by electroporation of CEM×174 cells18. Lentiviral vectors with CXCR4-using Env of the HIV-1 HXB2 strain or VSV glycoprotein were produced as follows. 2.2 x 10⁶ 293T cells were seeded in a T25 flask the day prior to transfection. The next day, medium was replaced with 2.2 ml medium without antibiotics. Subsequently, 16 μl lipofectamine-2000 and 1.5 ml OptiMEM (Invitrogen) was used to transfect lentiviral vector plasmid pRRLcpptpgkgfp presSin19 (2.4μg) with packaging plasmids pSYNGP (1.5μg), (a kind gift of Dr. S. Kingsman (Oxford Biomedia, Oxford, UK)20, RSV-rev (0.6μg) and pVSV-G (0.8μg)21,22 or with pSV7D plasmid encoding HXB2 gp160 (0.8 μg). The pSV7D gp160 plasmid was a kind gift of Dr. J. Binley (Torrey Pines Institute for Molecular Sciences, La Jolla, CA, USA). Medium was replaced the next day with 4 ml of fresh medium. On day 3, medium was harvested in the morning and replaced with fresh medium. This procedure was repeated in the evening and the next day. Cellular debris from the lentiviral vector containing medium was removed by low speed centrifugation and supernatant was stored at 4°C. On the fourth day, supernatants were pooled and concentrated using an Amicon Ultra concentrator, MWCO 100,000 (100 kDa; Millipore Corporation, Bedford, MA, USA).

**Replication assays**

Single-cycle replication was assayed with LuSIV cells: 4 ng CA-p24 HIV-1 was pre-incubated at 0, 3.1, 12.5, 50 or 200 μM Dh-5 or Dhvar2-5 in a final volume of 50 μl at 37°C. After 30 min, 50×10⁴ LuSIV cells were added (200 μl) and luciferase production was determined 24 hr later, as previously described23. The final concentrations of the peptides were 0.6, 2.5, 10 or 40 μM, respectively. Fusion inhibitor T20 was added at different time points before or after infection, as indicated in the text (end concentration 1, 10, 100, 500 or 1000 ng/ml). Pre-
incubation of different mixtures of HIV-1, LuSIV and Dhvar2 was performed as follows. Equal volumes (80 µl) of HIV-1, LuSIV cells and Dhvar2 were pre-incubated in pairs at 37°C. All components were mixed after 30 min and co-cultured for 24 hr, followed by luciferase measurement. Single-cycle replication assays with lentiviral vectors were performed with concentrated supernatant containing the lentiviral vector with HIV-1 Env or VSV-G, which was pre-incubated with 200 µM Dh-5, Dhvar2 or Dhvar4 in 250 µl. After 30 min, 500×10^3 T cells were added (LuSIV, PM1 or SupT1), followed by co-culture for 3 days in a final volume of 1 ml (50 µM final peptide concentration). At day 3, cells were harvested, fixated in 4% paraformaldehyde and analyzed by FACS for the expression of GFP (FACScan, BD Biosciences). Replication assay with PM1 cells was initiated with 0.1 or 1 ng CA-p24 HIV-1, which was pre-incubated with 200 µM Dhvar2 or mock treated for 30 min at 37°C, followed by co-culture with 80×10^3 PM1 cells in a final volume of 250 µl (final concentration Dhvar2: 40 µM). Viral replication was followed by CA-p24 ELISA of the supernatant.

**gp120 ELISA**

ELISAs were performed as described previously. LAI gp120 (100 ng/ml) was captured onto the solid phase using antibody D7324 to the C5 region (Aalto Bio Reagents, Dublin, Ireland). Bound gp120 was detected with purified immunoglobulin from pooled serum of HIV-1-infected individuals (HIVlg), CD4-IgG2, or one of the monoclonal antibodies 17b (+/- soluble CD4 (scD4)), 2G12 or IgG1b12. Competition experiments were performed at half maximal binding concentrations of the respective reagents. These were 3 (HIVlg), 0.15 (CD4-IgG2), 0.02 (17b+scD4), 0.1 (17b), 0.2 (2G12) and 0.02 (IgG1b12) µg/ml. LAI gp120, CD4-IgG2, and scD4 were kindly donated by Drs. M. Franti and W. Olson (Progenics Pharmaceuticals, Inc., Tarrytown, NY, USA). 17b and HIVlg were a kind gift of Dr. J. Binley (Torrey Pines Institute for Molecular Sciences, La Jolla, CA, USA) and IgG1b12 was provided by Dr. D.R. Burton (the Scripps Research Institute, La Jolla, CA, USA). 2G12 from Dr. H. Kattinger was obtained from the NIH AIDS Research and Reference Reagent Program.
Peptide depletion assay
PM1 T cells were infected with HIV-1, cells were spun down at peak infection (1020 ng CA-p24/ml) and virus containing supernatant was used to infect fresh PM1 cells. Supernatant with virus (990 ng CA-p24/ml) was harvested 40 hr later and spun down in the ultracentrifuge for 2 hr at 33,000×g. Virus was resuspended in PBS (final concentration 950 ng CA-p24/ml) and incubated with Dh-5 or Dhvar2 (40 μM) for 30 min at 37°C. Alternatively, 2.5×10⁶ LuSIV, PM1 or SupT1 cells were incubated in 1 ml PBS with Dh-5 or Dhvar2. Cells and virus were subsequently removed by centrifugation (cells 400×g, virus 33,000×g) and the remaining concentration of peptide in the supernatant was determined by capillary zone electrophoresis (CZE) and RP-HPLC. CZE separations were conducted on a Biofocus 2000 instrument (BioRad, Hercules, CA, USA) equipped with an uncoated fused silica capillary of 24 cm and 50 μm ID, essentially according to the instruction of the manufacturer. Briefly, the capillary was rinsed for 60 sec with the low pH BioRad CZE phosphate buffer (0.1 M phosphate, supplemented with polymer modifier). Appropriate dilutions of the supernatants were mixed with 10 times diluted BioRad CZE phosphate buffer, containing 40 μM imidazol as internal standard. Samples were injected by pressure injection at 20 psi/sec. Separation was performed at a voltage of 15 kV (cathode at the detector site) and a temperature of 20°C. On-line UV detection of the samples was accomplished at 200 nm. Run time of the separation was 10 min. RP-HPLC separations were performed by HPLC (Jasco, Tokyo, Japan) on a reversed phase C-18 column (Vydac 218TP54, 24 cm and 4.6 mm ID, 5 μm particle size) developed with a lineair gradient of acetonitrile containing 0.1% TFA. On-line UV detection of the samples was accomplished at 214 nm. Run time of the separation was 20 min. Relative peptide concentrations in the samples were determined based on the relevant peak areas.
RESULTS

Dh-5 inhibits, but derivatives enhance HIV-1 replication
Amino acids in the active domain (Dh-5) of salivary peptide Histatin 5 have previously been replaced to increase the fungicidal activity of this peptide against *Candida albicans* (Table 1). Dhvar2 to -4 are more amphipatic, while Dhvar5 is less amphipatic than Dh-5\(^14,15\). Because *Candida* is associated with immunocompromised patients like HIV-1 infected persons progressing towards AIDS\(^12\), we wanted to explore the antiviral properties of Dh-5. We furthermore investigated whether the variant peptides displayed enhanced antiviral activity, which was expected due to their increased antifungal properties. We incubated HIV-1 with these peptides and tested viral infectivity in a single-cycle replication assay with LuSIV reporter cells. These reporter cells contain the firefly luciferase gene downstream of the LTR promoter, resulting in Tat-mediated luciferase expression, which is a measure of infectivity\(^16\). Dh-5 was able to reduce the infectivity of HIV-1, but modification of Dh-5 did not enhance the anti-HIV-1 properties of this peptide: none of the peptide variants decreased luciferase production more than Dh-5 (Fig. 1).

![Figure 1](image_url)

Figure 1. Modified antimicrobial peptides with improved antifungal properties enhance HIV-1 infectivity. The active domain (Dh-5) of salivary peptide Histatin 5 was modified in order to increase the antifungal activity. Dh-5 and derivatives were tested for their anti-HIV-1 activity in a single-cycle replication assay. HIV-1 was incubated with the peptides and subsequently co-cultured with LuSIV cells. These reporter cells express luciferase after HIV-1 infection, which was detected 24 hr later.
The active domain (Dh-5) of salivary peptide Histatin 5 was modified in order to increase the antifungal activity. Charges at neutral pH are depicted above the amino acids.

(a) calculated mean hydrophobic moment, a measure for amphipathicity of the peptide in \( \alpha \)-helical conformation (14). (b) LC50-values, the concentration causing 50% cell death, for Candida albicans (4, 14).

Surprisingly, the Dhvar2, -3 and -4 peptides with increased amphipathicity stimulated HIV-1 infection. In the absence of HIV-1, none of these peptides influenced the background luciferase levels of the LuSIV cells, excluding that the peptides trigger signaling pathways in the cell that lead to LTR activation and luciferase production (data not shown). Dhvar2 showed the highest enhancing effect by increasing luciferase production on average ten-fold.

Dhvar2 also stimulated HIV-1 replication in T cells over a prolonged period of time. PM1 T cells were infected with Dhvar2-treated or mock-incubated HIV-1 and viral replication was followed by CA-p24 ELISA on the supernatant. The results are very obvious when a non-infectious dose of HIV-1 was used, in which case Dhvar2 rescued HIV-1 replication (Fig. 2). When a high viral input was used, we found no stimulation by Dhvar2 in comparison to the mock treated sample (results not shown). Cell toxicity was observed at higher concentrations of Dhvar2 (>60 \( \mu \)M), which obviously decreased viral replication (data not shown).
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Figure 2. Dhvar2 can rescue HIV-1 replication at low viral inputs. A non-infectious HIV-1 dose of 0.4 ng CA-p24/ml was mock treated or incubated with Dhvar2, followed by co-culture with PM1 T cells. Viral replication was subsequently followed by measuring CA-p24 production in the supernatant by ELISA.

Dhvar2 binds to cells

To elucidate the mechanism by which Dhvar2 enhances HIV-1 infection, we pre-incubated the peptide (or medium as a mock treatment) either with HIV-1 or the LuSIV cells for 30 min at 37°C. Both treatments yielded the approximately 10-fold enhancement (Fig. 3). Pre-incubation of virus and cells prior to addition of Dhvar2 yielded the same level of stimulation. This experiment therefore does not identify the target for Dhvar2 stimulation, but it does show that the peptide has a broad time span in which it can stimulate HIV-1 infection.

Figure 3. Dhvar2 pre-incubation experiments. Black bars: HIV-1, LuSIV and Dhvar2 were pre-incubated in different pairs, as indicated on the x-axis. After 30 min, all were incubated together for an additional 24 hr. White bars: the same experiment was also performed with medium instead of Dhvar2 (mock treatment). Luciferase was measured after 24 hr. Error bars represent standard deviations.
We next investigated whether Dhvar2 (or Dh5 as a control) affects the binding of a broad range of reagents to the HIV-1 envelope (Env) gp120 protein in a gp120 ELISA (Fig. 4A). We focused on reagents that bind to the CD4 and co-receptor binding sites, since binding to these sites might provide a plausible mechanism for stimulation of infectivity by Dhvar2. Purified Ig from HIV-1-infected individuals (HIV1g) was used, as well as several monoclonal antibodies. 2G12 binds to a glycan dependent epitope on the outer domain of gp12038, IgGb12 targets the CD4 binding site30, and 17b is directed to a CD4-induced site overlapping the co-receptor binding site27. 17b was therefore tested with and without soluble CD4 (sCD4). Furthermore, we tested CD4-IgG2, which is a tetrameric CD4-based molecule that is directed to the CD4 binding site26. Increasing amounts of peptide were allowed to compete with these respective reagents for binding to gp120, which was captured on an ELISA plate. The reagents were used at the pre-determined half maximal binding concentrations. CD4-IgG2 binding was also performed in the presence of sCD4 to provide a control for competition efficiency.

Although we observed efficient inhibition of CD4-IgG2 binding to gp120 by sCD4, we did not observe an effect of Dh-5 or Dhvar2 on binding of the reagents to gp120 (Fig. 4A). We tested a wide concentration range of the peptides (0; 3.7; 11.1; 33.3 and 100 μM) and 100 μM is twice the concentration that stimulates virus infectivity. Even this amount of peptide did not influence binding of the various reagents to gp120, indicating that the peptides mode of action does not involve binding to the CD4 or co-receptor binding sites on gp120.

To further identify the target of Dhvar2, we tested whether Dhvar2 or Dh-5 could be depleted from a PBS solution by virus or T cells. High concentrations of virus (950 ng CA-p24/ml) did not deplete the peptides from the solution by virus centrifugation. In contrast, T cells (LusIV, PM1 or SupT1) could deplete both peptides from the solution (Fig. 4B). The amount of Dh-5 that remained in solution was on average 5 times higher than Dhvar2, indicating that Dhvar2 binds more efficiently to T cells than Dh-5. This observation was confirmed with FITC-labeled peptides that can be detected by FACS analysis. The amount of FITC-labeled Dhvar2 that bound to LuSIV or SupT1 T cells was on average three times
higher than the amount of Dh-5 (Fig. 4C). Combined, these experiments suggest that the target of Dhvar2 is the T cell rather than the virus.

Figure 4. Dhvar2 binds T cells. (A) gp120 ELISA: Increasing amounts of peptide (Dh-5 or Dhvar2) were allowed to compete with several reagents for binding to captured gp120. The reagents (HIVig, 2G12, IgGb12, 17b (+/- soluble CD4 (sCD4)) and CD4-IgG2) target the CD4 or co-receptor binding sites on gp120 and were used at their pre-determined half maximum binding capacity. Competition of sCD4 with CD4-IgG2 was performed in parallel to provide a control for competition efficiency. Results obtained with the highest concentration (100 µM) of peptide are depicted. nd: not done. (B) Peptide depletion assay: HIV-1 or cells (LuSIV, PM1, SupT1) were added to a stock of Dh-5 or Dhvar2 in PBS. After incubation for 1 hr, virus and cells were spun down and the remaining concentration of peptide was determined. (C) FACS analysis: Dh-5 and Dhvar2 were labeled with FITC. After incubation with LuSIV or SupT1 T cells and subsequent washing, cells were analyzed by FACS. The mean fluorescence intensity is indicated.
Stimulation by Dhvar2 is dependent on HIV-1 Env

To investigate whether the stimulatory properties of Dhvar2 are also applicable to other viruses, we studied whether the peptide could enhance LuSIV infection by CCR5-using SIV-mac239. Although not as efficient as the stimulation observed with CXCR4-using HIV-1, Dhvar2 enhanced SIV infection by a factor of 3.5 (Fig. 5).

Figure 5: Dhvar2 enhances infectivity of both HIV-1 and SIV. LuSIV cells were infected with HIV-1 or SIV-mac239 in the presence of 40 μM Dhvar2. Luciferase was determined after 24 hr. Error bars represent standard deviations.

To study whether Dhvar2 stimulates viruses that use other entry mechanisms, we compared a lentiviral vector that is pseudotyped with either the vesicular stomatitis virus glycoprotein (VSV-G) or HIV-1 Env. We used a standard lentiviral vector with the green fluorescent protein (GFP) reporter gene. After entry of this pseudovirus into the cell and subsequent reverse transcription, GFP will be produced, which can be detected by FACS three days later. Dhvar2 could elevate the transduction efficiency of the HIV-1 Env-based vector 2-fold (Fig 6A, left). In contrast, the percentage of GFP-positive SupT1 T cells was not elevated by Dhvar2 when the vector was bearing VSV-G (Fig. 6A, right). This result clearly demonstrates that the enhancing effect of Dhvar2 is dependent on the HIV-1 Env protein and excludes that the peptide affects other steps of the retroviral replication cycle shared by both vectors, which includes reverse transcription, integration, transcription and translation. The FACS results with SupT1 T cells are quantified in Fig. 6B. Similar results were obtained when the vector was used to infect LuSIV cells (Fig. 6B). As a control, we included Dh-5 and Dhvar4. No stimulation was observed with Dh-5 and a slight stimulation with Dhvar4.
**Figure 6. Stimulation by Dhvar2 is dependent on HIV-1 Env.** (A) SupT1 T cells were infected with a lentiviral vector in the presence of Dhvar2. The vector with the GFP gene was pseudotyped with either HIV-1 Env or VSV-G. After three days, the percentage of GFP positive cells was determined by FACS. Representative FACS plots are shown, with the percentage of GFP positive cells indicated. (B) SupT1 or LuSIV cells were infected with the lentiviral vector bearing HIV-1 Env, which was mock treated or incubated with Dh-5, Dhvar2 or Dhvar4. Three days later, the percentage GFP positive cells was determined.

**Dhvar2 promotes HIV-1 Env-mediated entry**

VSV and HIV-1 use different routes of cellular entry: VSV enters through endocytosis, whereas HIV-1 enters through receptor-mediated fusion of viral and cellular membranes\(^{31,32}\). The experiment using VSV-G and HIV-1 Env pseudotyped lentiviral vectors indicates that the effect of Dhvar2 is HIV-1 Env-dependent (Fig 6). From these results, one cannot discriminate between effects on Env-mediated membrane fusion or post-entry steps preceding reverse transcription (e.g. uncoating). To make this distinction, we employed the entry inhibitor T20. This peptide stabilizes a transient structural Env intermediate, thereby blocking subsequent conformational changes and the fusion of viral and cellular
membranes\textsuperscript{33,34}. First, we investigated whether Dhvar2 interferes with the inhibitory properties of T20 by titrating both peptides on LuSIV cells that were subsequently infected with HIV-1. Addition of Dhvar2 did not alter the inhibition profile of T20 (Fig. 7A). This result demonstrates that T20 inhibition is dominant over Dhvar2 stimulation, justifying further usage of T20 in these assays.

![Graph showing luciferase activity](image)

**Figure 7. Dhvar2 promotes HIV-1 Env-mediated cell entry.** (A) The inhibitory properties of fusion inhibitor T20 is unaffected by Dhvar2. LuSIV cells were infected with HIV-1 in the presence of varying concentrations Dhvar2 and T20. Luciferase production was determined 24 hr later. (B) Left panel: LuSIV cells were HIV-1 infected, followed by T20 addition 240 min later to stop the ongoing infection process. Right panel: Dhvar2 was added at t=255, with or without prior (t=240) T20 application. As a control, T20 was added 5 min prior to HIV-1 infection. Error bars represent standard deviations.
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We next infected LuSIV cells with HIV-1 and added T20 after 240 min to stop the ongoing infection process. Compared to the control infection, T20 partially reduced the luciferase production, demonstrating that a substantial amount of virus, but not all, had already entered the cells within 240 min (Fig. 7B, left panel). As a control, T20 was added 5 min before HIV-1 infection (t=−5), which totally abolished luciferase production. When Dhvar2 was added at t=255, this expectedly led to a considerable increase in luciferase production. However, this stimulation was not observed when T20 was supplied 15 min prior to Dhvar2 addition (Fig. 7B, right panel). In case Dhvar2 would stimulate a post-entry step, one would expect an increase in luciferase production despite the presence of T20 because this drug inhibits new entry events, but not virus that has already been fused with the cell at the time of T20 addition. We therefore conclude that Dhvar2 promotes the Env-mediated entry step.

DISCUSSION

Vaginal, rectal, or oral transmission of HIV-1 involves crossing of mucosal tissue, a process that is not yet entirely understood. One of the complicating factors for the virus are antimicrobial peptides that are secreted at mucosal surfaces as part of the innate immune system. Their use as candidate forms of prophylaxis against HIV-1 has therefore been proposed. There are several studies on antimicrobial peptides and inhibition of HIV-1 replication, and their mode of action is diverse, including disruption of the viral membrane, inhibition of reverse transcription or prevention of viral entry. Previously, we reported that human saliva contains several compounds that inhibit HIV-1 at different stages of the replication cycle. We now investigated the active domain of one salivary peptide (Histatin 5), and derivatives thereof. Amino acids in the active domain (Dh-5) were substituted, yielding peptide variants (Dhvar2-5) with increased fungicidal activity. We report that Dh-5 decreases the infectivity of HIV-1 in a single-cycle replication assay. Unexpectedly, none of the peptide variants were more
potent antivirals. Interestingly, the Dhvar2 variant significantly stimulated HIV-1 up to 30-fold in the single-cycle replication assay.

We found that Dhvar2 binds to T cells and that it enhances Env-mediated viral entry. Stimulation was observed with CCR5-using SIV, CXCR4-using HIV-1, and pseudotyped viruses, but not when this pseudovirus was bearing the VSV-G protein. VSV and HIV-1 have different routes of entry: VSV enters a cell through endocytosis, whereas HIV-1 enters through receptor-mediated fusion of viral and cellular membranes\textsuperscript{31,32}. In a pulse-chase experiment with the T20 entry inhibitor, we could demonstrate that Dhvar2 acts early at the entry step (Fig. 7B). Membrane interaction is a very general property of antimicrobial peptides\textsuperscript{1}. Possibly, Dhvar2 facilitates fusion of the viral and cellular membrane, due to the fact that this amphipatic peptide inserts in membranes and weakens the structure of the lipid bilayer. We observed a strong correlation between amphipaticity and the stimulatory or inhibitory properties of the different peptides (Table I). We therefore do not think that Dhvar2 has a specific protein-based receptor on T cells or the virus.

The difference between Dh-5 and the variants is the predicted 3-dimensional $\alpha$-helical conformation: Dhvar2, 3 and 4 have a more pronounced separation between the hydrophilic and hydrophobic amino acids than Dh-5, leading to an optimal amphipaticity in the $\alpha$-helical conformation (Table I). While in the context of Candida the derivatives have an increased fungicidal activity, this apparently does not apply to the HIV-1/human cell interaction. At the concentrations used in our study, Dhvar2 seems to affect the membrane such that Env-mediated membrane fusion and subsequent viral entry is promoted. This also applies to Dhvar3 and 4, but to a lesser extent.

Interestingly, one of the Dh-5 variants (Dhvar5) shows sequence similarity to the C-terminus of a recently described papillomavirus capsid protein\textsuperscript{43}. This peptide mediates escape of viral genome degradation by destabilizing the membrane of the endocytic compartment. Remarkably, Dhvar5 is the only Dh-5 variant that did not stimulate HIV-1 infection. Furthermore, Dhvar5 is the only variant that is less amphipatic than Dh-5. These results demonstrate that the stimulatory properties of a peptide on viruses is context dependent and therefore
difficult to predict. This papillomavirus study combined with our results suggest that minor membrane destabilization may be beneficial to a broad range of viruses.

Recently, another study showed that antimicrobial peptides with comparable bactericidal effects have differential effects on HIV-1 replication. One peptide, cryptdin 3 (Crp3), stimulated HIV-1 in an unidentified step preceding reverse transcription\(^{41}\). Crp3 is a member of the \(\alpha\)-Defensin family, and has been described to form anion-selective pores in mammalian cell membranes\(^{44}\). It is therefore possible that the mode of action is comparable to that of Dhvar2. We are the first to show that peptides with minor amino acid substitutions can have an unexpected reverse effect on HIV-1 replication.

Our findings are relevant for the design and clinical application of antimicrobial peptides. \textit{Candida albicans} is the predominant species of yeast isolated from patients with oral candidiasis, which is a frequent symptom of HIV-1 infection and a criterion for staging and progression of AIDS\(^{12}\). Treating candidiasis patients with the peptide variants with increased antifungal activity seems tempting, but would be unadvisable and potentially dangerous if this patient is HIV-1 infected. In the absence of HIV-1 infection, people who receive treatment for oral candidiasis with the variant peptides should be aware of the increased susceptibility to HIV-1 infection.

In conclusion, our study shows that modification of antimicrobial peptides in order to improve their activity may have unwanted and potential dangerous side effects on other pathogens. Since these effects are difficult to predict, this should be considered in the development of antimicrobial peptides for clinical application.

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REFERENCES


CHAPTER SEVEN

General discussion

Microbicides to prevent sexual HIV-1 transmission
CHAPTER SEVEN

1 THE NEED FOR CONDOM ALTERNATIVES

Although the probability of sexual HIV-1 transmission is rather small (0.001-0.009 per coital act)\textsuperscript{1-2}, humankind is experiencing a dramatic HIV-1 pandemic. To slow the virus from spreading, treating as many HIV-1 seropositive patients as possible with antiretroviral medicines is helpful, since the viral load of the infected partner correlates with the probability of transmission\textsuperscript{14}. However, from a medical, ethical, practical, and financial point of view, preventing HIV-1 from establishing a persistent infection after sexual intercourse is obviously preferred. In this respect, condoms are the most effective prophylaxis\textsuperscript{5}, but unfortunately this is complicated by several factors. For instance, many men are unwilling to use condoms, and many women simply cannot negotiate usage of this contraceptive\textsuperscript{6,7}. Furthermore, in some circles or cultures, a stigma is attached to persons that use condoms\textsuperscript{8-10}. Thus despite the fact that condoms are preferred and should be promoted, alternatives have to be considered, especially in developing countries where the HIV infection rate is high and women may hold a weak position.

One of the alternatives, and proven to be very effective against other viruses, is vaccination. Our current knowledge indicates that an effective HIV-1 vaccine should induce antibody responses as well as a vigorous protective cytotoxic T-lymphocyte (CTL) response (reviewed in \textsuperscript{11}). Since dendritic cells (DC) are the most potent antigen-presenting cells, and mediate immunity to many viral infections, a current view is that exploitation of the immunostimulatory potential of DC will be key to achieve a protective immune response to prevent HIV-1 infection\textsuperscript{12-20}. Given the fact that DC also play a crucial role in establishment of a productive HIV-1 infection, this demonstrates the possible dual role of DC in HIV pathogenesis\textsuperscript{14}. However, despite extensive efforts, no effective and safe HIV-1 vaccine has been developed and no breakthrough is expected for the near future\textsuperscript{21,22}. In the meantime, microbicides form an attractive alternative, which is the scope of this chapter.
2 MICROBICIDES AGAINST HIV-1

Microbicides are agents used topically in the vagina or rectum to prevent infection by sexually transmitted pathogens. Such an approach may be successful as research indicates that women demonstrate interest in products that can be used with or without their partner’s knowledge. In addition, men who have sex with men frequently use lubricants during unprotected anal sex, suggesting that lubricants with microbicides could be an acceptable strategy. Some microbicides also inhibit transmission of other pathogens like herpes simplex virus (HSV). This is a beneficial side-effect since HIV-1 susceptibility correlates with the presence of other sexually transmitted diseases (STD’s).

Currently, several phase III trials of candidate microbicides against HIV-1 are in progress or will commence shortly. A definite answer on safety and efficacy of those microbicides is not expected until 2008.

Three classes of microbicides against HIV-1 can be distinguished: 1) surfactants that destroy the viral envelope, 2) compounds that block HIV-1 binding to its receptor(s), and 3) antiretrovirals that block reverse transcription in infected cells. These three different classes of microbicides will be discussed below.

2.1 Microbicides that disrupt the viral membrane

Microbicides in this class target the virus in a non-specific manner. The most (in)famous representative of this class is probably nonoxinol-9, an over-the-counter spermicide that has in vitro anti-HIV-1 activity by destroying lipid bilayers. Nonoxinol-9 was the first vaginal microbicide tested in a phase III trial. In 1992, a randomized trial was performed with 892 female sex workers from four countries. Unfortunately, the study did not show a protective effect of nonoxinol-9 on HIV-1 transmission. Repeated use of the microbicide caused toxic effects that even enhanced HIV-1 infection, probably by breaching the epithelial barrier and causing inflammations.

Other agents of this class are compounds that lower the pH below 4.5, which results in HIV-1 inactivation. Several microbicides with this mode of action are
currently in phase I/II/III trials\textsuperscript{37}. It should be noted however that semen is alkaline and can cause a significant rise in pH that allows HIV-1 infection\textsuperscript{38}.

The study with nonoxinol-9 demonstrates that microbicides may have an unpredictable effect. A comparable case was demonstrated \textit{in vitro} by us in chapter 6 of this thesis (ref 39). We tested recently developed antifungal peptides for their impact on HIV-1 infection. The opportunistic fungus \textit{Candida albicans} causes oropharyngeal candidiasis in a majority of immunocompromised patients, including HIV-infected persons progressing towards AIDS\textsuperscript{40}, and can even use the same receptor (DC-SIGN) as HIV-1\textsuperscript{41}. Previously, modifications of the active domain of Histatin 5, a salivary peptide with antimicrobial properties, resulted in highly active peptides with increased amphipathicity and activity against this yeast\textsuperscript{42,43}. On basis of their amphipatic structure, the peptides were expected to possess antiviral activity as well. Unexpectedly, the peptide derivatives promoted viral entry and replication, probably by weakening the lipid bilayers of viral and cellular membranes. The usage of these peptides to treat candidiasis should therefore be limited to HIV-negative immunocompromised patients, \textit{e.g.} transplantation patients on immune suppressive medication.

There are many more examples of compounds that have virucidal properties against HIV-1 \textit{(i.e. disrupt the virion) in vitro}, consequently preventing infection of target cells\textsuperscript{30,44\textendash}48. Each of these compounds in principle could be used as a microbicide to destroy HIV-1 during sexual transmission. However, the two examples above demonstrate that microbicides that target the virus in a non-specific manner potentially can do more harm than good. Monitoring clinical safety is therefore a priority in current microbicide research\textsuperscript{30}, and the development of agents that more specifically target a step in the HIV-1 infection process may be a safer strategy.

\textbf{2.2 Microbicides that prevent virus binding}

Binding of HIV-1 to its receptor and subsequent infection of the cell can be prevented with compounds that either bind the virus or the receptor. Sulphated polysaccharides were the first microbicides reported to inhibit HIV-1 binding to target cells \textit{in vitro}\textsuperscript{49}. Due to their negative charge, they bind to the HIV-1
envelope (Env) glycoprotein (gp120)\textsuperscript{7,30,50}, which has also been demonstrated with acylated milk proteins\textsuperscript{51}. Candidate agents in this class of microbicides that are already in, or scheduled for phase III trials in women at risk in Africa include high molecular weight negatively charged sulphated polymers such as PRO 2000, carageenan (a naturally occurring sugar polymer), and cellulose sulphate\textsuperscript{7,26,30,52,53}.

Another strategy, which has been proven to be successful in macaques, is the vaginal application of neutralizing antibodies against SHIV (simian immunodeficiency virus within HIV-1 Env)\textsuperscript{54}. Two enormous drawbacks to the use of neutralizing antibodies are their cost and the variability of the viral Env protein to which they bind\textsuperscript{35}. Only a couple of broadly cross-reactive neutralizing antibodies have been developed in the past 20 years, and none of these recognize all virus strains\textsuperscript{55,56}.

In another macaque study, two HIV-1 Env-binding compounds could protect macaques against challenge with SHIV\textsuperscript{57}. These compounds bind to gp120\textsuperscript{58,59}, or block fusion by binding to gp41\textsuperscript{57,60,61}. Yet another example of HIV-binding molecules that inhibit infection of T cells and also DC-mediated HIV-1 transmission \textit{in vitro} are soluble lectins. These compounds bind and neutralize the virus and possibly induce phagocytosis and viral breakdown\textsuperscript{62-64}. These are just a few examples of HIV-1-binding molecules that can inhibit the infection of (S)HIV \textit{in vitro} or in a primate model\textsuperscript{57}.

As mentioned however, the variability of HIV-1 Env is a consistent problem with all molecules that target the virus in a specific manner\textsuperscript{55,55,66}, and it would therefore be wise to target the cellular receptors instead of the virus. In sexual transmission, HIV-1 can bind to a wide range of possible receptors expressed on different cells like dendritic cells (DC), T cells and epithelial cells. These receptors include DC-SIGN\textsuperscript{67-70}, langerin\textsuperscript{67,71}, CD4 and/or CCR5\textsuperscript{67,72-75}, the mannose receptor (MR)\textsuperscript{67,71} and possibly other unidentified C-type lectin receptors\textsuperscript{67,76}. The relative contribution of all HIV-1 receptors in viral capture and transmission \textit{in vivo} remains to be determined\textsuperscript{71,77-80}, stressing the need for microbicide mixtures that target distinct receptors.

A small molecule that binds to CCR5 (CMPD167\textsuperscript{81}) could protect a significant percentage of macaques if this molecule was applied in the vagina prior to
challenge. A comparable study was performed with a RANTES-analogue, and confirmed that CCR5-blocking molecules can protect macaques from challenge with SHIV. Given the fact that in the macaque studies described above not all animals were protected, we should develop microbicide gels that contain several different compounds. One potential microbicide that has a desired broad activity, is bovine lactoferrin (bLF). This natural milk protein inhibits HIV–1 replication in T cells by two independent mechanisms: bLF strongly binds to HIV–1 Env, and it binds to the (co)–receptors on the T cell. Another feature of bLF is the capacity to prevent DC-mediated HIV–1 transmission (chapter 5 and ref). bLF strongly binds to DC-SIGN, thus preventing virus capture and subsequent transmission. In addition to this broad activity spectrum, bLF is non-toxic, available in large quantities, and inexpensive. It therefore forms an interesting candidate microbicide against HIV–1, and further research on the possible application for this purpose seems warranted.

2.3 Microbicides that prevent viral replication

A third possibility is blocking productive HIV–1 infection even if the virus has already entered a T cell. Nucleotide analogues inhibit reverse transcription of viral RNA into DNA by the reverse transcriptase (RT) enzyme. The analogues compete with the natural RT ligand for binding, and are normally orally administered to treat HIV–infected individuals. To paralyze incoming virus during sexual transmission, a gel formulation has been developed with the RT inhibitor tenofovir. The safety and tolerability of tenofovir in a vaginal gel has recently been demonstrated in women, and effectiveness in preventing HIV–1 transmission will now be tested in follow-up studies.

Another way of blocking reverse transcription is the administration of non-nucleoside inhibitors. These molecules inhibit RT by binding to an allosteric site on this enzyme, and some are being formulated as vaginal gels with the aim to enter phase III trials in 2007. Resistance of the virus is a problem with both types of RT inhibitors, and there is a serious concern for women who are unaware of their HIV–positive status, yet use these compounds as microbicide. Absorption
of these antiretrovirals by the body could select for resistant variants that would prevent future antiretroviral therapy\(^\text{26}\).

In reference 94 and chapter 4 of this thesis, we describe our research on the HIV-inhibitory compounds that are secreted by plasmacytoid DC (PDC). PDC are thought to play an important role in innate immune responses to different types of viruses, including HIV-1\(^\text{95-98}\). We could confirm that PDC secrete large amounts of interferon alpha (IFN\(\alpha\)), which potently inhibits HIV-1 replication. We furthermore found that PDC secrete a small molecule that efficiently inhibits HIV-1 replication. Whether these compounds can be used to prevent HIV-1 transmission by blocking replication remains to be determined. IFN\(\alpha\) is an antiviral cytokine that is already administered to persons to treat hepatitis C virus infection, but it has unpleasant side-effects\(^\text{99-101}\). The small antiviral molecule secreted by PDC has to be identified first in order to consider its prophylactic potential. Due to its size (<3 kDa) and heat-sensitivity, it may well be an antimicrobial peptide. These small peptides are active against a broad range of bacteria, fungi and viruses\(^\text{47,102}\). With respect to HIV-1, they have been described to disrupt the viral membrane, inhibit replication, and block entry by binding to cell surface molecules\(^\text{46,103-105}\). Note that all of these properties of antimicrobial peptides correspond with the three classes of microbicides described in this chapter. More research on the small unidentified molecule for use in microbicides, and antimicrobial peptides in general, seems therefore justified.

Another possibility of preventing intracellular HIV-1 from spreading is blocking DC-mediated transmission after the virus has been captured in the mucosa. HIV-1 transmission is thought to take place across a so-called ‘infectious synapse\(^\text{106-108}\)’, and formation of such a structure depends on strong cell-cell contact through ICAM-1—LFA-1 binding (ref 109 and chapter 2). If this interaction can be blocked \(\text{in vivo}\), we could prevent transmission and subsequent spreading of HIV-1. The fungal metabolite lovastatin, which belongs to the statin compounds used in the treatment of hypercholesterolemia, was shown to bind LFA-1 and inhibit the interaction with ICAM-1\(^\text{110}\). Since this compound is already administered to individuals, thereby proven to be a safe drug, it forms an interesting candidate microbicide against HIV-1. In our assays, lovastatin could
inhibit HIV-1 transmission by DC, but inhibition was due to a toxic effect on the DC and T cells. Given the importance of LFA-1 in HIV-1 transmission by DC, future research should focus on the development of less toxic derivatives or other small molecule inhibitors of the ICAM-1—LFA-1 interaction\textsuperscript{111-113}.

3 CONCLUDING REMARKS

The development of microbicides to slow the HIV-1 pandemic seems the most effective strategy on the short term. One concern is that offering access to partially effective microbicides may offset successful condom promotion campaigns\textsuperscript{30}. However, mathematical projection suggests that consistent usage of a low-effective microbicide provides greater protection over time than less consistently used high-effective products like condoms\textsuperscript{114}.

There are several important matters concerning the development of microbicides. Resistance of the virus and the potential hazardous and unexpected side-effects of compounds are two of them. A safe and effective microbicide therefore preferentially is a mixture of compounds that target different steps of the viral infection process, thereby spreading the risks. When targeting cellular elements instead of the virus, one has to keep in mind that a microbicide may disturb desired cellular processes as well. The long-term presence of the compound is necessary to protect an individual, but the compound should not block DC-SIGN or the ICAM-1—LFA-1 interaction for too long.

Given the importance of DC in HIV-1 transmission, a microbicide mixture should at least contain one compound that prevents DC-mediated HIV-1 capture. This strategy is supported by a recent study using cervical tissue explants, which demonstrates that simply blocking CD4 and co-receptors is not sufficient. Although local replication of the virus was inhibited, it could not be prevented that DC carrying infectious HIV-1 migrated out of the tissue\textsuperscript{115}. HIV-1 may remain in an infectious state in DC for a sufficient amount of time\textsuperscript{68,106,116,117} to reach areas where microbicides do not penetrate, e.g. the lymph node. Finally, since different DC subsets can capture HIV-1 through a range of different receptors\textsuperscript{67}, not only
DC-SIGN should be the target, but also other receptors like the mannose receptor and CD4.

In the absence of an effective HIV-1 vaccine, we are forced to consider alternatives. Our increasing knowledge on the HIV-1 infection pathway may provide new leads for the development of microbicides that prevent sexual acquisition of HIV-1. This might prompt means for the containment of this rapidly spreading virus.

REFERENCES


CHAPTER SEVEN


GENERAL DISCUSSION: MICROBICIDES TO PREVENT HIV TRANSMISSION


SUMMARY

HIV-1 can infect cells of the immune system that bear the appropriate receptors: CD4 and an additional co-receptor. Cells that are susceptible to HIV-1 infection are amongst others CD4+ T cells, macrophages and dendritic cells (DC). CCR5 is the major co-receptor used by HIV-1, and in some patients the virus evolves to use CXCR4 as co-receptor, which coincides with faster disease progression. By infecting cells of the immune system, HIV-1 causes a gradual collapse of this system and finally death.

Sexual transmission of HIV-1 is hindered by a mucosal barrier that has to be crossed, a process that is not yet entirely understood. DC are thought to play an important role in this process by capturing the virus in the mucosa and transporting it to the lymph nodes where HIV-1 is presented to CD4+ T cells. In addition to this, DC can facilitate local HIV-1 replication in mucosal T cells.

DC are professional antigen-presenting cells that sample the environment at sites of pathogen entry and play an essential role in the induction of adaptive immune responses. Upon activation by micro-organisms or inflammatory signals, immature DC migrate to the draining lymph nodes while up regulating co-stimulatory molecules and developing into mature effector DC. The scope of this thesis is on the mechanism of DC-mediated HIV-1 transmission, which DC and T cell subsets are involved, and whether we can interfere with this process.

In Chapter 2 of this thesis, our research on the importance of strong DC-T cell interaction through ICAM-1—LFA-1 binding is described. We have previously identified ICAM-1 on DC as a crucial factor for HIV-1 transmission. ICAM-1 binds LFA-1 on T cells, an integrin responsible for adhesion and signaling at the immunological synapse. To corroborate the importance of the ICAM-1—LFA-1 interaction, we made use of LFA-1 negative leukocytes from Leukocyte Adhesion Deficiency type 1 (LAD-1) patients. We show in Chapter 2 that DC-mediated HIV-1 transmission to LAD-1 T cells is impaired in comparison to healthy controls. Furthermore, HIV-1 transmission to T cells from a unique LAD-1 patient with a well characterized LFA-1 activation defect was impaired as well, demonstrating that activation of LFA-1 is crucial for efficient transmission.
In Chapter 3 and 4, we describe our research on the different cell types involved in transmission. Several T cell subsets can be identified in humans: naïve T cells (T_N) that initiate an immune response to new antigens, and memory T cells that respond to previously encountered pathogens. The memory T cell pool comprises central memory (T_CM) and effector memory cells (T_EM), which are characterized by distinct homing and effector functions. The T_EM cell subset, which can be further divided into effector Th1 and Th2 cells, has been shown to be the prime target for viral replication after HIV-1 infection, and is abundantly present in mucosal tissues. In Chapter 3, we performed transmission experiments to these different T cell types. We found that co-receptor expression on the respective T cell subsets is a decisive factor for transmission. Accordingly, CCR5-using (R5) HIV-1 was most efficiently transmitted to T_EM cells, suggesting that mucosal T cells are an important target for DC-mediated transmission. This may contribute to the initial burst of virus replication that is observed in these cells. In contrast, CXCR4-using (X4) HIV-1 was preferentially transmitted to T_N cells, which are considered to inefficiently support HIV-1 replication. These results indicate that DC may play a decisive role in the susceptibility of T_N cells to X4 tropic HIV-1.

In Chapter 4, the DC were the subject of variation. In humans, 2 main DC types are identified: CD11c⁺ myeloid DC (MDC) and CD11c⁻ CD123⁺ plasmacytoid DC (PDC). MDC include Langerhans cells, dermal DC and interstitial DC. They have been associated with HIV-1 capture and sexual transmission, whereas PDC play an important role in the innate immune responses to different types of viruses, including HIV-1. To compare the influence of MDC and PDC on HIV-1 infection of T cells, we isolated donor-matched MDC and PDC from peripheral blood, activated them by adding different maturation-inducing compounds, and co-cultured them with T cells and HIV-1. We found that MDC enhance HIV-1 infection through capture of the virus and subsequent transmission to T cells, and that differently matured MDC subsets have different HIV-1 transmission efficiencies. These differences were not due to soluble factors, viral capture differences or the expression of integrins ICAM-1, -2, -3 or LFA-1. In contrast, regardless of their state of maturation, PDC inhibit HIV-1 replication in T cells.
through the secretion of IFNα and an additional, unidentified small molecule. In this chapter, we thus show that the two main types of DC have opposing roles in HIV-1 infection of T cells.

Given the important role of MDC in sexual HIV-1 transmission, we explored the possibility of preventing this process in **Chapter 5**. We tested proteins from milk and serum for their ability to block DC-mediated HIV-1 transmission, of which bovine lactoferrin (bLF) is the most potent inhibitor. bLF binds strongly to DC-SIGN, thus preventing virus capture and subsequently transmission. Interestingly, the bovine protein is a much more efficient inhibitor of transmission than human LF (hLF). Since bLF is non-toxic and easy to purify in large quantities, it forms an interesting candidate microbicide against HIV-1. Another advantage of bLF is its ability to block HIV-1 replication in T cells. This broad spectrum of activity underscores the usefulness of bLF as a microbicide to prevent HIV-1 transmission, and research on such usage of bLF seems justified.

The development of microbicides can also have unexpected and potential dangerous side-effects, as shown in **Chapter 6**. We have tested the antiviral properties of variants of an antimicrobial peptide from saliva (Histatin 5). This peptide inhibits *Candida albicans*, an opportunistic fungus that causes oropharyngeal candidiasis in a majority of HIV-infected patients progressing towards AIDS. The peptide variants are more amphipatic versions of the active domain of Histatin 5 (Dh-5), and displayed increased fungicidal activity. Although Dh-5 inhibited HIV-1 replication, none of the peptide variants was more effective in this respect. In contrast, three out of four derivatives significantly increased HIV-1 replication by promoting the envelope-mediated cell entry process. Most likely, the derivatives affect membranes, thereby facilitating fusion of viral and cellular membranes.

Finally, in **Chapter 7** we have put the main findings of the thesis in perspective with recent literature on microbicide development. In the absence of an effective HIV-1 vaccine, we have to consider alternatives to slow the virus from spreading.
SAMENVATTING

HIV-1
Humaan Immunodeficiëntie Virus type I (HIV-1) is een virus dat ‘Acquired Immune Deficiency Syndrome’ (AIDS) veroorzaakt. Op dit moment zijn meer dan 40 miljoen mensen geïnfecteerd met HIV-1, waarvan 60% in Afrika leeft ten zuiden van de Sahara. Voor zijn vermenigvuldiging infecteert HIV-1 CD4+ T cellen. Hiervoor bindt HIV-1 aan receptoren op de T cel, namelijk CD4 en een co-receptor. Grofweg zijn er twee types HIV-1: virussen die naast CD4 CCR5 als co-receptor gebruiken, of virussen die CXCR4 als co-receptor gebruiken. Om onbekende redenen is CCR5-gebruikend HIV-1 de variant die seksueel wordt overgedragen. Dit type HIV-1 kan vervolgens evolueren naar het gebruik van CXCR4, wat gepaard kan gaan met versnelde progressie richting AIDS. Omdat T cellen een cruciaal onderdeel zijn van het immuunsysteem, zal dit belangrijke beschermingsmechanisme langzaam ineenstorten, met de dood als gevolg.

Dendritische cellen en HIV-1 transmissie
Tijdens de seksuele overdracht van HIV-1 vormen de epitheelcellen in de slijmvliesen een barrière voor het virus. Dendritische cellen (DC) spelen zeer waarschijnlijk een belangrijke rol in de seksuele overdracht van HIV-1, omdat zij het virus kunnen opnemen in de slijmvliesen. DC zijn ‘antigeen presenterende cellen’ die normaal gesproken ziekteverwekkers (pathogenen) herkennen. Ze bevinden zich overal in het lichaam, en worden geactiveerd op het moment dat ze een pathogen tegenkomen. Vervolgens zullen ze zich naar de lymfeknopen begeven en (stukjes van) het pathogen presenteren aan T cellen. Op deze manier wordt een adequate immuunreactie opgewekt.

HIV-1 maakt ingenieus misbruik van de DC, als een soort paard van Troje: het virus wordt netjes afgeleverd bij de T cel, die vervolgens geïnfecteerd wordt. Hoe dit gebeurt, en waarom er geen adequate immuunrespons wordt opgewekt tegen HIV-1 is voor een groot deel onbekend. De overdracht van HIV-1 via DC noemen we ‘transmissie’, en daar gaat dit proefschrift over.
In **Hoofdstuk 1** staat een uitgebreide introductie over HIV, dendritische cellen en seksuele transmissie.

**Hoofdstuk 2** gaat over ons onderzoek naar het belang van sterke DC-T cel interactie via het binden van ICAM-1 aan LFA-1. Dit zijn ‘integrines’, moleculen die belangrijk zijn voor de interactie tussen cellen. In een vorig onderzoek hebben we ICAM-1 expressie op de DC als een cruciale factor voor DC—HIV-1 transmissie geïdentificeerd. ICAM-1 bindt aan LFA-1 op T cellen, wat belangrijk is voor adhesie en signalering tussen een DC en een T cel in ‘normale’ immuunreacties. Deze interactie is dus ook van belang voor HIV-1 transmissie door DC. Om dit verder te onderzoeken hebben we gebruik gemaakt van uniek patientenmateriaal. ‘Leukocyte Adhesion Deficiency type 1’ (LAD-1) is een zeldzaam syndroom dat gekenmerkt wordt door de afwezigheid van LFA-1 op de cellen van deze patiënten. In hoofdstuk 2 laten we zien dat HIV-1 transmissie naar T cellen van deze patiënten zo goed als onmogelijk is. We hebben ook gebruik gemaakt van de T cellen van een unieke LAD-1 patiënt die door een mutatie LFA-1 tot expressie brengen dat niet functioneel is. Ook naar deze cellen was transmissie onmogelijk, waarmee we laten zien dat niet alleen LFA-1 aanwezigheid van belang is voor DC—HIV-1 transmissie, maar ook de activatie van dit molecul.

In **Hoofdstuk 3 en 4** inventariseerden we welke cel types allemaal een rol kunnen spelen in DC—HIV-1 transmissie. Er zijn namelijk meerdere T cell types identificeerbaar: naïeve T cellen (Tn) zijn belangrijk voor de immuunreacties tegen nieuwe pathogenen. ‘Memory’ T cellen reageren juist op pathogenen die al eens de persoon in kwestie hebben geïnfecteerd. De memory T cel groep bestaat uit zgn. ‘central memory’ (T<sub>CM</sub>) en ‘effector memory’ T cellen (T<sub>EM</sub>). T<sub>CM</sub> cellen zijn belangrijk voor het bewaren van immunititeit op de lange termijn, en T<sub>EM</sub> cellen leveren actief die bescherming op de korte termijn. Deze twee types memory T cellen bevinden zich op verschillende plaatsen in het lichaam. De T<sub>EM</sub> cellen bestaan op hun beurt weer uit T helper type 1 en 2 cellen, en blijken na seksuele overdracht van HIV-1 het eerste doelwit te zijn voor het virus. In Hoofdstuk 3 beschrijven we onze DC-transmissie experimenten naar deze verschillende T cel types. We laten zien dat de expressie van HIV-1 co-receptoren (CCR5 en CXCR4)
niet hetzelfde is op de verschillende T cellen. Het gevolg hiervan is dat HIV-1 dat gebruik maakt van CCR5 zeer efficiënt overgedragen wordt aan T_{EM} cellen. Dit zou een rol kunnen spelen bij de observatie dat juist deze cellen het eerste doelwit zijn van HIV-1 na seksuele transmissie. CXCR4-gebruikend HIV-1 werd juist zeer efficiënt overgedragen aan T_{N} cellen. Over het algemeen wordt aangenomen dat HIV-1 slechts replieert in deze cellen, maar wij laten zien dat DC een belangrijke rol kunnen spelen in het toegankelijk maken van deze cellen voor HIV-1.

In **Hoofdstuk 4** zijn niet de T cellen, maar de DC het onderwerp van variatie. In het lichaam komen twee groepen van DC voor: myeloïde DC (MDC) en plasmacytoïde DC (PDC). De MDC groep wordt verantwoordelijk geacht voor seksuele HIV-1 transmissie, en is erg heteroogeen van aard. PDC spelen juist een belangrijke rol in de niet-specifieke, aangeboren afweer tegen allerlei virussen, inclusief HIV-1. Om de invloed van deze twee DC hoofdgroepen op HIV-1 replicatie in T cellen met elkaar te vergelijken, isoleerden we MDC en PDC direct uit het bloed van gezonde donoren. De DC werden op meerdere manieren geactiveerd, en vervolgens gecultiveerd met HIV-1 en T cellen. Zoals verwacht stimuleerden MDC de HIV-1 infectie van T cellen, aangezien MDC het virus kunnen overdragen aan T cellen. Echter, we vonden verschillen in de mate van stimulatie tussen MDC die op verschillende wijze geactiveerd waren. Deze verschillen zijn niet toe te schrijven aan uitgescheiden factoren, verschillen in virus opname, of de expressie van integrines op het DC oppervlak. In tegenstelling tot de MDC, remden PDC juist de HIV-1 replicatie in T cellen, ongeacht welke activatie stimulus we gebruikten. Dit effect kon volledig worden toegeschreven aan de uitscheiding van stoffen door de PDC. Eén stof konden we identificeren als interferon-alfa, en één is een tot dusver ongeïdentificeerd klein molecule. Met deze studie hebben we laten zien dat de twee DC hoofdgroepen zich fundamenteel anders gedragen in relatie tot HIV-1 replicatie in T cellen.

Gezien het feit dat MDC zo’n belangrijke rol spelen in de seksuele overdracht van HIV-1, onderzochten we in **Hoofdstuk 5** de mogelijkheid om dit proces te blokkeren. We testten meerdere eiwitten uit melk en serum, en vonden dat lactoferrine uit koeiemelk (bLF) zeer efficiënt de DC—HIV-1 transmissie kon voorkomen. bLF bindt aan de belangrijkste HIV-1 receptor op DC (DC-SIGN) en
voorkomt daarmee virus opname en transmissie. Aangezien bLF niet toxisch is en gemakkelijk in grote hoeveelheden te isoleren valt, is het een interessante microbicide-kandidaat om seksuele HIV-1 transmissie te voorkomen. Microbicides zijn stoffen die vaginaal of rectaal gebruikt kunnen worden om seksuele overdracht van HIV-1 te voorkomen. Een extra voordeel van bLF is dat het naast transmissie ook HIV-1 replicatie in T cellen kan voorkomen (via een ander mechanisme). Deze brede werking tegen HIV-1 maakt bLF extra interessant, en we zijn daarom van mening dat er meer onderzoek zou moeten plaatsvinden naar de toepassing van dit molecuul als microbicide.

Dat de ontwikkeling van microbicides ook onverwacht gevaarlijke effecten kan hebben, laten we zien in Hoofdstuk 6. Daarin testten we de anti-virale werking van enkele varianten van een anti-microbiëel peptide uit speksel (Histatin 5). Anti-microbiële peptides zijn kleine eiwitjes die onderdeel zijn van de niet-specifieke afweer tegen bacteriën, schimmels en virussen. Histatin 5 remt de groei van Candida albicans, een schimmel die ‘candidiasis’ veroorzaakt in personen met een verminderde afweer, zoals HIV-1 patienten. De ontworpen peptide varianten waren actiever tegen Candida, doordat ze meer amfipatisch van aard waren (dwz, ze hebben zowel hydrofobe als hydrofiele delen). Tot onze verbazing stimuleerden 3 van de 4 varianten de HIV-1 replicatie in T cellen, terwijl het originele peptide (Histatin 5) HIV-1 wèl remt. Waarschijnlijk beïnvloeden de peptidedevarianten de celmembran op een dusdanige manier dat HIV-1 efficiënter de cel kan infecteren. We laten hiermee zien dat de ontwikkeling van microbicides onverwachte problemen kan opleveren. De ontworpen peptides waren weliswaar actiever tegen Candida, maar zullen niet gebruikt kunnen worden in HIV-1 patienten.

In Hoofdstuk 7 plaatsen we de belangrijkste bevindingen van dit proefschrift in een context van recente ontwikkelingen op het gebied van microbicides. In de afwezigheid van een goed werkend HIV-1 vaccin en de culturele barrières die condoomgebruik bemoeilijken, kunnen microbicides als alternatief gebruikt worden om de verspreiding van HIV-1 te remmen.
LIST OF ABBREVIATIONS

APC  antigen-presenting cell
βLG  β-lactoglobulin
bLF  bovine lactoferrin
CA-p24 capsid-protein 24
CD  cluster of differentiation
CCR5 / 7 chemokine (C-C motif) receptor 5 / 7
CTL  cytotoxic T-lymphocyte
CXCR4 chemokine (CXC motif) receptor 4
DC  dendritic cell
DC-SIGN DC-specific ICAM-3-grabbing nonintegrin
DNA deoxyribonucleic acid
ELISA enzyme-linked immunosorbent assay
Env  envelope
FACS fluorescent-activated cell sorting
FCS fetal calf serum
FITC fluorescein isothiocyanate
GFP green fluorescent protein
GM-CSF granulocyte-macrophage colony-stimulating factor
gp120 glycoprotein 120
hBD human β-defensin
HIV-1 / 2 human immunodeficiency virus type 1 / 2
hLF human lactoferrin
HSA human serum albumin
HSV herpes simplex virus
ICAM-1 / 2 / 3 intercellular adhesion molecule-1 / 2 / 3
iDC immature DC
IFN interferon
Ig immunoglobulin
IL interleukin
kDa kilo Dalton
ABBREVIATIONS

LAD-1  leukocyte adhesion deficiency type 1
LF     lactoferrin
LFA-1  leukocyte function-associated molecule-1
LPS    lipopolysaccharide
LTR    long terminal repeat
mDC    mature DC
MDC    myeloid DC
MF     maturation factors
MFI    mean fluorescence intensity
MHC-I / II major histocompatibility class I / II
MR     mannose receptor
mRNA   messenger RNA
PAMP   pathogen-associated molecular patterns
PBL    peripheral blood leukocytes
PBMC   peripheral blood mononuclear cells
PCR    polymerase chain reaction
PDC    plasmacytoid DC
PE     phycoerythrin
PFA    paraformaldehyde
PgE2   prostaglandin E2
PRR    pattern recognition receptors
R5 HIV CCR5-using HIV
RLU    relative light units
RNA    ribonucleic acid
RT     reverse transcriptase
RT-PCR reverse transcription PCR
SAC    (fixed) Staphylococcus Aureus Cowan strain I bacteria
sCD4   soluble CD4
SEB    Staphylococcus enterotoxin B
SHIV   a laboratory-created hybrid virus made from HIV and SIV
SIV    simian immunodeficiency virus
STD    sexually transmitted disease
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>SQV</td>
<td>saquinavir</td>
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<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>central memory T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;</td>
<td>effector memory T cell</td>
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<tr>
<td>T&lt;sub&gt;N&lt;/sub&gt;</td>
<td>naïve T cell</td>
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<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th1 / 2</td>
<td>T helper type 1 / 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>X4 HIV</td>
<td>CXCR4-using HIV</td>
</tr>
</tbody>
</table>
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Fedde
CURRICULUM VITAE

Fedde Groot (1978, the Netherlands) graduated in Biology from Utrecht University in 2001. During his first internship at the department of Microbiology (Utrecht University), Fedde studied LPS production in *E. coli*. His interest in viruses proved stronger during his second internship, when he investigated the initiation of HIV reverse transcription at the department of Human Retrovirology (AMC, University of Amsterdam). In 2002, Fedde started his PhD training at this latter group, in collaboration with the department of Cell Biology & Histology. The results of this research are described in this thesis. In September 2006, Fedde joined Quentin Sattentau’s group at The Sir William Dunn School of Pathology at the University of Oxford (UK). Supported by an amfAR fellowship, Fedde now studies ‘HIV-1 cell to cell spread’.
PUBLICATIONS


